ER export of ERGIC-53 is controlled by cooperation of targeting determinants in all three of its domains

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Summary
Selective export of proteins from the endoplasmic reticulum (ER) requires transport signals that have not been fully characterized. Here, we provide the first complete map of ER export determinants of a type I membrane protein, ERGIC-53, that cycles in the early secretory pathway. ER export requires a phenylalanine motif at the C-terminus, known to mediate coat protein II (COPII) interaction, that is assisted by a glutamine in the cytoplasmic domain. Disulfide bond-stabilized oligomerization is also required. Efficient hexamerization depends on the presence of a polar and two aromatic residues in the transmembrane domain (TMD). Oligomerization becomes independent on disulfide bonds when TMD hydrophobicity is increased. ER export is also influenced by TMD length, 21 amino acids being most efficient. When transferred to a signal-less construct, the established targeting motifs reconstitute full transport activity. The results suggest an ER-export mechanism in which transmembrane and luminal determinants mediate oligomerization required for efficient recruitment of ERGIC-53 into budding vesicles via the C-terminal COPII-binding phenylalanine motif.

Key words: COPII, Endoplasmic reticulum, ERGIC, Oligomerization, Transport signal

Introduction
Export of proteins from the endoplasmic reticulum (ER) is likely to involve default pathways as well as selective sorting (Pfeffer and Rothman, 1987; Kuehn and Schekman, 1997; Hauri et al., 2000). Selectivity is indicated by the fact that some membrane proteins are concentrated in vesicles budding from the ER, such as vesicular stomatitis virus (VSV) G protein, KDEL receptor, SNARE (SNAP receptor) proteins and ERGIC-53 (Balch et al., 1994; Bednarek et al., 1995; Klumperman et al., 1998; Martinez-Menarguez et al., 1999). Both budding of transport vesicles and selective recruitment of membrane proteins into the buds are mediated by COPII coat proteins (Aridor et al., 1998; Kuehn et al., 1998; Springer and Schekman, 1998) (for reviews, see Antonny and Schekman, 2001; Barlowe, 2002).

Selective ER export requires signals that have not been studied in detail. A di-acidic motif is required for efficient transport of the VSV-G protein in mammalian cells and of Sys1p in yeast (Nishimura and Balch, 1997; Sevier et al., 2000; Votsmeier and Gallwitz, 2001). Similarly, a di-phenylalanine motif (FF) and di-hydrophobic motifs in the cytoplasmic tail of ERGIC-53 (Kappeler et al., 1997) and p24 proteins (Fiedler et al., 1996; Dominguez et al., 1998; Nakamura et al., 1998; Belden and Barlowe, 2001) can mediate selective protein export from the ER. Di-acidic and hydrophobic motifs interact with COPII components. In some cases, accessory factors are required to direct transmembrane cargo into COPII vesicles (Kuehn et al., 1996; Herrmann et al., 1999; Powers and Barlowe, 2002). The ER export motifs characterized up until now are not easily transplantable to reporter proteins (Kappeler et al., 1997; Nishimura and Balch, 1997; Sevier et al., 2000; Nufer et al., 2002), with one exception. A single valine at the extreme C-terminus of type I membrane proteins required for correct localization of transforming growth factor α (TGFα), membrane type-1 matrix metalloproteinase (MT1-MMP) (Urena et al., 1999) and CD8 alpha (Iodice et al., 2001) to the plasma membrane is sufficient for efficient ER export in reporter proteins (Nufer et al., 2002). The fact that most known ER export motifs are not active when transferred to reporter proteins suggests that optimal recruitment of membrane proteins by the COPII budding machinery is controlled by multiple determinants.

We use ERGIC-53 as a model type I membrane protein to study mechanisms underlying protein traffic early in the secretory pathway (Hauri et al., 2000). ERGIC-53 is a major type I membrane protein of the tubulovesicular clusters of the ERGIC (Schweizer et al., 1988; Schindler et al., 1993) and rapidly cycles between ERGIC and ER (Lippincott-Schwartz et al., 1990; Aridor et al., 1995; Klumperman et al., 1998). Retrieval of ERGIC-53 to the ER is controlled by a di-lysine ER targeting signal (Itin et al., 1995), whereas anterograde transport is mediated by a FF motif at the C-terminus (Kappeler et al., 1997). However, the cytoplasmic domain of ERGIC-53 containing the FF motif is not sufficient for efficient ER export when appended to reporter proteins (Kappeler et al., 1997). In addition, a cycling-impaired ERGIC-53 variant lacking the FF motif still exhibits some slow transport (Itin et al., 1995; Kappeler et al., 1997). These results suggest that there are additional targeting determinants in the luminal and/or transmembrane domain of ERGIC-53.
The aim of the present study was to identify all the targeting determinants of ERGIC-53 in order to understand its mechanism of ER export. We show that efficient transport requires a phenylalanine at position –2 from the C-terminus and a glutamine in the cytoplasmic domain. Moreover, the TMD of ERGIC-53 contains a noncontiguous motif required for correct oligomerization of ERGIC-53. Proper oligomerization and disulfide-linked stabilization are a prerequisite for efficient ER export of ERGIC-53.

Materials and Methods

Antibodies and reagents

Mouse monoclonal antibodies (mAb): 9E10.2 (IgG1) against c-myc, 2/614/88 (IgG1) against sucrase-isomaltase (SI) (Hauri et al., 1985), HP2/6.1 (IgG1) against human CD4 (kindly provided by F. Sanchez-Madrid, Spain). Cell culture reagents were from GibcoBRL (UK) and Sigma (Switzerland).

