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Targeting of Protein ERGIC-53 to the ER/ERGIC/*cis*-Golgi Recycling Pathway

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Abstract. ERGIC-53 is a lectin-type membrane protein that continuously recycles between the ER, ER–Golgi intermediate compartment (ERGIC) and the *cis*-Golgi. To identify the targeting signals that mediate this recycling, N-glycosylated and myc-tagged variants of ERGIC-53 were constructed. By monitoring endoglycosidase H resistance, we measured the loss from the ER–ERGIC-*cis*-Golgi cycle of ERGIC-53. A domain exchange approach with the plasma membrane reporter protein CD4 showed that the transmembrane and the lumenal domains are not sufficient, while the cytoplasmic domain of ERGIC-53 is required and sufficient for pre-*medial*-Golgi localization. However, the ERGIC-53 cytoplasmic domain on CD4 lead to increased ER-staining by immunofluorescence microscopy indicating that this domain alone cannot provide for unbiased recycling through the ER–ERGIC-*cis*-Golgi compartments. Complete progress through the ER–ERGIC-*cis*-Golgi recycling pathway requires the cytoplasmic domain acting together with the lumenal domain of ERGIC-53. Dissection of the cytoplasmic domain revealed a COOH-terminal di-lysine ER-retrieval signal, KKFF, and an RSQQE targeting determinant adjacent to the transmembrane domain. Surprisingly, the two COOH-terminal phenylalanines influence the targeting. They reduce the ER-retrieval capacity of the di-lysine signal and modulate the RSQQE determinant.

LOCALIZATION of resident membrane proteins in the secretory pathway is achieved by two targeting mechanisms: protein retention and retrieval of transported proteins from a later compartment (Pelham and Munro, 1993; Nilsson and Warren, 1994).

Protein retention describes a process by which proteins are excluded from incorporation into transport vesicles. Such a process has been reported for Golgi membrane proteins: the M-protein of avian coronavirus forms large homooligomers in the *cis*-Golgi via its transmembrane domains leading to large aggregates unable to enter the coated bud (Swift and Machamer et al., 1991; Machamer et al., 1993; Weisz et al., 1993). A similar type of aggregate was proposed to form between glycosylation enzymes of the Golgi apparatus which are known to be retained by their *trans*membrane domains (Nilsson et al., 1991; Munro, 1991; Aoki et al., 1992; Burke et al., 1992; Colley et al., 1992; Russo et al., 1992; Tang et al., 1992; Teasdale et al., 1992; Wong et al., 1992). Since the concentration of Golgi glycosylation enzymes cannot reach that of a viral protein, Golgi enzymes are believed to form large heterooligomeric complexes by kin recognition (Nilsson et al., 1993). Although a direct heterooligomeric interaction among Golgi proteins remains to be shown, the kin recognition hypothesis is supported by the observation that artificial retention in the ER of the *medial*-Golgi enzyme GlcNAc-transferase results in the retention of another *medial*-Golgi enzyme, mannosidase II (Nilsson et al., 1994).

Localization by protein retrieval is a fundamentally different process. Here, membrane proteins enter budding vesicles and are transported to the next compartment, where they are recognized by a receptor and sorted into vesicles destined for recycling to the compartment of origin (Pelham and Munro, 1993). Such a process was described for type I membrane proteins of the ER that contain a COOH-terminal di-lysine ER-retrieval signal, KKXX or KXKXX, in their cytoplasmic domain (Jackson et al., 1990). In mammalian and in yeast cells these proteins can leave the ER and recycle from a post-ER compartment (Jackson et al., 1993; Gaynor et al., 1994; Townsley et al., 1994). Di-lysine signals were shown to bind coatomer in vitro (Cosson and Letourneur, 1994) and mutations in yeast coatomer subunits effect the intracellular retention of di-lysine proteins in vivo (Letourneur et al., 1994). These findings support a coatomer-mediated retrieval mechanism for di-lysine ER proteins.

The mannose-specific lectin ERGIC-53 (Schindler et al., 1993; Arar et al., 1995) is predominantly associated with the ER–Golgi intermediate compartment (ERGIC)[1] (Schwei-

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1. *Abbreviations used in this paper:* ERGIC, ER–Golgi intermediate compartment; endo H, endoglycosidase H.

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zer et al., 1988, 1990, 1991). Due to continuous recycling, ERGIC-53 and its rat homologue p58 (Saraste and Svensson, 1991) are also present at lower concentrations in the rough ER and in the first fenestrated *cis*-cisternae of the stacked Golgi (Foguet, M., and H.-P. Hauri, unpublished observation; Schweizer et al., 1988; Chavrier et al., 1990). ERGIC-53 is a 53-kD nonglycosylated type I transmembrane protein that assembles into equal amounts of disulfide-linked homodimers and homohexamers (Schweizer et al., 1988; Schindler et al., 1993). It has a large lumenal domain exhibiting homology to leguminous lectins and mammalian galectins, a single transmembrane segment, and a short 12–amino acid long cytoplasmic domain (Schindler et al., 1993; Fiedler and Simons, 1994; Arar et al., 1995). The cytoplasmic domain contains a COOH-terminal tetrapeptide, KKFF, matching the consensus for a di-lysine ER-retrieval signal.

We show that targeting of ERGIC-53 cannot be explained by a simple retrieval mechanism, since proper localization required the lumenal and the cytoplasmic domains. The cytoplasmic domain possesses three determinants all of which are required for correct targeting within the ER–ERGIC-*cis*-Golgi recycling pathway: a functional di-lysine ER-retrieval signal, KKFF, a targeting determinant adjacent to the membrane, RSQQE, and two COOH-terminal phenylalanines that reduce the retrieval efficiency of the di-lysine signal and modulate the RSQQE targeting determinant. Thus the ER-retrieval signal, although critical for retaining ERGIC-53 in the ER–ERGIC-*cis*-Golgi recycling pathway, is not sufficient for the correct distribution throughout these compartments. Targeting of ERGIC-53 depends on a complex interplay of multiple determinants.

Materials and Methods

Materials

Monoclonal antibodies used included HP2/6.1 (IgG2A)(Carrera et al., 1987) and 6D10 (IgG1) against human CD4. In addition the following mAb hybridoma supernatants (~10-fold concentrated by ammonium sulfate precipitation) were used: 9E10.2 (IgG1)(Evan et al., 1985) against a c-myc epitope (ATCC CRL 1729; American Type Culture Collection, Rockville, MD), G1/93 (IgG1) against ERGIC-53 (Schweizer et al., 1988), G1/221 (IgG1) against human transferrin receptor, G1/296 (IgG12A) against p63 (Schweizer et al., 1993), G1/133 (IgG1) against giantin (Linstedt and Hauri, 1993). [^{35}S]methionine was isolated from *Escherichia coli* grown under sulfur-limited conditions in the presence of [^{35}S]sulfate (New England Nuclear, Boston, MA). Protease inhibitors (used at the following final concentration 1 μg/ml pepstatin, 1 μg/ml aprotitin, 0.5 μg/ml leupeptin, 1 μg/ml benzamidin, 1 μg/ml antipain, and 0.2 mM phenylmethylsulfonyl fluoride), cycloheximide and nocodazole were from Sigma Chem. Co. (St. Louis, MO).

Recombinant DNAs

The c-myc epitope tagged and N-glycosylated form of ERGIC-53 designated GM is shown in Fig. 1. The DNA sequence coding for the c-myc epitope EQKLISEEDL was introduced after the signal sequence at the amino acid position 31 of ERGIC-53 by PCR-based splicing (Horton et al., 1989). The PCR amplified DNA was cloned back to the ERGIC-53 cDNA (Schindler et al., 1993) via the SacII site at position 116 leading to P53cmyc. In a second reaction aspartic acid at position 61 of wild-type ERGIC-53 was mutated to asparagine by PCR mutagenesis (Ho et al., 1989) leading to a NGT N-glycosylation consensus site. The PCR-amplified DNA was ligated to the ERGIC-53 cDNA via the BglI restriction site at position 269. This construct was termed P53G1. The relegated DNA was treated with Klenow and cloned into the EcoRV site of Bluescript SK vector (Stratagene, La Jolla, CA). Both constructs were cloned into pECE to test for expression in COS cells. To create the final GM construct the two mutations were combined by ligating the cmyc tagged 5′-end of P53cmyc via the SacI site at position 116 to P53G1. For expression in COS cells GM was cloned as a SalI/XbaI fragment into the pECE expression vector (Ellis et al., 1986). All mutations were confirmed by DNA-sequencing (Sequenase 2.0; US Biochemical Co., Cleveland, OH).

For further mutagenesis, GM was cloned as a ClaI/XbaI fragment into AccI/XbaI cut pECE. The resulting plasmid was designated pECE*. This cloning step eliminated the single AccI site in pECE so that the AccI site in GM at position 1429 became unique. All further constructs which contained the ERGIC-53 lumenal domain were generated by PCR using 3′-primers with a terminal XbaI restriction site. This allowed direct cloning of the PCR-amplified sequences as AccI/XbaI fragments into pECE* that were then confirmed by sequencing.

Chimeric proteins between ERGIC-53 and human CD4 (Maddon et al., 1985) were constructed by PCR based splicing by overlap extension (Horton et al., 1989). L4T53C53 was entirely generated by PCR introducing a EcoRI site at the 5′- and a BamHI site at the 3′-end of the coding sequence. This construct was fully sequenced. The PCR amplified sequences of all further constructs containing the CD4 lumenal domain were ligated back to the L4T53C53 DNA via either the TaqI site at position 1129 of the human CD4 cDNA or the AvaI site at position 1197. These DNAs were treated with Klenow, ligated into the EcoRV site of Bluescript SK, and confirmed by sequencing. For expression in mammalian cells these constructs were cloned into pCB6 (Brewer and Roth, 1991); CD4, L4T53C53 as EcoR/BamHI fragments, L4T4C53 as an EcoRI and L4T4C53 K(-4)stop as a EcoRI/XbaI fragment.

Cell Culture and Transfection

COS-1 cells were grown in DME supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml fungizone. COS cells were transfected using the DEAE-Dextran method (Cullen, 1987). 2 μg DNA per 35-mm dish was used.

Immunofluorescence

All immunofluorescence experiments were carried out in eight-well multichamber glass slides (Miles Labs, Naperville, IL). 42–48 h after transfection the slides were fixed with paraformaldehyde and processed for indirect immunofluorescence. Saponine-permeabilized cells were stained as described (Schweizer et al., 1988). Primary antibody dilutions were as follows: anti-ERGIC-53 (G1/93) 1:50 for endogenous ERGIC-53; 1:2,000 for overexpressed ERGIC-53; anti–c-myc epitope (9E10.2) 1:50; anti human CD4: HP2/6.1 1:150 (10× concentrated culture supernatant), HP2/6.1 ascites 1:4000, 6D10 1:50; anti-giantin (G1/133) 1:50; anti-p63 (G1/296) 1:50; anti-transferrin receptor (G1/221) 1:50.

For surface staining of ERGIC-53 and mutants the cells were cooled to 4°C for 20 min followed by an incubation with G1/93 or 9E10.2 at a 1:10 dilution in 250 μl culture medium for 30 min. After six washes with culture medium the cells were fixed with 3% paraformaldehyde, pH 8.3, for 30 min. Thereafter the cells were rewarmed to room temperature, fixed for another 30 min, washed twice with PBS and twice with PBS containing 20 mM glycine, and then permeabilized for 20 min with PBS-glycine containing 0.1% saponine. The cells were then subjected to indirect immunofluorescence microscopy as described below. For differential staining of the cell surface and intracellular structures of CD4 and CD4/ERGIC-53 chimeric proteins the following double-immunofluorescence protocol was applied. The cells were cooled to 4°C for 20 min followed by an incubation with mAb 6D10 at a dilution of 1:10 in culture medium for 30 min. After six washes with culture medium the cells were reincubated with IgG1-specific FITC-labeled affinity-purified goat anti–mouse secondary antibody (Cappel, West Chester, PA) for 30 min, washed again six times with culture medium, and fixed with 3% paraformaldehyde, pH 8.3, for 30 min. Thereafter the cells were rewarmed to room temperature, fixed for another 30 min, washed twice with PBS and twice with PBS containing 20 mM glycine, and then permeabilized for 20 min with PBS-glycine containing 0.1% saponine. The cells were then incubated with HP2/6.1 at a 1:150 dilution in PBS-saponine for 30 min, washed four times with PBS-saponine, and incubated with rhodamine (TRITC)-labeled IgG2a-specific affinity-purified goat anti–mouse secondary antibody (Cappel) for 30 min. After four final washes with PBS-saponine the cells were mounted in 90% glycerol, 10% PBS, and 0.1 mg/ml phenylenediamine. The cells were analyzed by a Reichert Polyvar immunofluorescence microscope.

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Pulse–Chase Experiments and Immunoprecipitation

42 h after transfection the cells were washed once with PBS, incubated in labeling medium (PBS, 20% dialyzed FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1% nonessential amino acids) for 15 min at 37°C and pulsed for 5 min at 37°C with 20 μCi [^{35}S]methionine in 200 μl labeling medium per 35-mm dish. The cells were washed once with cold DMEM containing 10 mM methionine and then chased in DMEM containing 10 mM methionine at 37°C. The dishes were transferred to ice and all the subsequent steps were carried out on ice. The cells were washed twice with PBS, once with 100 mM sodium phosphate, 1% bovine serum albumin, pH 8, scraped off with a rubber policeman in 1 ml solubilization buffer (100 mM sodium phosphate, 1% Triton X-100, pH 8, and protease inhibitors), and passed five times through a 25-gauge needle. After incubation for 60 min the lysate was centrifuged at 100,000 *g* for 60 min, the resulting supernatant was incubated with either 2 μl G1/93 ascites, 20 μl 9E10.2, or 20 μl HP2/6.1 for 60 min followed by an incubation with 2 μl rabbit anti-mouse IgG (Cappel) for 30 min. Immunocomplexes were isolated by adding 10 μl protein A–Sepharose (Pharmacia, Uppsala, Sweden), rotated over night at 4°C, washed four times with solubilization buffer, once with 100 mM sodium phosphate, pH 8, and once with 10 mM sodium phosphate, pH 8. Proteins were eluted from the protein A beads by boiling in electrophoresis sample buffer. For nonreduced samples 4–10%, for reduced samples 7–10% SDS–polyacrylamide gradient slab gels (Laemmli, 1970) were run, salicylated, and visualized by fluorography using Kodak X-omat AR or Fuji RX films.

Endoglycosidase H Digestion

Immunoprecipitates were released from the protein A–Sepharose beads by boiling for 3 min in 30 μl 200 mM Na-citrate, pH 5.5, 2% SDS in the presence of protease inhibitors, frozen at −20°C for 30 min, and boiled again for 3 min. Then the samples were diluted with an equal volume of distilled water containing protease inhibitors and incubated with 4 mU endoglycosidase H (endo H) for 22 h at 37°C.

Results

Characterization of the Epitope-tagged and N-glycosylated ERGIC-53

ERGIC-53 has no posttranslational modifications that would allow to monitor changes in intracellular localization (Schweizer et al., 1988). To study loss of pre-*medial*-Golgi targeting directly we constructed a N-glycosylated, epitope-tagged variant of ERGIC-53 (Fig. 1, *GM*). We used this construct to measure ER to *medial*-Golgi transport rates in pulse–chase experiments based on the acquisition of endo H resistance of its glycan moiety. Acquisition of endo H resistance is a *medial*-Golgi event, and therefore reflects the fraction of ERGIC-53 that has escaped the ER–ERGIC-*cis*-Golgi cycle.

To determine if ERGIC-53 (GM) behaves identical to overexpressed wild-type ERGIC-53, COS-1 cells were transiently transfected with the corresponding DNAs and analyzed by pulse-labeling with [^{35}S]methionine followed by immunoprecipitation, or by indirect immunofluorescence microscopy. The introduced c-myc epitope tag was recognized by the epitope-specific mAb 9E10 (Evan et al., 1985) both in indirect immunofluorescence experiments (Fig. 2, *e* and *f*) and immunoprecipitation (Fig. 3). In pulse–chase experiments GM and overexpressed wild-type ERGIC-53 displayed virtually identical expression levels and oligomerization (Fig. 3), and the localization revealed by immunofluorescence microscopy indicates that the modifications did not alter the intracellular distribution compared to overexpressed wild type (Fig. 2). However, both overexpressed proteins showed a staining of the cell periphery

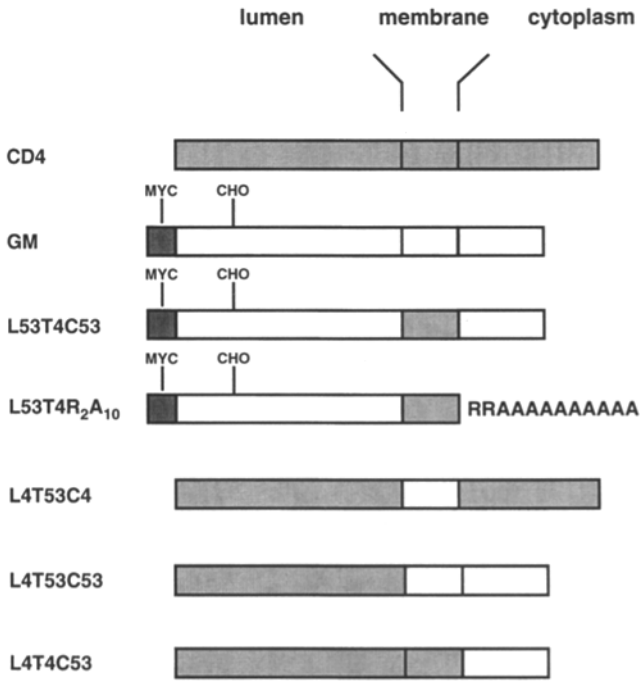

Figure 1. Schematic representation of ERGIC-53 domain-exchanged constructs. ERGIC-53 constructs contain a N-glycosylation site introduced at amino acid position 61 of ERGIC-53, (*CHO*), and a c-myc epitope tag introduced after the signal sequence cleavage site, (*MYC*). The amino acid sequence of the cytoplasmic domain of the ERGIC-53 constructs is given in the single letter code. *L*, lumenal domain; *T*, *trans*-membrane domain; *C*, cytoplasmic domain; *4*, CD4; *53*, ERGIC-53. The transmembrane domain T53 consists of the hydrophobic residues 481-498 of ERGIC-53 (Schindler et al., 1993) and T4 comprises the hydrophobic residues 375-395 of human CD4 (Maddon et al., 1985).

typical for ER, and led to a comparable fraction of transfected cells exhibiting surface staining (compare Fig. 2, *c* and *d* with *e* and *f*). Cells expressing identical levels of ERGIC-53 or GM bound equal amounts of radio-iodinated mAb to ERGIC-53 suggesting identical loss of both proteins from the ER–ERGIC-*cis*-Golgi recycling pathway (data not shown). After immunoprecipitation of GM with mAb 9E10 against the c-myc epitope, digestion with endo H confirmed that the introduced N-glycosylation consensus site was glycosylated and that the oligomerization state of GM was identical to that of ERGIC-53 (Fig. 3). The kinetics of oligomerization of endogenous ERGIC-53 in nontransfected cells and that of overexpressed GM or ERGIC-53 in transfected cells was identical (not shown), indicating that the overall folding of GM is indistinguishable from ERGIC-53, and overexpressed ERGIC-53 and GM showed the same half-life in pulse-chase experiments (not shown). In Fig. 3 hardly any endogenous ERGIC-53 is coprecipitated with GM demonstrating that disulfide-linked heterooligomers between GM and endogenous ERGIC-53 should not influence the interpretation of a mutational analysis. We conclude that overexpression of the GM construct in COS-1 cells fulfills the criteria required for a mutational analysis of the targeting determinant(s) of ERGIC-53.