Recombinant DNA

Standard molecular biology protocols were adapted from Ausubel et al. (Ausubel et al., 1997). Oligonucleotides were from Microsynth (Switzerland). ERGIC-53 constructs containing a myc-tag and an artificial N-glycosylation site, termed GM, have been described (Itin et al., 1995; Kappeler et al., 1997). Selected amino acids were substituted by oligonucleotide-directed PCR-mutagenesis or sequence-overlap-extension PCR (Ho et al., 1989). Two silent mutations were introduced into GM to generate unique restriction sites. First, the codon of Y498 was changed to tac, creating a BsrGI site. Second, the codon of R499 was changed to aga, creating a BglII site. PCR fragments generated for substitution of cysteines C466 and C475 were recloned via EcoRI and AccI sites into GM constructs. Fragments encoding TMD mutants were recloned via AccI/BsrGI, AccI/BglII or AccI/XbaI. Fragments with cytosolic domain mutants were recloned via AccI/XbaI. Chimeric GM constructs with substitution of the cytoplasmic domain or TMD were generated by annealing complementary oligonucleotides as described previously (Nufer et al., 2002) and recloned into GM. Constructs with CD4 TMD or L18 TMD have been described (Itin et al., 1995; Nufer et al., 2002). GM constructs with L18 TMD and polyalanine or serine tails have been described (Nufer et al., 2002). Starting from the L18 TMD mutant, extensions to 20, 21, 22, 24 and 26 leucines were generated by PCR-based site-directed mutagenesis and introduced as AccI/BglII fragments. All constructs were confirmed by sequencing using standard methods.

Cell culture and transfection

COS-1 cells were transfected by the diethylaminoethyl-dextran (DEAE-dextran) method (Kappeler et al., 1997) and Lec-1 cells by the Lipofectamine method (Ausubel et al., 1997). Oligonucleotides were from Microsynth (HP2/6.1 (IgG1) against human CD4 (kindly provided by F. Sanchez-Madrid, Spain). Cell culture reagents were from GibcoBRL (UK) and Sigma (Switzerland).

Metabolic labeling and immunoprecipitation

Forty to sixty hours after transfection the cells were washed twice with PBS, starved in labeling medium (Modified Eagle’s Medium (MEM) without methionine, 10% dialyzed fetal calf serum (FCS) and pulsed with 100 μCi/ml [35S]-methionine/cysteine (PerkinElmer Life Sciences, USA). Chase was in Dulbecco’s Modified Eagle’s Medium containing 10% FCS and 10 mM L-methionine. For immunoprecipitation, the cells were washed twice with cold PBS, resuspended in lysis buffer [100 mM Na phosphate, 1% Triton-X100, pH 8, 0.2 mM phenyl methyl sulphonyl fluoride (PMSF)] and centrifuged at 100,000 g for 1 hour. The supernatant was added to protein A-Sepharose beads (Amersham Pharmacia Biotech) carrying prebound antibodies. After 1 hour on a rotary shaker, the beads were washed four times with lysis buffer, once with 100 mM Na phosphate (pH 8) and once with 10 mM Na phosphate (pH 8).

Endoglycosidase (endo H) digestion

Immunoprecipitates were boiled for 3 minutes in 50 mM Tris/HCl, 1% SDS and 0.1 M β-mercaptoethanol (pH 6.8). An equal volume of 0.15 M Na citrate (pH 5.3) containing protease inhibitors was added, and the sample was digested with 10 mU of endo H (Roche) at 37°C overnight.

Density gradient centrifugation

Lec-1 cells stably expressing GM or transiently expressing CD4 or sucrase-isomaltase were labeled with 100 μCi/ml [35S]-methionine/cysteine, harvested in PBS containing 0.2 mM PMSF and 20 mM iodoacetamide, and centrifuged at 1500 g for 5 minutes. The cells were lysed in 0.4 ml 20 mM HEPES, 150 mM NaCl, 1% Triton-X100, pH 7.4, 0.2 mM PMSF, 20 mM iodoacetamide. After 1 hour on ice, the lysate was cleared by centrifugation at 100,000 g for 1 hour. The supernatant was loaded on a 4 ml continuous 5-30% (w/v) sucrose density gradient (prepared in lysis buffer containing 0.1% Triton-X100) and centrifuged in a SW56ti rotor for 18 hours at 42,000 rpm. Twelve fractions of 350 μl were collected from the bottom by a peristaltic pump. Low- and high-molecular-weight markers (Amersham Pharmacia Biotech) were diluted in homogenization buffer and centrifuged at 100,000 g before fractionation. GM, CD4 and sucrose-isomaltase were immunoprecipitated using antibodies prebound to protein G Sepharose (Sigma). Aliquots of the fraction gradients containing molecular weight markers were precipitated with trichloroacetic acid, washed with ethanol/ether (1:1) and analyzed by SDS-PAGE. Densities were analyzed by refractometry.

Gel electrophoresis and fluorography

Samples were separated by SDS-PAGE and radioactivity was visualized by fluorography using sodium salicylate and BioMax MR-1 films (Rochester, MN). Fluorograms were quantified with a ChemImageTM device and AlphaEaseTM software (Alpha Inotech Corporation, USA).

Computer analysis

Basic sequence analysis was carried out with the GCG programs. Other software used is available at ExPaSy server (http://www.expasy.org/). For detection of putative TMDs the programs HMMTOP (Tusnady and Simon, 1998) and TMPred (Hofmann and Stoffel, 1993) were used in combination with structure predictions. Representation of TMD as helical wheel was generated by using GCG program HelicalWheel.