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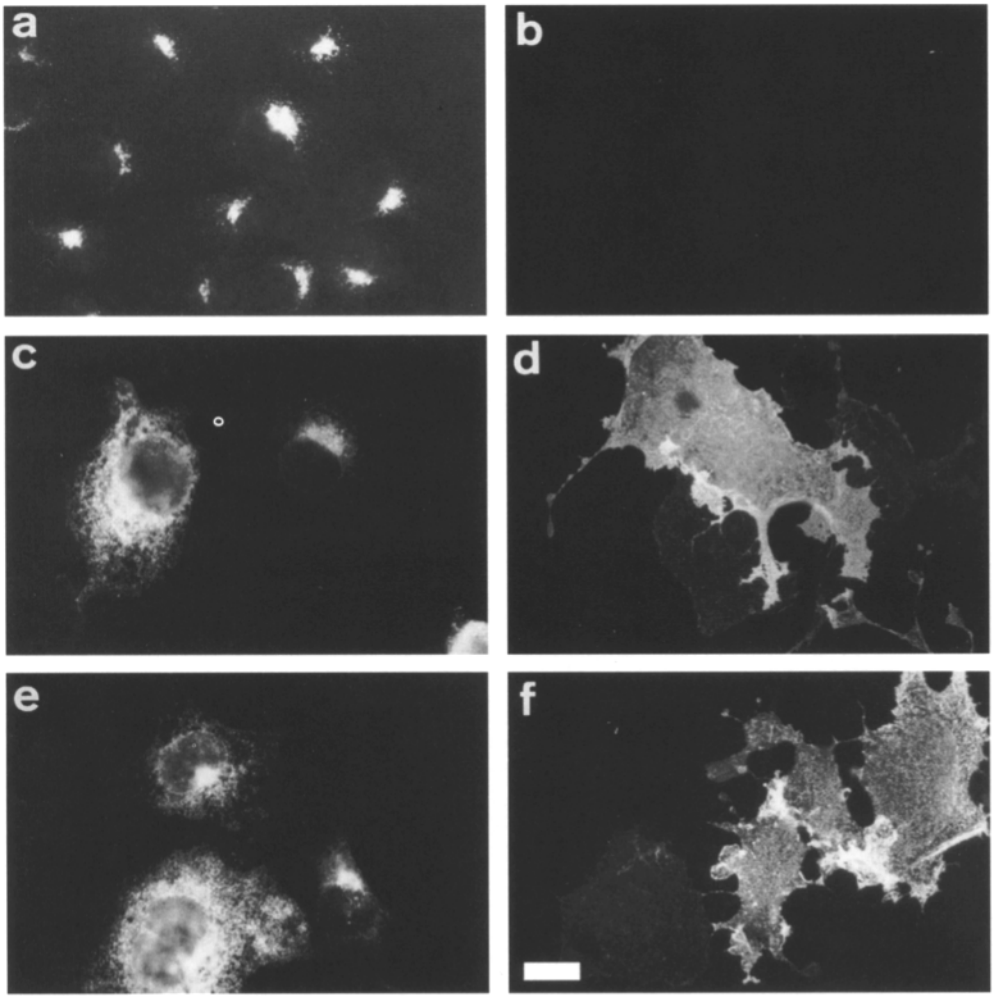

Figure 2. Immunofluorescence localization of overexpressed ERGIC-53 and GM. COS cells were mock-transfected (*a* and *b*), or transfected with ERGIC-53 cDNA (*c* and *d*) or GM cDNA (*e* and *f*). 44 h after transfection the cells were stained before fixation and permeabilization at 4°C (*b*, *d*, and *f*), or after fixation and permeabilization (*a*, *c*, and *e*) using mAbs G1/93 against ERGIC-53 or 9E10 against GM (mAb concentrations: *a*, G1/93 1:50; *b*, G1/93 1:10; *c*, G1/93 1:2,000; *d*, G1/93 1:10; *e*, 9E10 1:50; *f*, 9E10 1:10) followed by goat anti-mouse FITC. Note that both cells in *c* are transfected, endogenous ERGIC-53 is not stained at this dilution of mAb G1/93. Overexpression of ERGIC-53 or GM leads to cell surface expression in some cells whereas all of the endogenous ERGIC-53 is intracellular in mock-transfected cells. The intracellular patterns of overexpressed ERGIC-53 and GM are very similar. Bar, 10 μm.

Neither the Lumenal Nor the Transmembrane Domain Is Sufficient for Pre–medial-Golgi Localization

Within the secretory pathway there is precedence for targeting mediated by lumenal, transmembrane, and cytoplasmic domains acting alone or in combination (Nilsson et al., 1989; Munro, 1991; Sweet and Pelham, 1992; Ponnambalam et al., 1994; Schweizer et al., 1994). To test the contribution to targeting of each domain of ERGIC-53 we have chosen human CD4, a monomeric, N-glycosylated plasma membrane protein as a reporter. To test sufficiency for correct targeting of the lumenal domain of ERGIC-53 we replaced its transmembrane with that of CD4 and substituted the cytoplasmic domain with alanines (Fig. 1., *L53T4R$_2$A$_{10}$*). Targeting sufficiency of the transmembrane domain was tested by replacing the transmembrane domain in CD4 by that of ERGIC-53 (Fig. 1, *L4T53C4*). The requirement for the transmembrane domain in ERGIC-53 targeting was addressed by replacing the transmembrane domain with that of CD4 (Fig. 1, *L53T4C53*).

COS cells transfected with GM, L53T4C53 and L53T4R$_2$A$_{10}$ were pulsed for five min with [^{35}S]methionine and chased for up to 2 h. The immunoprecipitated mutant proteins were digested with endo H and the fraction of resistant protein determined (Fig. 4 *a*). GM representing overexpressed wild-type ERGIC-53 showed ~10% endo H resistance after 1 h of chase (Fig. 4 *a*). This value was identical to that obtained with an ERGIC-53 mutant containing the N-glycosylation site but no epitope tag (not shown). The replacement of the transmembrane domain in ERGIC-53 by CD4 (L53T4C53) did not change the acquisition of endo H resistance, suggesting that it is not required for targeting (Fig. 4 *a*). The inverse construct with the ERGIC-53 transmembrane domain in a CD4 background L4T53C4 showed similar kinetics of endo H resistance (Fig. 4b) and comparable localization by immunofluorescence (not shown) as overexpressed wild type CD4. This indicated that the transmembrane domain of ERGIC-53 was not sufficient for the targeting of ERGIC-53. Substitution of both the transmembrane and the cytoplasmic domains (L53T4R$_2$A$_{10}$) resulted in ~50% endo H resistance after 1 h of chase (Fig. 4 *a*). The lumenal domain of ERGIC-53 is not sufficient for targeting.