Results

COS-1 cells transfected by the dicistronic dextran (DEAE-dextran) method (Kappeler et al., 1997) and Lec-1 cells by the Ca-phosphate precipitation method (Ausubel et al., 1997) or by using FuGene6 reagent (Roche, Switzerland).
Previous studies have suggested that the RSQQE motif in the cytoplasmic tail is required for correct targeting of ERGIC-53, but the precise role of this motif is unknown (Itin et al., 1995). To study the importance of RSQQE in detail we first replaced it by alanines. This change reduced FF-mediated transport (Fig. 1 and data not shown). A subsequent alanine scan of the RSQQE sequence showed that Q501 but not the other amino acids were required for efficient FF-mediated transport (not shown).

Are F509 and Q501 in a poly-alanine tail sufficient to mediate ER-to-Golgi transport as efficiently as the wild-type tail of ERGIC-53? To test this, we appended different cytoplasmic tails (Fig. 1B) to a myc-tagged, glycosylated variant of ERGIC-53 termed GM (Itin et al., 1995) and replaced the di-lysine signal in GM by alanines to prevent recycling. The constructs were expressed in COS cells and their transport from ER to Golgi was studied by pulse-chase metabolic labeling with [35 S]-methionine, followed by endo H digestion of the immunoprecipitated mutant proteins (Kappeler et al., 1997). On the basis of our previous studies a 1 hour chase time was selected for analysis. It is noteworthy that all constructs exhibited normal topology and oligomerized normally indicating correct folding (not shown). Fig. 1 shows that the transport of the construct having both RSQQE and FF (GMA5 FF) was efficient, as indicated by an endo H resistance of more than 40% after a 1 hour chase. Polyalanine tails carrying no motif, only RSQQE or only Q501 were inefficient. The single presence of F509 partially reconstituted transport efficiency but was significantly below that observed for construct GMA5 FF. However, the combined presence of Q501 and F509 mediated transport in a way that was indistinguishable from that of GMA5 FF. We conclude that F509 is required and is largely sufficient for the transport of (oligomeric) ERGIC-53 and that the additional presence of Q501 augments transport efficiency.

Formation of disulfide-linked dimers by membrane proximal cysteine residues is required for transport of ERGIC-53

ERGIC-53 forms disulfide-linked homodimers and homohexamers immediately after synthesis (Schweizer et al., 1988). Oligomers are stabilized by the two conserved cysteines C466 and C475 close to the membrane (Lahtinen et al., 1999) (Fig. 2A).

To study the role of disulfide-bond-stabilized oligomerization in transport, a set of GMA7 and GMA5 FF constructs with single or double substitutions of the cysteines was prepared (Fig. 2A). The constructs exhibited the expected oligomeric state when analyzed by nonreducing SDS-PAGE (Fig. 2B). While metabolically labeled GMA7 carrying both cysteines rapidly formed dimers and hexamers, the mutants with single replacement of either C466 or C475 only formed dimers. A construct lacking both cysteines remained monomeric (Fig. 2B). Importantly, all constructs were stable, as indicated by unchanged trypsin sensitivity, and had the same turnover as the wild-type protein (not shown). Moreover, the lectin activity is preserved in these noncovalently linked constructs (Appenzeller et al., 1999). Obviously, the lack of the tested disulfide bonds has no impact on protein stability.

We then tested ER-to-Golgi transport of the constructs in a pulse-chase/endo H experiment. Because these constructs also lack the di-lysine retrieval signal, the assay measures forward transport only. GMA7 exhibited transport with about 20% endo H resistance (Fig. 2C). Mutating the cysteines in GMA7 did not further reduce transport. GMA5 FF was efficiently transported...
as expected (Fig. 2C). Substitution of C\textsubscript{466} in GMA\textsubscript{3}FF did not affect transport, but substitution of C\textsubscript{475} alone or in combination with C\textsubscript{466} reduced the transport of GMA\textsubscript{3}FF to the level of GMA\textsubscript{7} (Fig. 2C).

These results show that covalent dimerization via the membrane proximal C\textsubscript{475}, but not disulfide-linked hexamerization via C\textsubscript{466} and C\textsubscript{475}, is required for efficient transport of ERGIC-53, provided that the cytosolic ER export determinants are present.

Effect of TMD length on ER export

Previous studies showed that the slow transport of GMA\textsubscript{7} can be enhanced considerably when its TMD is replaced by that of CD4 (Kappeler et al., 1997) (Fig. 3). The TMD of CD4 has a length of 21 amino acids and is not known to contain any anterograde transport information. By comparison, the TMD of ERGIC-53 contains 18 amino acids. This raises the question of whether TMD length plays a role in ER export.