The Cytoplasmic Domain Is Sufficient for Pre–medial-Golgi Localization of a Reporter Protein

The above results strongly argued for a role of the cytoplasmic domain in intracellular targeting of ERGIC-53, and to analyze this directly two CD4-ERGIC53 constructs were generated: L4T4C53, containing only the cytoplasmic domain, and L4T53C53 containing the cytoplasmic and the transmembrane domain of ERGIC-53 (Fig. 1). Transfected

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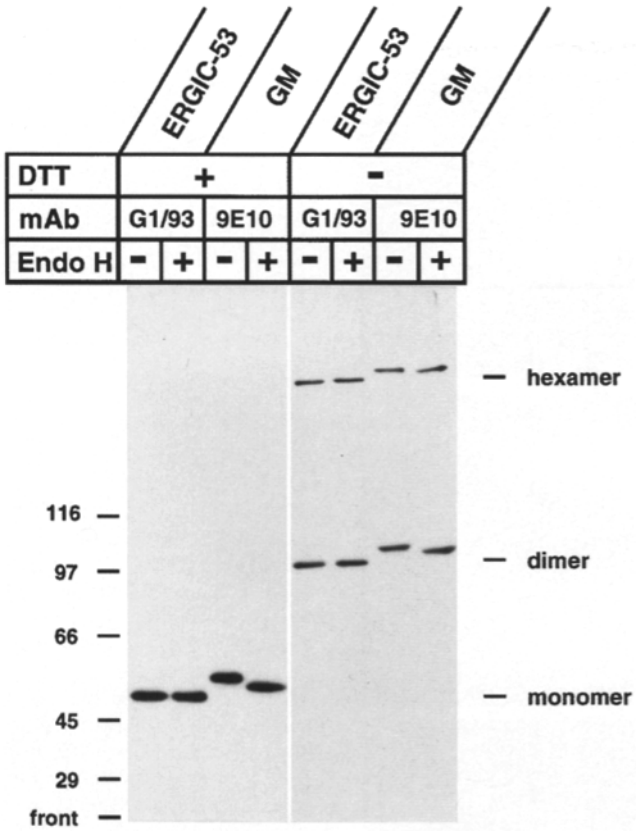

Figure 3. C-myc epitope tag and N-glycosylation site are functional in modified ERGIC-53 (*GM*) and do not interfere with oligomerization. COS cells were transfected with cDNAs encoding ERGIC-53 or GM. 44 h after transfection the cells were labeled for five min with [^{35}S]methionine followed by a chase of 15 min, and then subjected to immunoprecipitation with the indicated mAbs (G1/93 against ERGIC-53, 9E10 against the c-myc epitope). Where marked the samples were treated with endo H to remove N-linked high-mannose glycans and analyzed by SDS-PAGE (4–10% slab gel) under reducing (*DTT+*) or nonreducing (*DTT−*) conditions, followed by fluorography. Note that the expression levels of overexpressed ERGIC-53 and GM are identical and that no detectable endogenous ERGIC-53 is precipitated with the c-myc antibody. Numbers at the left margin indicate the position of molecular mass standards in kD.

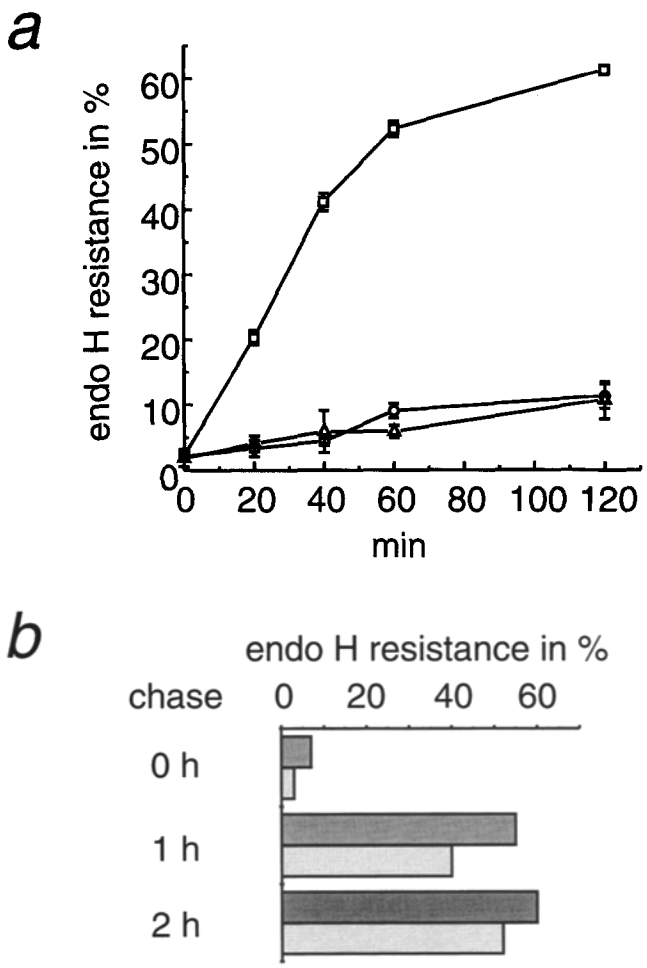

Figure 4. ERGIC-53 lumenal and transmembrane domain are not sufficient for targeting. (*a*) Transfected COS cells were pulsed for five min with [^{35}S]methionine and chased for the indicated times with unlabeled methionine in excess followed by an immunoprecipitation with mAb 9E10 against the c-myc epitope. The precipitates were digested with endo H and analyzed by SDS-PAGE and fluorography. The amount of endo H-resistant protein was determined by laser scanner densitometry of fluorograms. GM (○), L53T4C53 (△), L53T4R$_2$A$_{10}$ (□). The values given are means ± SD of triplicate cultures treated in parallel. (*b*) Transfected COS cells were pulsed for 15 min with [^{35}S]methionine and chased for the indicated times with unlabeled methionine in excess followed by an immunoprecipitation with mAb HP2/6 against CD4. The precipitates were digested with endo H and analyzed as described in *a*. Values are the average of two independent experiments. Dark bars, CD4; Light bars, L4T53C4.

COS cells were analyzed by a double immunofluorescence procedure which allowed differential staining of the cell surface and intracellular membranes (Fig. 5; see Materials and Methods). Overexpressed CD4 showed strong surface staining under nonpermeabilized and a perinuclear intracellular staining under permeabilized conditions. The perinuclear staining most likely represents newly synthesized protein in transit through the Golgi as well as internalized CD4 concentrated in endosomes and/or lysosomes. Overexpressed CD4 chimera L4T53C53 and L4T4C53, on the other hand, showed no surface staining but a strong staining indicative of the ER: reticular intracellular staining with a nuclear ring (Fig. 5, *c–f*). It is important to note that this staining is distinct from an ERGIC pattern (see also Fig. 8 *B*). For L4T4C53, a few cells exhibited surface staining (insert in Fig. 5, *e* and *f*). To confirm these results biochemically, transfected COS cells were pulse–chased and the immunoprecipitates were digested with endo H (data not shown). In contrast to wild-type CD4, the chimeras remained endo H sensitive after prolonged chase times indicating a pre-*medial*-Golgi localization, which is in line with the ER-staining observed in Fig. 5. We conclude that the cytoplasmic domain of ERGIC-53 is sufficient for pre-*medial*-Golgi targeting.

The Cytoplasmic Domain Contains Three Targeting Features

To define the targeting signal(s) in the cytoplasmic domain of ERGIC-53, amino acids were serially truncated from the COOH terminus. Surprisingly, deletion of up to seven amino acids, which eliminated the putative KKXX

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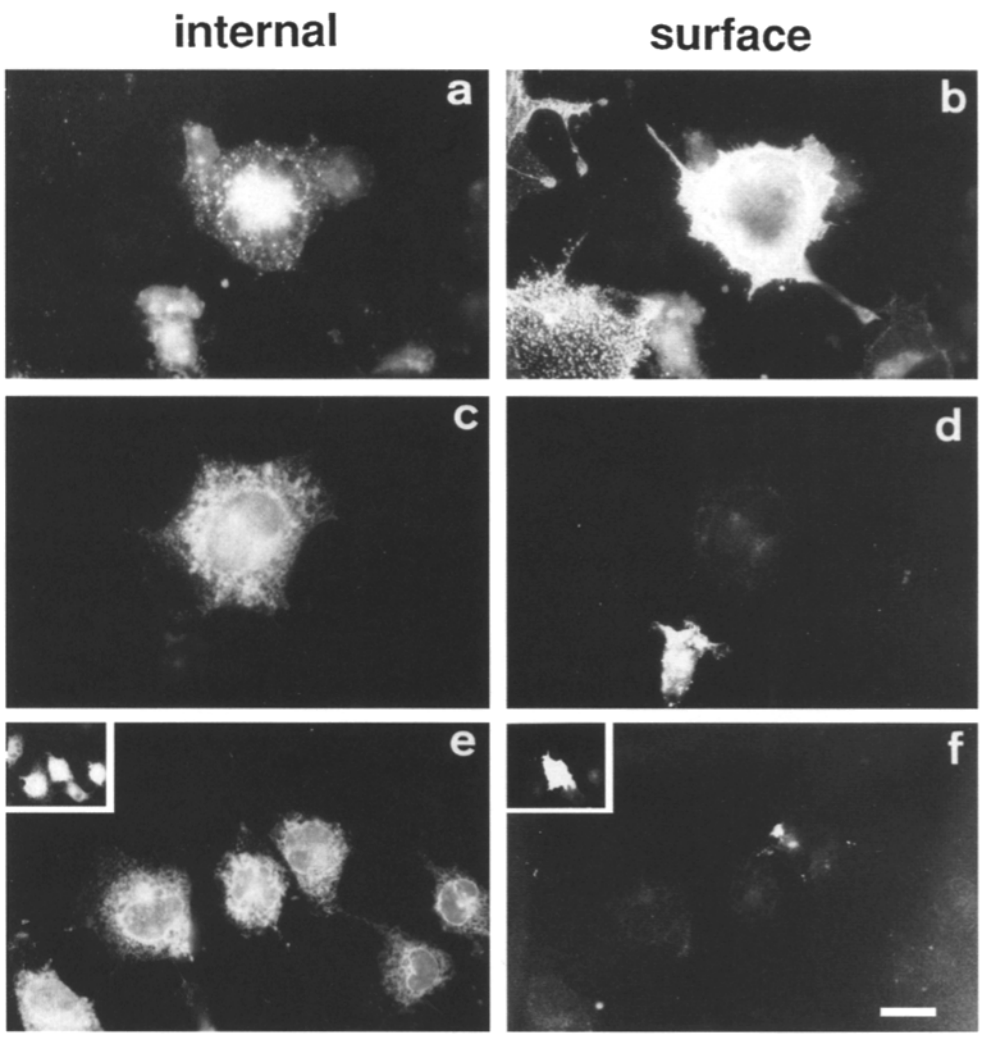

Figure 5. Double-immunofluorescence analysis of surface and intracellular CD4, L4T53C53 and L4T4C53. Transfected COS cells were cooled to 4°C and incubated with mAb 6D10 (IgG1), against CD4, followed by IgG1-specific goat anti-mouse-FITC to stain protein at the cell surface (*b*, *d*, and *f*). The cells were then fixed and permeabilized followed by an incubation with mAb HP2/6.1 (IgG2a), against CD4, and goat anti-mouse IgG2a-specific goat anti-mouse-TRITC staining the intracellular protein (*a*, *c*, and *e*). The DNA's used were CD4 (*a* and *b*), L4T53T53 (*c* and *d*) and L4T4C53 (*e* and *f*). Bar corresponds to 10 μm in *a*, *b*, *c*, *d* and 22 μm in *e* and *f*.