To address this question we substituted the entire TMD of GMA\textsubscript{7} with stretches of 18 to 26 leucines (L\textsubscript{18} to L\textsubscript{26}, Fig. 3). Leucine is the most abundant residue in \(\alpha\)-helical TMDs (Ulmschneider and Sansom, 2001) and acts as a strong helix former (Chou and Fasman, 1974). Replacing the TMD of ERGIC-53 with that of CD4 or a L\textsubscript{18} stretch did not affect membrane association as tested by the carbonate/pH 11.5 extraction. Moreover, the constructs had the correct topology, as tested by protease protection assays, and they still formed disulfide-linked dimers and hexamers and are therefore considered to be correctly folded (Kappeler et al., 1997; Nufer et al., 2002) (and not shown). The effect of membrane length on transport was tested by pulse-chase/endo H (Fig. 3). While

![Fig. 2](image1.png)

**Fig. 2.** Dimerization by disulfide bonds is required for efficient transport. (A) GM constructs. Luminal cysteines 466 and 475 of GMA\textsubscript{7} (– tail) and GMA\textsubscript{3}FF (+ tail) (also compare Fig. 1) were changed to alanines, individually or in combination. (B) Oligomer formation of GMA\textsubscript{7} constructs bearing cysteine substitutions. 42 hours after transfection, COS cells were labeled for 5 minutes with \([\text{\textsuperscript{35}S}]\)-methionine and chased as indicated. The cells were washed and lysed in the presence of 20 mM iodoacetamide and subjected to immunoprecipitation with anti-myc. Immunoprecipitates were separated by 4-10% gradient SDS-PAGE under nonreducing conditions followed by fluorography. Monomeric (1\times), dimeric (2\times) and hexameric (6\times) forms of GM forms are indicated by arrows at the right margin. The size of molecular weight markers is indicated at the left margin. (C) Transport of GM constructs probed by pulse-chase/endo H (Fig. 1). Black bars: GMA\textsubscript{7} (–) and GMA\textsubscript{3}FF (+) constructs; grey, white and hatched bars represent values of corresponding constructs with the indicated cysteine substitutions. Results are mean±s.e.m. of at least three independent experiments.

![Fig. 3](image2.png)

**Fig. 3.** TMD length affects ER export. GMA\textsubscript{7} constructs containing different TMDs were expressed in COS cells and transport was probed by pulse-chase/endo H (Fig. 1): T53, wild-type TMD of ERGIC-53; T33L\textsubscript{2} and T33L\textsubscript{3}, T53 elongated by two or three leucines, respectively; T4, TMD of CD4; L\textsubscript{18}-L\textsubscript{26}, TMDs consisting of leucine stretches of indicated length. Results are mean±s.e.m. of at least three independent experiments. * statistical significance to all bars except L\textsubscript{22} and T33L\textsubscript{2}; ** statistical significance to T53 (\(P\lt 0.05\), Student’s t-test).
the GMA7 construct with ERGIC-53’s TMD of 18 amino acids (T53) exhibited slow transport, the construct with CD4’s TMD of 21 amino acids (T4) was efficiently transported. Unexpectedly, replacing the TMD of ERGIC-53 with L18 significantly reduced transport when compared with T53. Lengthening the TMD to L20 increased the transport rate, and a further increase was observed with L21. Further extension up to L26 decreased transport again. We also tested the effect of lengthening ERGIC-53’s authentic TMD by two leucines (T53L2) or three leucines (T53L3). As shown in Fig. 3, transport of T53L2 and T53L3 was considerably faster than that of T53. Interestingly, transport of T53L2 (TMD of 20 amino acids) was faster than L20, but comparable to that of L21. Similarly, transport of T53L3 (TMD of 21 amino acids) was faster than L21. Lengthening ERGIC-53’s TMD by four leucines did not further increase transport (not shown).

These results suggest that the TMD of ERGIC-53, by virtue of its suboptimal length of 18 amino acids, contributes to ER retention. The optimal length for ER export appears to be 21 amino acids. However, additional features of the TMD must contribute to ER export given that TMDs consisting entirely of leucines were less efficient than the authentic TMDs of ERGIC-53 or CD4. These features may be the degree of hydrophobicity or the presence of anterograde transport determinants.

Polar and aromatic residues of the transmembrane domain of ERGIC-53 contribute to efficient oligomerization and transport

To test whether the more hydrophobic character of an L18 stretch mediates retention, we constructed additional mutants in GMA7 and GMA5FF with a TMD length of 18 amino acids.

Fig. 4. Polar and aromatic residues in the TMD are required for efficient transport. (A) Replacement of ERGIC-53’s TMD slows transport. The TMD of ERGIC-53 (T53) in GM constructs with (+) or without (−) the FF motif was substituted with different amino acid repeats with a total length of 18 residues. Constructs were expressed in COS cells and transport was probed by pulse-chase/endo H (Fig. 1). Black bars: GMA7 (−) and GMA5FF (+) constructs with ERGIC-53’s TMD (T53). Grey, white and hatched bars: constructs with TMD substitutions as indicated. Results are mean±s.e.m. of at least three independent experiments. (B) Representation of ERGIC-53’s TMD as helical wheel. Linear sequence of TMD (residues 481-498) of human ERGIC-53 is given below the wheel. Polar amino acids and glycine 494 are in italics. Polar and aromatic residues facing one side of TMD helix are in bold. Residues influencing transport of GMA5FF are encircled in the helical wheel and marked by arrowheads in the linear sequence. (C) Transport of TMD mutants (fluorogram). COS cells were transfected with GM constructs containing either wild-type TMD (lane 1, GMA7; lane 2, GMA5FF) or amino acid substitutions in TMD of GMA5FF (lanes 3 to 6 as indicated in D). 42 hours after transfection, cells were subjected to pulse-chase/endo H analysis. (D) Quantification of fluorograms including that shown in C. Results are mean±s.e.m. of at least three independent experiments. Grey bars, GMA5FF constructs with substitutions in the TMD as indicated; white and black bars, GMA7 (wt−) and GMA5FF (wt+) with wild-type TMD. *Statistical significance to bar 2 (P<0.05, Student’s t-test).
but different features (Fig. 4A). The (LA)$_9$ TMD has a hydrophobicity comparable to that of ERGIC-53, whereas the (ILV)$_6$ TMD has maximal hydrophobicity. Both TMDs were properly associated with the membrane as tested by carbonate/ pH 11 extraction (not shown). When tested by pulse-chase/endo H, we observed that these TMDs reduced transport efficiency, both in GMA$_7$ and GMA$_{5}$FF, as compared with the wild-type TMD of ERGIC-53 (Fig. 4A). Clearly, the reduction in transport was independent of hydrophobicity. The results led us to conclude that the TMD of ERGIC-53 must contain anterograde targeting information that supports the FF motif.

A notable feature of the ERGIC-53 TMD is the unusual presence of polar residues (Q$_{488}$ and T$_{489}$) (Fig. 4B). It also contains some aromatic residues. Tyrosines and tryptophanes are often found at the lipid-water interphase of $\alpha$-helical TMDs, and phenylalanines are common in TMDs (Ulmschneider and Sansom, 2001). If the TMD is plotted as a helical wheel, it becomes apparent that Q$_{488}$ lies close to aromatic residues at one face of the helix. Therefore, our search for targeting determinants in the TMD focused on these residues. Q$_{494}$ was also examined because glycines tend to increase helix flexibility and allow for close packing of TMD helices (Javadpour et al., 1999; Ubarretxena-Belandia and Engelman, 2001). Using GMA$_{5}$FF, we changed several TMD residues to leucine, either alone or in combination, and probed transport efficiency of the constructs by pulse-chase/endo H. None of the single substitutions had an effect on the transport rate of GMA$_{5}$FF (not shown). Substitution of the T$_{489}$ or the G$_{494}$ in combination with the Q$_{488}$ or any of the internal phenylalanines also had no effect (not shown). However, the substitution of the Q$_{488}$ together with either F$_{484}$ or Y$_{498}$ reduced the transport rate, as did the double substitution of F$_{484}$ and Y$_{498}$ (Fig. 4C,D). The most prominent reduction in transport of GMA$_{5}$FF was observed when the TMD contained the triple mutation F$_{484}$L/Q$_{488}$L/Y$_{498}$L. Transport of this construct was as inefficient as that of GMA$_7$ with a wild-type TMD (Fig. 4C,D). Interestingly, neither substituting F$_{481}$ nor Y$_{495}$ affected transport, although these residues are located on the same side of the helix (Fig. 4B).

How can the determinants F$_{484}$/Q$_{488}$/Y$_{498}$ in the TMD influence the transport of ERGIC-53? One possibility is that they are required for efficient oligomerization. To test this, we analyzed the kinetics of disulfide-linked oligomerization in a pulse-chase experiment (Fig. 5). After a short pulse of 5 minutes, the GM reporter with wild-type TMD exhibited predominantly disulfide-linked dimers. With time, hexamers were also formed. The hexamer/dimer ratio was 0.5 after 60 minutes. By contrast, the GM reporter with the triple mutation F$_{484}$L/Q$_{488}$L/Y$_{498}$L in the TMD (mt TMD) formed dimers efficiently, but formation of hexamers was inefficient. The maximal hexamer/dimer ratio never exceeded a value of 0.3.

These experiments indicate that polar and aromatic residues facing one side of the TMD helix are required for transport because they contribute to the oligomerization of ERGIC-53.

Anterograde targeting determinants in a signal-less reporter promote full transport activity

Are the identified anterograde transport determinants sufficient for efficient ER export? Our experiments suggest that ER export requires an interplay of cytosolic, transmembrane and luminal determinants, rendering it difficult to address this question. Indeed, transplantation of cytosolic anterograde determinants, alone or together with the TMD of ERGIC-53, to the monomeric reporter proteins CD4 and Tac-1, or to the oligomeric reporters CD8 and VSV-G, did not promote efficient ER export (Kappeler et al., 1997) (results not shown). Obviously, the determinants are only effective in a defined, presumably hexameric, context. We tested, therefore, whether efficient anterograde transport can be reconstituted by introducing the determinants into the signal-less ERGIC-53 variants L$_{53}$L$_{18}$R$_{2}$A$_{10}$ and L$_{53}$L$_{18}$R$_{2}$S$_{10}$ (Nufer et al., 2002).
which particularly only consist of the authentic luminal part of ERGIC-53. These variants have a myc-tag and an artificial N-linked glycosylation site like GM, but their TMD is replaced by a L18 stretch and the cytoplasmic domain consists of a string of polyalamines or serines separated from membrane by two arginines. Both signal-less reporters are properly folded and correctly inserted into the membrane bilayer. They exhibit a very slow transport rate below that of GMA7 (Nufer et al., 2002) (Fig. 6). For the reconstitution of the signal-less ERGIC-53 reporters with a minimal set of anterograde transport determinants, the cytosolic determinants Q501 and F509, as well as the transmembrane determinants F484/Q488/Y498, were reintroduced at their proper positions (Fig. 6A). In addition, all the signal-less (sl) and reconstituted (rec) reporters were prepared without or with substitution of the C466 alone or together with C475.

All constructs were inserted into the membrane with correct topology (not shown). They exhibited disulfide-stabilized oligomerization on nonreducing gel as expected. Constructs having both C466 and C475 formed disulfide-linked dimers and hexamers (6x); constructs with C466A substitution only formed dimers (2x) (Fig. 6B). The constructs with the double substitution C466A/C475A did not form intermolecular disulfide bridges and remained monomeric (1x). Oligomerization of the signal-less constructs was identical to their GM counterparts (Nufer et al., 2002) (and not shown).