ER-retrieval signal, did not substantially change the rate of endo H resistance compared to wild type (Fig. 6, lines *2*, *3*, *4*, and *6*). These results suggest that the inner five amino acids RSQQE adjacent to the transmembrane domain act as a targeting determinant. To exclude conformational artifacts due to truncation, which might slow down exit from the ER, we restored the authentic tail length by re-adding seven alanines to the RSQQE, and we deleted the KKXX-motif in L53T4C53. Both constructs showed equal endo H resistance and oligomerization equal to wild type, strongly arguing against a truncation artifact (Fig. 6, lines *5* and *7*). We conclude that the five residues RSQQE adjacent to the transmembrane domain constitute a targeting determinant.

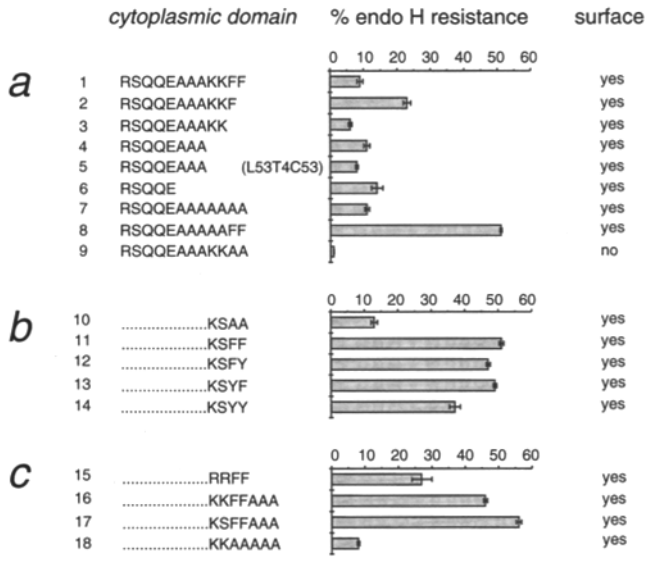

Figure 6. The cytoplasmic domain of ERGIC-53 contains a KKXX-retrieval signal and a second targeting signal adjacent to the membrane. Endo H resistance was determined after 5 min of pulse with [^{35}S]methionine followed by 60 min of chase as described in Fig. 4 *a*. The mutations indicated were all constructed in a GM background, except mutation 5 that was constructed in L53T4C53 (see Fig. 1). The amino acid sequence of the cytoplasmic domain is given in the single letter code with construct 1 representing the wild-type sequence. The values given are means ± SD of triplicate cultures treated in parallel. Surface appearance of the overexpressed proteins was analyzed by indirect immunofluorescence microscopy staining the cell surface with mAb 9E10 at 4°C before fixation and permeabilization as described in Fig. 2. (*a*) The cytoplasmic domain of ERGIC-53 contains two targeting determinants. (*b*) The RSQQE determinant is suppressed by aromates in the -1/-2 position from the COOH terminus. (*c*) Lysine-specificity and position-dependence identify KKFF as a KKXX ER-retrieval signal.

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We next studied the role of the lysines in the cytoplasmic domain of ERGIC-53 (Fig. 6, line *9*). A construct with two correctly positioned lysines (−3/−4 from the COOH terminus) but lacking the terminal phenylalanines did not acquire endo H resistance and was not transported to the cell surface. This construct is significantly different from construct 1 (Fig. 6, *wt*), containing the terminal phenylalanines and from construct 7 (Fig. 6) that contains only the RSQQE determinant. It also confirmed that transport of ERGIC-53 to the Golgi and the cell surface was not simply a question of enforced overexpression but an intrinsic property of the cytoplasmic domain.

Introduction of the two COOH-terminal phenylalanine residues in absence of the lysines showed an unexpected increase in endo H resistance (Fig. 6, line *8*) reflecting comparable rates of transport as shown for $L53T4R_2A_{10}$ and CD4 (Fig. 4). The phenylalanines suppress the targeting mediated by the RSQQE determinant. Comparison of a mutant with one lysine changed to serine, and the phenylalanines substituted by alanines (KSAA) to mutants with phenylalanines or tyrosines present in the −1/−2 position from the COOH terminus (KSFF, KSFY, KSYF, KSYY) showed that terminal phenylalanine/tyrosine residues suppress the RSQQE determinant (Fig. 6, lines *10–14*).

To assess whether the KKFF sequence is a functional di-lysine ER-retrieval signal, we tested two criteria: lysine dependence and position dependence from the COOH terminus (Jackson et al., 1990). Both, the lysine dependence tested by substitution with arginines (Fig 6, line *15*) and the position dependence from the COOH terminus tested by addition of three alanines to the COOH terminus (Fig. 6, lines *16* and *17*, and compare line *9* to *18*) identified the KKFF-tetrapeptide as a di-lysine ER-retrieval signal. We conclude that the cytoplasmic domain of ERGIC-53 contains three targeting determinants: a di-lysine ER-retrieval signal, a RSQQE determinant adjacent to the membrane and two COOH-terminal phenylalanines which influence the RSQQE determinant. It is important to note that the overall folding of all mutants generated in a GM background was controlled by comparing their kinetics of oligomerization to that of ERGIC-53. All mutants shown in this paper oligomerize identical to ERGIC-53.

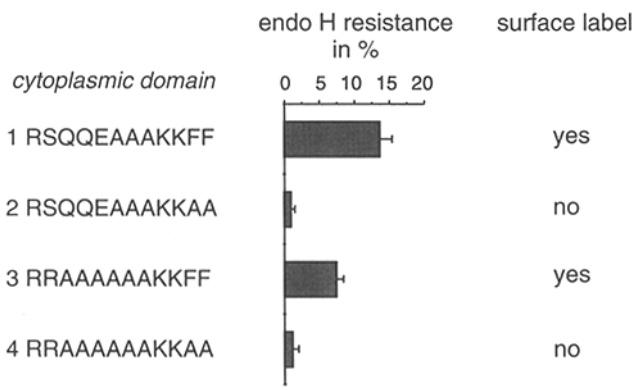

Figure 7. The retrieval capacity of the di-lysine signal is reduced by the phenylalanines. Endo H resistance was determined as in Fig. 5. Chase time was 5 h. All constructs were in a GM background containing the indicated cytoplasmic domains. Construct 1 corresponds to GM, and construct 2 is identical to construct 9 in Fig. 5. The values given are means ± SD of triplicate cultures treated in parallel.

The COOH-terminal Phenylalanines Modulate the ER-retrieval Signal

Besides suppressing the inner targeting determinant do the COOH-terminal phenylalanines also influence the di-lysine signal? Construct 9 in Fig. 6, which had the terminal phenylalanines replaced by alanines in the wild-type cytoplasmic domain, was not transported to the *medial*-Golgi after 5 h of chase (Fig. 7, line *2*). This result, however, did not allow us to conclude that the terminal phenylalanines influence the di-lysine motif since in that construct the inner targeting determinant was not suppressed and could have contributed to the intracellular targeting efficiency (compare Fig. 7, lines *1* and *2*). However, substitution of the phenylalanines in RRAAAAAAKKFF by alanines enabled us to determine the influence of the phenylalanines on the di-lysine motif. The resulting construct with the cytoplasmic amino acid sequence RRAAAAAAKKAA (Fig. 7, line *4*) showed no significant ER to *medial*-Golgi transport after 5 h of chase comparable to RSQQEAAAKKAA (Fig. 7, line *2*). Both constructs showed expression levels identical to GM and displayed no surface staining by immunofluorescence microscopy (data not shown).

We conclude that COOH-terminal phenylalanines reduce the ER-retrieval efficiency mediated by the di-lysine motif.