**Fig. 6.** Reconstitution of efficient transport by a minimal number of transport determinants in a signal-less reporter. (A) GM construct and signal-less GM constructs reconstituted with a minimal set of anterograde transport determinants. The signal-less constructs possess a poly-leucine TMD and a poly-alanine or poly-serine cytoplasmic domain. The minimal anterograde transport determinants are in bold. All constructs were prepared without and with alanine substitution of cysteine 466 alone, or cysteine 466 together with cysteine 475. (B) Oligomerization of reconstituted constructs. The reconstituted constructs with alanine (ala tail) or serine tail (ser tail) were tested for disulfide-linked oligomerization as described in Fig. 2, except that only one time point was analyzed (15 min chase). 1x, cysteines 466 and 475 have been substituted; 2x, cysteine 466 has been substituted; 6x, both cysteines are present. (C,D) Transport of GM constructs expressed in COS cells probed by pulse-chase/endo H (Fig. 1). rec, reconstituted constructs with cysteine substitutions indicated; sl, signal-less constructs. The ‘sl’ and ‘rec’ constructs contain alanine tails in panel C, and serine tails in panel D. Mean±s.e.m. of at least three independent experiments.
The reconstituted constructs were then tested for their transport ability by pulse-chase/endo H. Fig. 6 shows that they were as efficiently transported as GMA3FF, irrespective of whether alanine tails (C) or serine tails (D) were used. By contrast, the signal-less reporters without the transport determinants exhibited a slow transport rate, even below that of GMA7. Surprisingly, reconstituted reporters not able to form disulfide-linked oligomers were transported as efficiently as the corresponding dimeric and hexameric constructs. This is in contrast to the result obtained with GMA5 FF in which the double substitution C466A/C475A drastically reduced transport (see Fig. 2).

When examining the disulfide-linked oligomerization of some constructs presented in this study by nonreducing SDS-PAGE, we noticed monomeric forms and large aggregates that had an Mr higher than hexamers for constructs with TMD substitutions to L18, (LA)9 or (ILV)6, in addition to correctly oligomerized proteins (Fig. 7). By contrast, constructs with wild-type TMD or with a leucine stretch containing the reconstituted TMD determinants oligomerized correctly and did not show aggregates. We conclude that reconstitution of the TMD determinants F484/Q488/Y498 into the leucine stretch promotes correct oligomerization. A TMD consisting of a leucine stretch adopts a more pronounced α-helix than the TMD of ERGIC-53 and is highly hydrophobic. This may explain why the reconstituted constructs exhibited efficient transport, even in the absence of disulfide linkages.

Collectively, a minimal set of anterograde transport determinants can mediate efficient transport of signal-less variants of ERGIC-53. Moreover, determinant-driven transport in an artificial background does not necessarily require disulfide-linked oligomerization.

ERGIC-53 is present in a complex both in the presence or the absence of disulfide linkages

Because our reconstitution experiments suggested that ERGIC-53 could form oligomers in the absence of intermolecular disulfide bridges, we tested the oligomeric state of GM and GM with C466A/C475A substitutions by rate-zonal centrifugation. Lec-1 cells stably expressing either GM or GM with C466A/C475A substitutions were labeled with [35S]-methionine for 5 minutes and lysed immediately or chased for 1 hour before lysis. Cleared lysates were subjected to rate-zonal centrifugation using continuous 5-30% (w/v) sucrose gradients (Fig. 8). Fractions were immunoprecipitated with anti-myc. After a 1 hour chase, GM was found in fractions 6 and 7 (Fig. 8B). This position corresponds to the size of the 158 kDa marker protein (Fig. 8A). Interestingly, dimers and hexamers of fractionated GM appeared in the same fractions, despite the considerable size difference on nonreducing SDS-gels. The GM construct with C466A/C475A substitution was found in fractions 7 to 9 (Fig. 8C), corresponding to a marker size of about 200 kDa. Importantly, both GM variants were found in these fractions already after the 5 minute pulse (not shown), suggesting that complex formation is rapid. It is difficult to correctly predict the size of the GM complexes from density gradients because the marker proteins are all globular, whereas oligomerization of the GM constructs may lead to fibrillar structures via the coiled-coil domains. Density gradient analysis may, therefore, underestimate the actual size of the GM complexes. Nevertheless, these experiments show that ERGIC-53 forms complexes even in the absence of intermolecular disulfide bonds. We confirmed the disulfide-bond-independent oligomeric state of the GM constructs by chemical cross-linking of membrane fractions in conjunction with SDS-PAGE (not shown).

Discussion

This study provides the first complete map of ER export determinants of a type I membrane protein. We show that cytoplasmic targeting motifs interacting with the COPII budding machinery are not sufficient for correct trafficking of ERGIC-53 unless they are presented in an oligomeric form. Oligomerization is mediated, in turn, by the cooperation of luminal and TMD determinants.

The minimal cytosolic determinants of ERGIC-53 are the penultimate F509 known to mediate COPII binding (Nufer et al., 2002) and Q501 (Fig. 9A). F509 is conserved in ERGIC-53 of most species except in yeast and Dictyostelium (Fig. 9B). In the ERGIC-53 homolog of Schizosaccharomyces pombe the phenylalanine is replaced by a tyrosine, and in Emp47p and Emp46p of Saccharomyces cerevisiae two leucines are present.
Fig. 8. ERGIC-53 is present in a complex of high density, irrespective of the presence or absence of intermolecular disulfide bonds. (A) Separation of soluble and membrane-bound marker proteins by rate-zonal centrifugation. Shown is a representative sucrose-density gradient with the position of marker proteins in kDa. The molecular sizes of the membrane proteins CD4 (48 kDa) and sucrase-isomaltase (209 kDa) are in italics. (B) Analysis of sucrose density gradient fractions by SDS-PAGE/fluorography. Lec-1 cells stably expressing GM were pulse-labeled with [35S]-methionine for 5 minutes. After a 60 minute chase, the cells were lysed and cleared lysates were fractionated by sucrose gradient centrifugation. Fractions were immunoprecipitated with anti-myc and immunoprecipitates were separated by nonreducing SDS 4-10% PAGE followed by fluorography. Arrows indicate dimeric (2x) and hexameric (6x) forms. (C) Fractionation of GM with C466A/C475A substitution (fluorogram of a 7% SDS gel). The experiment was performed as in panel B but with Lec-1 cells stably expressing a GM variant, with C466 and C475 mutated to alanines. 1x, monomeric form.

Fig. 9. Targeting determinants are conserved in ERGIC-53 orthologs. (A) Human ERGIC-53. Amino acid sequences of TMD and cytoplasmic domain are shown by a single-letter code. The di-lysine retrieval signal is in bold italic. Residues required for efficient ER exit are in bold. SS, cleavable signal sequence. (B) Partial sequence alignment of ERGIC-53 orthologs and homologs. Shown are the sequences of human ERGIC-53 (residues 453 to 510; HSapiens, SwissProt Acc P49257), and the orthologs and homologs of monkey (Caentiops, Q9TU32), rat (RNorvegicus, Q62902), mouse (MMusculus, Q9D0F3), frog (XLaevis, Q91671), fly (DMelanogaster, Q9V3A8), worm (CElegans, P90913), tunicata (PMisakiesi, Q9GR90), slime mold (DDiscoidum, Q87287), baker’s yeast (SCer proteins Emp47p and Emp46p, P42555 and Q12396) and fission yeast (ScPombe, O42707). The TMD is gray boxed. The conserved di-lysine ER targeting signal interacting with COPI is shown in bold italic, and the conserved ER exit motifs interacting with COPII are underlayed by black box. Residues that contribute to efficient transport are in bold, and the most membrane proximal cystein residue (C475 in human ERGIC-53) responsible for essential dimer stabilization is framed.
in place of the two terminal phenylalanines. We previously showed that a single tyrosine at position –2, as well as two leucines or isoleucines at positions –1 and –2 can functionally substitute for the phenylalanine motif in human ERGIC-53 (Nufer et al., 2002). Although the di-leucine motif is not essential for the transport of yeast Emp47p (Schoroder et al., 1995), its substitution in Emp46p results in the loss of ER exit (Sato and Nakano, 2002). A leucine-isoleucine motif, as found in Dictyostelium, has not been experimentally tested but probably also functions as an ER export determinant.

Transport efficiency of ERGIC-53 carrying only F509 in a polyanaline tail was already high, but the additional presence of Q501 further accelerated transport. Given that an immobilized ERGIC-53 tail peptide lacking Q501 showed the same COPII binding in vitro as the wild-type peptide (not shown), we conclude that Q501 is not directly involved in COPII binding but rather assists in the optimal presentation of F509 to the COPII machinery. Q501 is not strictly conserved among ERGIC-53 proteins (Fig. 9B). In these instances an optimal presentation of the F509 determinant may depend on the overall hydrophilicity and structure of the tail, rather than on a single amino acid.

A significant conclusion from the present study is that efficient ER export requires an optimal TMD length, 21 amino acids being most efficient. Lengthening the TMD of C-terminally anchored ER proteins results in release from the ER (Rayner and Pelham, 1997; Bulbarelli et al., 2002). Our experiments with the type 1 membrane protein ERGIC-53 anchored by poly-leucine TMDs confirm and extend this observation. They show that TMDs longer than 21 residues progressively slow down export. Moreover, while it was unclear in previous studies if reduced retention in the ER was indirectly due to movement of some sequence-specific residues relative to the boundaries of the lipid bilayer rather than membrane length, our results with poly-leucine TMDs show that membrane length is indeed an important factor. The requirement for optimal TMD length may reflect the fact that a TMD stretch of 21 residues can most efficiently be diverted to lipid domains active in COPII vesicle formation. In fact, the lipid composition at budding sites changes on activation of enzymes involved in lipid metabolism (Liscovitch et al., 1994; Ohashi et al., 1995). This change may alter membrane thickness and thereby lead to the exclusion of membrane proteins with short TMDs from budding vesicles. The relatively short 18 residues in the TMD of ERGIC-53 may aid in retaining monomeric ERGIC-53 in the ER until it is correctly oligomerized. Interestingly, CD4, a protein not known to form oligomers, has an optimal TMD length of 21 amino acids.