Correct Targeting of ERGIC-53 Requires the Entire Cytoplasmic Domain and the Presence of the Lumenal Domain

As the endo H assay cannot monitor localization within the ER–ERGIC-*cis*-Golgi cycle we used immunofluorescence microscopy of cells expressing moderate levels of protein to distinguish ER from ERGIC and Golgi. High expression levels lead to saturation of the recycling pathway and result in staining of the cell surface and the endosomal system, a condition that renders a morphological analysis impossible (see also Fig. 2). To determine the role of the cytoplasmic targeting signals in maintaining ERGIC-53 within the ER–ERGIC-*cis*-Golgi cycle, COS cells were transfected with the cDNAs corresponding to the constructs in Fig. 7 with the cytoplasmic domains: RSQQEAAAKKFF (wild type), RSQQEAAAKKAA, RRAAAAAAKKFF, and RRAAAAAAKKAA. 16 h after transfection, corresponding to a threefold increased expression of mutant versus endogenous ERGIC-53 (data not shown), mutant protein was selectively visualized with the mAb against the c-myc epitope. Overexpression of GM with the cytoplasmic sequence RSQQEAAAKKFF lead to the characteristic concentrated perinuclear pattern (Fig. 8 *A*, *a*) observed for endogenous ERGIC-53 (Fig. 8 *B*, *b*), whereas substitution of the terminal phenylalanines by alanines (RSQQEAAAKKAA) lead to a typical ER pattern with a pronounced staining of the nuclear envelope and less concentration in a perinuclear area (Fig. 8 *A*, *b*). This finding emphasizes the importance of the phenylalanines in targeting. Proteins terminating in RRAAAAAAKKFF and RRAAAAAAKKAA were localized to the nuclear envelope and a reticular network in the cell

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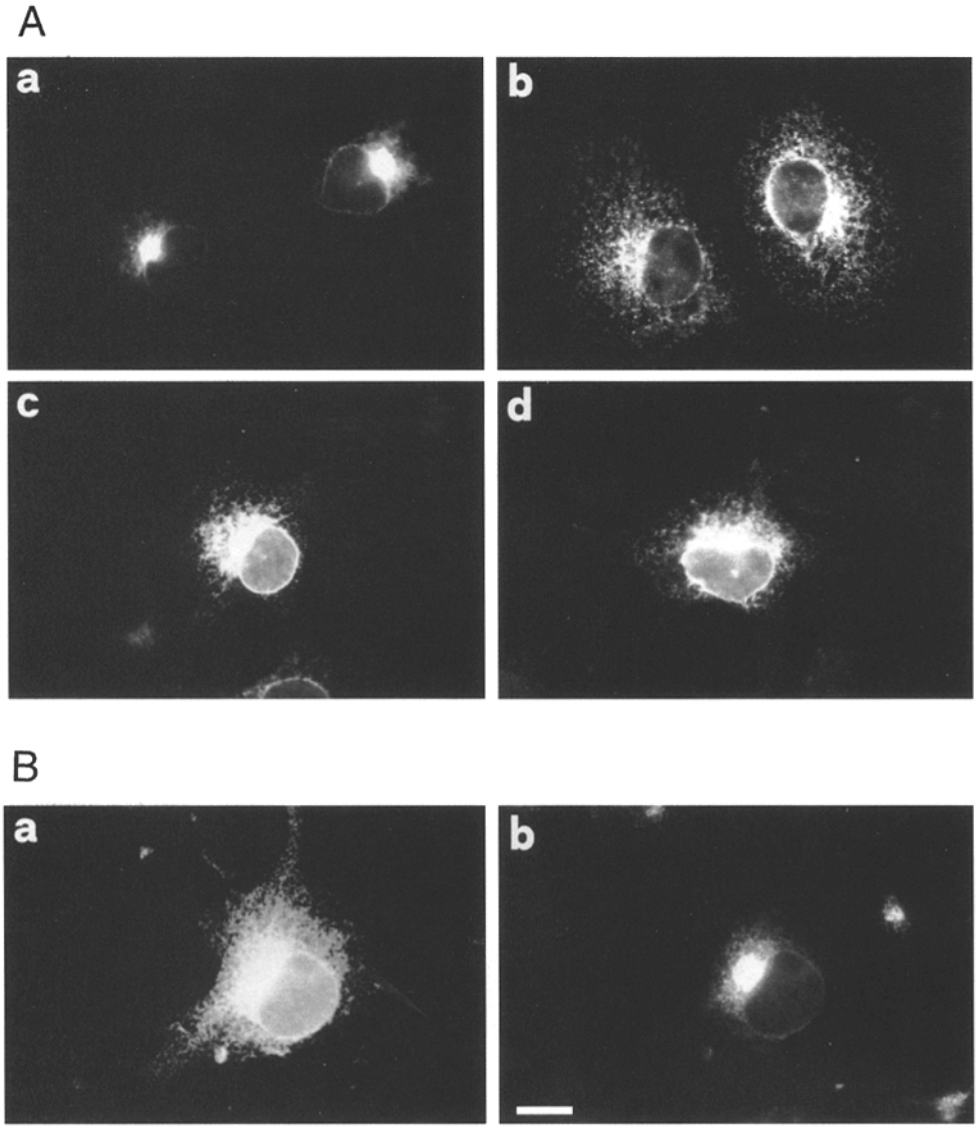

Figure 8. (*A*) Immunofluorescence localization of GM (*a*), RSQQEAAAKKAA (*b*), RRAAAAAAKKFF (*c*), RR-AAAAAAKKAA (*d*). (*B*) Double immunofluorescence localization of L4T4C53 (*a*) with endogenous ERGIC-53 (*b*). Transfected COS cells were subjected to indirect immunofluorescence 16 h after transfection. Staining was as described in Fig. 2 with mAbs 9E10 (*A*, *a–d*) against the c-myc-epitope, HP2/6 (*B*, *a*) against CD4 and G1/93 (*B*, *b*) against endogenous ERGIC-53. Bar, 10 μm.

periphery (Fig. 8 *A*, *c* and *d*). The finding that neither of the two ER-retrieval signals alone was capable of mediating wild-type targeting showed the requirement for the RSQQE determinant for targeting. Therefore, ERGIC-53 localization requires all three features in the cytoplasmic domain of ERGIC-53: a di-lysine ER-retrieval signal, a RSQQE targeting determinant and two COOH-terminal phenylalanines modulating both targeting determinants. That the different mutants show differences in their distribution also argues against binding of the mutants to calnexin or even endogenous ERGIC-53 via their carbohydrate moiety as an explanation for their targeting within the ER–ERGIC-*cis*-Golgi cycle.

We have shown that virtually the entire cytoplasmic domain is required for targeting of ERGIC-53, but is the cytoplasmic domain of ERGIC-53 also sufficient for correct targeting of a reporter protein within the ER–ERGIC-*cis*-Golgi cycle? By double immunofluorescence microscopy the pattern of L4T4C53 was compared with endogenous ERGIC-53 16 h after transfection. L4T4C53 showed mostly an ER pattern with a strong nuclear ring clearly distinct from endogenous ERGIC-53 (Fig. 8 *B*, *a* and *b*), while localization of a construct containing both the cytoplasmic and the lumenal domain of ERGIC-53, L53T4C53, was indistinguishable from GM (not shown). This difference in localization may be due to the reduced influence of the RSQQE determinant in the CD4-chimera L4T4C53. Indeed a CD4-chimera lacking the KKFF-retrieval signal, L4T4C53 (K-4stop), acquired 32% endo H resistance after 1 h of chase (not shown) considerably more than the 12% for the corresponding mutation in L53T4C53 (Fig. 6, line 5). We conclude therefore that the presence of the ERGIC-53 lumenal domain is required for a functional RSQQE determinant.

Discussion

Based on the acquisition of endo H resistance and the localization of ERGIC-53 mutants expressed in COS cells we have established that both the cytoplasmic and the lumenal domain of the protein are required for correct tar-

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geting of ERGIC-53. We have further shown under high and confirmed under low expression conditions that the cytoplasmic domain contains three targeting determinants: a functional di-lysine ER-retrieval signal, a determinant RSQQE adjacent to the membrane and two COOH-terminal phenylalanines that modulate both.

Acquisition of Endo H Resistance Reflects Loss of Intracellular Retention of ERGIC-53

Since endogenous ERGIC-53 has not been found in compartments beyond the *cis*-Golgi we have exploited the acquisition of endo H resistance of the glycosylated ERGIC-53 variant GM as a principal measure for the rate of loss from the ER–ERGIC-*cis*-Golgi system. This assay reveals subtle changes in the overall retention of GM mutants in the pre–*medial*-Golgi recycling pathway, provided the expression levels of the different constructs are comparable, a condition that was met in our experiments. However, the assay does not discriminate between recycling of overexpressed ERGIC-53 (GM) from post-*medial*-Golgi compartments and transport to the cell surface. Three findings argue against a major contribution of recycling from late Golgi sites. First, ER-localized GM in highly overexpressing COS cells failed to costain with FITC-ricin, a lectin recognizing -(1,4)-linked galactose added in the *trans*-Golgi (not shown). Jackson et al. (1993) reported ER staining by Helix pomatia lectin (recognizing terminal GalNAc) and peanut lectin (recognizing terminal Gal-(1,3)-GalNAc) in cells overexpressing an O-glycosylated CD8/E19 chimeric protein possessing a di-lysine ER-retrieval signal. While this result is evidence for recycling of a di-lysine protein via the Golgi apparatus it does not necessarily indicate recycling from the *trans*-Golgi. Helix pomatia lectin recognizes terminal GalNAc added both in the *cis*-Golgi (Roth et al., 1994) and the *trans*-Golgi (Roth, 1984), and peanut lectin binds glycans in the *medial*-Golgi (Roth, 1987). Second, we found a strict correlation between the acquisition of endo H resistance and mislocalization to the cell surface of the different mutant proteins. Third, we have shown that ERGIC-53 contains a functional di-lysine ER-retrieval signal, and a recycling pathway from the ERGIC and the *cis*-Golgi is consistent with the major localization of coatomer, the putative receptor for di-lysine signals (Oprins et al., 1993; Cosson and Letourneur, 1994; Letourneur et al., 1994). Collectively, our data argue against ERGIC-53 recycling from the *trans*-Golgi to biosynthetically earlier compartments. We conclude therefore that the acquisition of endo H resistance reflects loss of intracellular retention of ERGIC-53.