Our findings that transport of ERGIC-53 was reduced when its TMD was replaced by artificial TMDs of the same length led to the discovery of the noncontiguous transport determinant F484/Q488/Y498. This motif positioned on one side of the α-helix was found to be required for efficient hexamORIZATION but not dimerization, as observed indirectly by analyzing the extent of disulfide-linked oligomers formed. Dimerization may be mediated by the coiled-coil domains of the stalk. Polar residues, such as glutamine and asparagine, are poorly represented in TMDs (Ulmschneider and Sansom, 2001), most probably because of energetic constraints, but, when present, they tend to be more conserved than in aqueous environments, suggesting structural or functional roles (Jones et al., 1994; Tourasse and Li, 2000). Although polar side-chain-driven membrane association is quite common for multispanning membrane proteins, polar residues are virtually absent from TMDs of single-spanning membrane proteins. Studies with model transmembrane helices show that an asparagine located in the middle of the single TMD, as well as other residues with two polar atoms, can drive strong transmembrane helix homooligomerization via interhelical bonding (Choma et al., 2000; Zhou et al., 2000; Gratkowski et al., 2001; Zhou et al., 2001). Interestingly, a high tendency for helix trimerization was observed when a glutamine was present in the single-spanning TMD. These findings are consistent with our observation that substitution of the glutamine together with two aromates in the TMD of ERGIC-53 reduced the formation of disulfide-linked hexamers, although mutating the Q488 alone did not significantly change transport. F484 and Y498 may contribute to helix stabilization of the TMD (Butterfield et al., 2002), and thereby ensure proper presentation of Q488. Alternatively, the two aromates may contribute to helix-helix packaging and stability (Burley and Petsko, 1985; Eilers et al., 2000; Adamian and Liang, 2001). In support of such a function, double substitution of F484 and Y498 led to a marked reduction in transport.

It was proposed that polar residues within single TMDs provide thermodynamic stability rather than oligomerization specificity (Choma et al., 2000; Zhou et al., 2001). By contrast, our data favor the notion that polar residues determine specificity of oligomerization. ERGIC-53 constructs with an all-leucine TMD stretch oligomerized inefficiently and formed aggregates unless the F484/Q488/Y498 motif was reintroduced.

To our knowledge, ERGIC-53 is the first example of a normal single-spanning membrane protein for which the requirement of a polar residue within TMD for proper oligomerization has been established. Previous mutagenesis studies with a CD8-p24 reporter noted the requirement of TMD glutamine for efficient ER export but its relationship to oligomerization was not studied (Fiedler and Rothman, 1997). In vitro experiments pointed to a role of polar side chains within hydrophobic stretches of α-helical TMDs for oligomerization (Choma et al., 2000; Zhou et al., 2000; Gratkowski et al., 2001; Zhou et al., 2001), but its in vivo relevance remained speculative. An interesting case of polar side-chain-driven TMD association in pathophysiology is a lethal mutation of a valine to a glutamate in the TMD of the neu oncogene that results in specific dimer formation and constitutive activation of this protein (Bargmann et al., 1986; Weiner et al., 1989; Hynes and Stern, 1994).

Unlike Q488, the aromatic residues of the motif are not strictly conserved among ERGIC-53 orthologs, although at least one such residue is present at the C-terminal end of the TMD near the lipid-water interface in most orthologs (Fig. 9B). When comparing the predicted structures of the TMDs, a slightly stronger helix potential is observed for the orthologs lacking the aromates. In these instances, the Q488 may be presented already in an optimal way and helix stabilization may no longer be required. None of the yeast ERGIC-53 homologs bears the glutamine or another residue with two polar atoms within the TMD. These TMDs exhibit the same hydrophobicity as human ERGIC-53 but have a higher potential for helix formation. The yeast proteins contain
predicted coiled-coil regions in their luminal stalk, but lack the conserved cysteine residues that are required for disulfide-linked oligomer formation. It is not known if the yeast homologs oligomerize. We assume they do, given that the putative cytoplasmic ER export motifs of yeast (that is a single tyrosine or two leucines) are not active in the monomeric reporter CD4 (Nufer et al., 2002). Interestingly, Sato and Nakano (Sato and Nakano, 2002) reported that the aromatic residues YYT/ MF in the TMD of Emp47p and Emp46p (Fig. 9B) are required for efficient transport. Although substitution of the analogous GYIMY motif in the TMD of human ERGIC-53 (Fig. 9B) had no influence on transport (not shown) it is possible that the YYT/ MF motif contributes to oligomerization of the yeast proteins.

It seems confusing that the TMD of CD4 was more efficient in mediating transport of chimeric ERGIC-53 than a leucine stretch of the same length. The TMD of CD4 does not carry any known transport motif or polar side chain. However, it has a Gx, xG motif known to promote helix-helix association in other single-spanning membrane proteins (Brosig and Langosch, 1998; Ubarretxena-Belanda and Engelman, 2001). In our chimera this motif may stabilize hexamers in a similar way as F484/Q488/Y498 motif stabilizes the authentic TMD of ERGIC-53. The wild-type CD4 protein, however, does not oligomerize where exogenously expressed (Fig. 8A). This is probably because the TMD alone is not sufficient and it also explains why CD4 reporters with the tail of ERGIC-53, or even with both the tail and TMD of ERGIC-53, are not efficiently transported. Efficient transport of ERGIC-53 required disulfide-linked oligomerization mediated by the most membrane proximal cysteine (C475), whereas disulfide-linked hexamer stabilization was not essential. It appears that TMD interaction can sufficiently stabilize the hexamers. We propose that stabilization of dimers by C475 may be a prerequisite for hexamerization mediated by TMD. Accordingly, the cysteine residue is conserved among ERGIC-53 proteins (Fig. 9B), with the exception of yeast and Dictyostelium homologs, which do not have a corresponding cysteine. Our sucrose-density gradient analysis showed that an ERGIC-53 construct unable to form intermolecular disulfide bridges is present in a large gradient. This suggests that oligomerization does not suffer from these limitations. We propose that transport competence of membrane proteins, in many cases, is achieved by the glycericolic presentation of an ER export motif leading to efficient recruitment of the protein into budding vesicles by interaction with COP II coat proteins.

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References


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