An alternative approach to characterize loss of intracellular targeting would be to record cell surface appearance of overexpressed ERGIC-53 mutants. However, transient overexpression of wild-type ERGIC-53 leads to cell surface mislocalization in 5–10% of the transfected cells (Kappeler et al., 1994). Surface-mislocalized ERGIC-53 protein efficiently internalizes and internalization is strongly affected by mutations in the cytoplasmic domain (Kappeler et al., 1994; Itin et al., 1995). As a consequence the amount of ERGIC-53 at the cell surface is a combined result of loss of intracellular targeting and internalization both of which can be differentially affected by mutations. These considerations invalidate surface appearance of ERGIC-53 mutants as a measure for loss of intracellular targeting.

Targeting of ERGIC-53

We have analyzed the targeting of ERGIC-53 in two steps. First, we identified the signals involved in pre–*medial*-Golgi localization by a biochemical approach under high expression conditions, and second, we tested whether the signals defined by this assay were required for the correct localization of ERGIC-53 within the ER–ERGIC-*cis*-Golgi system. For this purpose we analyzed by immunofluorescence microscopy the distribution of mutant ERGIC-53 proteins expressed at moderate expression levels, which is three times on average the level of endogenous ERGIC-53. This second analysis confirmed that the signals identified under high expression conditions are true targeting determinants and not simply an artefactual result due to excessive overexpression.

Our data indicate that pre–*medial*-Golgi targeting of ERGIC-53 depends to a large extent on the retrieval capacity of the di-lysine signal in its cytoplasmic domain, even though an RSQQE determinant and two COOH-terminal phenylalanines are required for correct localization within the ER–ERGIC-*cis*-Golgi recycling pathway. However, the cytoplasmic domain alone is not sufficient to target a monomeric reporter protein, L4T4C53, identically to endogenous ERGIC-53. A comparison of the acquisition of endo H resistance between monomeric L4T4C53 K(-4)stop and oligomeric L53T4C53 (K-4)stop, both lacking the KKFF ER-retrieval signal, is most consistent with the idea that the RSQQE determinant is reduced or nonfunctional in the monomeric background. But how could oligomerization influence the RSQQE determinant? Two of the four cysteine residues, which could potentially link ERGIC-53 oligomers by disulfide bridges, are located within a 15–amino acid distance from the transmembrane domain. Provided these cysteines covalently link the ERGIC-53 oligomers in the immediate vicinity of the membrane, this could actually lead to a close apposition of the RSQQE determinants on the cytoplasmic side of the membrane. It is conceivable that such an apposition is required for the RSQQE determinant to be functional, a situation not mimicked in the monomeric reporter protein.

It is not clear what kind of interaction is mediated by the RSQQE determinant. It could act as an imperfect retention signal slowing transit through the ERGIC and thereby contribute to the steady-state distribution of the protein. The fact that RSQQE is also functional when the transmembrane domain in GM is exchanged with that of CD4 in L53T4C53 indicates that this signal is not part of a transmembrane retention determinant, and hence the retention mechanism appears to be unrelated to that of Golgi proteins (Nilsson and Warren, 1994).

A different explanation for the involvement of the lumenal domain in the targeting of ERGIC-53 could be a lumenal interaction with other proteins as a consequence of ERGIC-53's function. The finding of Arar et al. (1995) that ERGIC-53 is identical to an intracellular mannose-specific lectin points to the possibility that ERGIC-53 may facilitate ER to Golgi transport of newly synthesized gly-

coproteins. Alternatively, ERGIC-53 may act as a chaperone downstream of calnexin (Hammond et al., 1994; Hammond and Helenius, 1994; Helenius, 1994) adding an additional level of quality control in the maturation process of glycoproteins. Either of these functions would involve the lumenal domain. Ligand binding may exert an allosteric effect on the cytoplasmic domain modulating the interaction with coatomer and thereby regulate the cycling of ERGIC-53. If so, a contribution of the lectin domain to sorting would only be apparent at moderate expression levels when enough ligand is available. The strong ER staining of highly overexpressed ERGIC-53 and GM as compared to the concentrated pattern seen under moderate expression would be consistent with this notion.

Role of the Phenylalanines

We observed that the COOH-terminal phenylalanines reduce the ER-retrieval capacity of the di-lysine signal and substitution of the phenylalanines in ERGIC-53 by alanines resulted in ER localization. Furthermore, consistent with a pre-Golgi localization, this construct when expressed in LEC-1 cells, which lack the *medial*-Golgi enzyme *N*-acetylglucosaminyl transferase, did not become endoglycosidase D sensitive and hence did not reach the *cis*-Golgi site where mannosidase I trimming occurs (F. Kappeler, C. Itin, and H.-P. Hauri, unpublished observations). This is in contrast to endogenous ERGIC-53, a minor fraction of which is localized to the *cis*-Golgi (Schweizer et al., 1988; Foguet, M., and H.-P. Hauri, unpublished observation). These observations suggest that in absence of the phenylalanines ERGIC-53 protein is efficiently retrieved before reaching the *cis*-Golgi, while in the presence of the phenylalanines transport to the *cis*-Golgi can occur. Subcellular fractionation experiments indeed showed that endogenous ERGIC-53 is retrieved to the ER from two sites. A major fraction directly recycles from the ERGIC without prior passage through the *cis*-Golgi, while a minor fraction has access to the *cis*-Golgi (Foguet, M., and H.-P. Hauri, unpublished observation).

Based on our finding that the phenylalanines reduce the pre-Golgi targeting efficiency of the di-lysine ER-retrieval signals, we propose that they reduce the efficiency of recognition of the di-lysine signal by coatomer in the ERGIC, and thereby allow a fraction of ERGIC-53 to be transported to the *cis*-Golgi.

Mechanism of ERGIC-53 Targeting

In summary our data suggest a model for ERGIC-53 targeting that is based on an active di-lysine ER-retrieval signal that acts together with the COOH-terminal phenylalanines, the RSQQE determinant and the lumenal domain to allow a post-ER localization. Localization of ERGIC-53 in the ER/ERGIC/*cis*-Golgi system is a result of retrieval and retention. In line with the most recent suggestion that COP's may not be involved in anterograde transport but in selective retrieval of di-lysine-signal–bearing proteins (Letourneur et al., 1994; Pelham, 1994) it is conceivable that KKFF is a retrieval signal whose binding to COPs is weak due to the presence of two COOH-terminal phenylalanines. Reduced COP binding may result in less efficient retrieval that in turn would shift the overall distribution from ER to ERGIC. In contrast, RSQQE together with the lumenal domain may function as a retention signal leading to the known concentration of protein ERGIC-53 in the ERGIC. Subcellular fractionation and COP binding studies will be required to test the role of the individual targeting determinants.

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References

Aoki, D., N. Lee, N. Yamaguchi, C. Dubois, and M. N. Fukuda. 1992. Golgi retention of a trans-Golgi membrane protein, galactosyltransferase, requires cysteine and histidine residues within the transmembrane domain. *Proc. Natl. Acad. Sci. USA.* 89:4319–4323.

Arar, C., V. Carpentier, J.-P. Le Cear, M. Monsigny, A. Legrand, and A.-C. Roche. 1995. ERGIC-53, a membrane protein of the ER-Golgi intermediate compartment is identical to MR60 an intracellular mannose-specific lectin of myelomonocytic cells. *J. Biol. Chem.* 270:3551–3553.

Brewer, C. B., and M. G. Roth. 1991. A single amino acid change in the cytoplasmic domain alters the polarized delivery of influenza virus hemagglutinin. *J. Cell Biol.* 114:413–421.

Burke, J., J. M. Pettitt, H. Schachter, M. Sarkar, and P. A. Gleeson. 1992. The transmembrane and flanking sequences of 1,2-N-acetylglucosaminyltransferase specify medial Golgi localization. *J. Biol. Chem.* 266:24433–24440.

Carrera, C. A., F. Sanchez-Madrid, M. Lopez-Botet, C. Berabeu, and M. O. De Landazuri. 1987. Involvement of the CD4 molecule in a post-activation event on T-cell proliferation. *Eur. J. Immunol.* 17:179–186.

Chavrier, P., R. G. Parton, H.-P. Hauri, K. Simons, and M. Zerial. 1990. Localization of low molecular weight GTP-binding proteins to exocytic and endocytic compartments. *Cell.* 63:317–329.

Colley, K. J., E. U. Lee, and J. C. Paulson. 1992. The signal anchor and stem regions of the beta-galactoside alpha 2,6-sialyltransferase may each act to localize the enzyme to the Golgi apparatus. *J. Biol. Chem.* 267:7784–7793.

Cosson, P., and F. Letourneur. 1994. Coatomer interaction with di-lysine endoplasmic reticulum retention motifs. *Science (Wash. DC).* 263:1629–1631.

Cullen, B. R. 1987. Use of eukaryotic expression technology in the functional analysis of cloned genes. *Methods Enzymol.* 152:684–704.

Ellis, L., E. Clauser, D. O. Morgan, M. Edrey, R. A. Roth, and W. J. Rutter. 1986. Replacement of insulin receptor tyrosine residue 1162 and 1163 comprises insulin-stimulated kinase activities and uptake of 2-deoxyglucose. *Cell.* 45:721–732.

Evan, G. I., G. K. Lewis, G. Ramsay, and J. M. Bishop. 1985. Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell. Biol.* 5:3610–3616.

Fiedler, K., and K. Simons. 1994. A putative new class of animal lectins in the secretory pathway homologous to leguminose lectins. *Cell.* 77:625–626.

Gaynor, E. C., S. T. Heesen, T. R. Graham, M. Aebi, and S. D. Emr. 1994. Signal-mediated retrieval of membrane protein from the Golgi to the ER in yeast. *J. Cell Biol.* 127:653–665.

Hammond, C., and A. Helenius. 1994. Folding of VSV G protein: sequential interactions with BiP and calnexin. *Science (Wash. DC).* 266:456–458.

Hammond, C., I. Braakman, and A. Helenius. 1994. Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. *Proc. Natl. Acad. Sci. USA.* 91:913–917.

Helenius, A. 1994. How N-linked oligosaccharides affect glycoprotein folding in the endoplasmic reticulum. *Mol. Biol. Cell.* 5:253–265.

Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene (Amst.).* 77:51–59.

Horton, R. M., H. D. Hunt, S. N. Ho, J. K. Pullen, and L. R. Pease. 1989. Engineering genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene (Amst.).* 77:61–68.

Itin, C., F. Kappeler, A. D. Linstedt, and H.-P Hauri. 1995. A novel endocytosis signal related to the KKXX ER-retrieval signal. *EMBO J.* 14:2250–2256.

Jackson, M. R., T. Nilsson, and P. A. Peterson. 1990. Identification of a consensus motif of transmembrane proteins in the endoplasmic reticulum. *EMBO J.* 9:3153–3163.

Jackson, M. R., T. Nilsson, and P. A. Peterson. 1993. Retrieval of *trans*membrane proteins to the ER. *J. Cell Biol.* 121:317–333.

Kappeler, F., C. Itin, R. Schindler, and H.-P. Hauri. 1994. A dual role for COOH-terminal lysine residues in pre-Golgi retention and endocytosis of ERGIC-53. *J. Biol. Chem.* 269:6279–6281.

Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Letourner, F., E. C. Gaynor, S. Hennecke, C. Démollière, R. Duden, S. D. Emr, H. Riezman, and P. Cosson. 1994. Coatomer is essential for retrieval of dilysine-tagged proteins to the ER. *Cell.* 79:1199–1207.

Linstedt, A. D., and H.-P. Hauri. 1993. Giantin, a novel conserved Golgi membrane protein containing a cytoplasmic domain of at least 350 kDa. *Mol. Biol. Cell.* 4:679–693.

Machamer, C. E., M. E. Grim, A. Esquela, S. W. Chung, M. Rolls, K. Ryan, and A. M. Swift. 1993. Retention of a cis-Golgi protein requires polar residues on one face of a predicted α-helix in the transmembrane domain. *Mol. Biol. Cell.* 4:695–704.

Maddon, P. J., D. R. Littman, M. Godfrey, D. E. Maddon, L. Chess, and R. Axel. 1985. The isolation and nucleotide sequence of a cDNA encoding the T cell surface protein T4: a new member of the immunoglobulin gene family. *Cell.* 42:94–104.

Munro, S. 1991. Sequence within and adjacent to the transmembrane segment of alpha-2,6-sialyltransferase specify Golgi retention. *EMBO J.* 10:3577–3588.

Nilsson, T., M. R. Jackson, and P. A. Peterson. 1989. Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the ER. *Cell.* 58:707–718.

Nilsson, T., and G. Warren. 1994. Retention and retrieval in the endoplasmic reticulum and the Golgi apparatus. *Curr. Opin. Cell. Biol.* 6:517–521.

Nilsson, T., J. M. Lucocq, D. Mackay, and G. Warren. 1991. The membrane-spanning domain of beta-1,4-galactosyltransferase specifies trans-Golgi localization. *EMBO J.* 10:3567–3575.

Nilsson, T., P. Slusarewicz, M. H. Hoe, and G. Warren. 1993. Kin recognition. A model for retention of Golgi enzymes. *FEBS Lett.* 330:1–4.

Nilsson,T., M. H. Hoe, P. Slusarewicz, C. Rabouille, R. Watson, F. Hunte, G. Watzele, E. G. Berger, and G. Warren. 1994. Kin recognition between medial Golgi enzymes in HeLa cells. *EMBO J.* 10:3567–3575.

Oprins, A., R. Duden, T. E. Kreis, H. J. Geuze, and J. W. Slot. 1993. Beta-COP localizes mainly to the *cis*-Golgi side in exocrine pancreas. *J. Cell Biol.* 121: 49–59.

Pelham, H. R. B. 1994. About turn for the COPs? *Cell.* 79:1125–1127.

Pelham, H. R. B., and S. Munro. 1993. Sorting of membrane proteins in the secretory pathway. *Cell.* 75:603–605.

Ponnambalam, S., C. Rabouille, J. P. Luzio, T. Nilsson, and G. Warren. 1994. The TGN38 glycoprotein contains two nonoverlapping signals that mediate localization to the *trans*-Golgi network. *J. Cell Biol.* 125:253–268.

Roth, J. 1984. Cytochemical localization of terminal *N*-acetyl-D-galactosamine residues in cellular compartments of intestinal Goblet cells: Implications for the topology of *O*-glycosylation. *J. Cell Biol.* 98:399–406.

Roth, J. 1987. Subcellular organization of glycosylation in mammalian cells. *Biochem. Biophys. Acta.* 906:405–436.

Roth, J., Y. Wang., A. Eckhardt, and R. L. Hill. 1994. Subcellular organization of the UDP-N-acetyl-D-galactosamine:polypeptide-N-actelygalactosaminyl-transferase-mediated O-glycosylation reaction in the submaxillary gland. *Proc. Natl. Acad. Sci. USA.* 91:8935–8939.

Russo, R. N., N. L. Shaper, D. J. Taatjes, and J. H. Shaper. 1992. 1,4-galactosyltransferase: a short NH_2-terminal fragment that includes the cytoplasmic and transmembrane domain is sufficient for retention. *J. Biol. Chem.* 267: 9241–9247.

Saraste, J., and K. Svensson. 1991. Distribution of the intermediate elements operating in ER to Golgi transport. *J. Cell Sci.* 100:415–430.

Schindler, R., C. Itin, M. Zerial, F. Lottspeich, and H.-P. Hauri. 1993. ERGIC-53, a membrane protein of the ERGIC, carries an ER retention motif. *Eur. J. Cell. Biol.* 61:1–9.

Schweizer, A., J. A. M. Fransen, T. Bächi, L. Ginsel, and H.-P. Hauri. 1988. Identification, by a monoclonal antibody, of a 53-kD protein associated with a tubular-vesicular compartment at the *cis*-side of the Golgi apparatus. *J. Cell Biol.* 108:1643–1653.

Schweizer, A., J. A. M. Fransen, K. Matter, T. E. Kreis, L. Ginsel, and H.-P. Hauri. 1990. Identification of an intermediate compartment involved in protein transport from the ER to the Golgi apparatus. *Eur. J. Cell Biol.* 53:185–196.

Schweizer, A., K. Matter, C. M. Ketcham, and H.-P. Hauri. 1991. The isolated ER–Golgi intermediate compartment exhibits properties that are different from ER and *cis*-Golgi. *J. Cell Biol.* 113:45–54.

Schweizer, A., M. Ericsson, T. Bächi, G. Griffiths, and H.-P. Hauri. 1993. Characterization of a novel 63kDa membrane protein: implications for the organization of the ER-to-Golgi pathway. *J. Cell Sci.* 104:671–683.

Schweizer, A., J. Rohrer, H.-P. Hauri, and S. Kornfeld. 1994. Retention of p63 in the ERGIC depends on the presence of all three of its domains and its ability to form oligomers. *J. Cell Biol.* 126:25–39.

Sweet, D. J., and H. R. B. Pelham. 1992. The Saccharomyces cerevisiae SEC20 gene encodes a membrane glycoprotein which is sorted by the HDEL retrieval system. *EMBO J.* 11:423–432.

Swift, A. M., and C. E. Machamer. 1991. A Golgi retention signal in a membrane-spanning domain of coronavirus E1 protein. *J. Cell Biol.* 115:19–30.

Tang, B. L., S. H. Wong, S. H. Low, and W. Hong. 1992. The transmembrane domain of N-glucosaminyltransferase I contains a Golgi retention signal. *J. Biol. Chem.* 267:10122–10126.

Teasdale, R. D., G. D'Agostaro, and P. A. Gleeson. 1992. The signal for Golgi retention of bovine β1,4-galactosyltransferase is in the transmembrane domain. *J. Biol. Chem.* 267:4084–4096.

Townsley, F. M., and H. R. B. Pelham. 1994. The KKXX signal mediates retrieval of membrane proteins from the Golgi to the ER in yeast. *Eur. J. Cell Biol.* 64:211–216.

Weisz, O. A., A. M. Swift, and C. E. Machamer. 1993. Oligomerization of a membrane protein correlates with its retention in the Golgi complex. *J. Cell Biol.* 122:1185–1196.

Wong, S. H., S. H. Low, and W. Hong. 1992. The 17-residue transmembrane domain of beta-galactoside alpha 2,6-sialyltransferase is sufficient for Golgi retention. *J. Cell Biol.* 117:245–258.