The Golgi-localization of Yeast Emp47p Depends on Its Di-lysine Motif But Is Not Affected by the *ret1-1* Mutation in α-COP

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Abstract. The *Saccharomyces cerevisiae EMP47* gene encodes a nonessential type-I transmembrane protein with sequence homology to a class of intracellular lectins defined by ERGIC-53 and VIP36. The 12–amino acid COOH-terminal cytoplasmic tail of Emp47p ends in the sequence KTKLL, which conforms with the consensus for di-lysine–based ER-localization signals. Despite the presence of this motif, Emp47p was shown to be a Golgi protein at steady-state. The di-lysine motif of Emp47p was functional when transplanted onto Ste2p, a plasma membrane protein, conferring ER localization. Nevertheless, the di-lysine motif was required for Golgi-localization of Emp47p and showed the same charge-independent, position-dependent characteristics of other di-lysine motifs.

α-COP has been shown to be required for ER localization of di-lysine–tagged proteins. Consistent with

this finding, the Ste2p-Emp47p hybrid protein was mislocalized to the cell surface in the α-COP mutant, *ret1-1*. Surprisingly, the Golgi-localization of Emp47p was unaffected by the *ret1-1* mutation. To investigate whether Emp47p undergoes retrograde transport from the Golgi to the ER like other di-lysine–tagged proteins we developed an assay to measure this step after block of forward transport in a *sec12* mutant. Under these conditions retrograde transport led to a specific redistribution of Emp47p from the Golgi to the ER. This recycling occurred from a Golgi subcompartment containing α1,3 mannose-modified oligosaccharides suggesting that it originated from a *medial-* or later Golgi compartment. Thus Emp47p cycles between the Golgi apparatus and the ER and requires a di-lysine motif for its α-COP–independent, steady state localization in the Golgi.

THE secretory pathway in eukaryotic cells is topologically separated into several distinct membrane-bound compartments performing different functions. Newly synthesized secretory proteins are transported through the compartments in a vectorial fashion. For some steps in this pathway specific positive signals have been identified that ensure proper delivery of a particular polypeptide to its final destination. Transport of secretory proteins from one compartment to the next is generally believed to be mediated by vesicles with characteristic coats, an increasing number of which are being characterized (Ladinsky et al., 1994; Narula and Stow, 1995).

The concept of secretory proteins passing through stable compartments must also account for the proper localization of those proteins that accomplish the specific functions characteristic for any given compartment. This localization problem for resident proteins can be solved by one or a combination of the following mechanisms: (*a*) passive retention because forward transport requires a signal, (*b*) active re-

tention via some intermolecular interaction, and (*c*) retrieval/recycling after transport to another compartment. Proteins that function in sorting and transport between compartments will also have to be recycled if they are used for more than one round of transport.

For the localization of ER proteins two mechanisms conserved from yeast to mammalian cells have been uncovered in recent years, both belonging to class *c* as defined above. The first is the HDEL/KDEL retrieval system for ER-proteins. The COOH-terminal peptide sequence HDEL (*Saccharomyces*) or KDEL (mammalian cells) on lumenal, but also on a few transmembrane proteins, is recognized by a specific receptor, Erd2p, present in the Golgi apparatus. Ligand transported out of the ER is bound by the receptor and the receptor/ligand complex then returns to the ER (Dean and Pelham, 1990; Lewis and Pelham, 1990, 1992; Lewis et al., 1990; Semenza et al., 1990). For transmembrane proteins of the ER another short COOH-terminal peptide sequence was identified that conferred ER localization (Pääbo et al., 1987; Nilsson et al., 1989; Jackson et al., 1990). This motif contains two critical lysine residues at either position $-3, -4$ or $-3, -5$ with respect to the COOH terminus of the protein. Using chimeric proteins, evidence was obtained in mammalian cells (Jackson et al., 1993) that the di-lysine motif, similarly to the

Table 1. Strains of Saccharomyces cerevisiae Used in This Study

Strain	Genotype	Source
RH448	MATa, his4, leu2, ura3, lys2, bar1-1	Lab strain
RH732	RH448 except pep4::URA3	Lab strain
RH1201	MATa/α, his4/his4, leu2/leu2, ura3/ura3, lys2/lys2, bar1/bar1	Lab strain
RH1491	MATa, sec12-4, ura3, his4, leu2, lys2,bar1-1	Lab strain
RH2825	RH732 except emp47::LYS2	Lab strain
RH2826	RH2825 except myc-EMP47::LEU2	This study
RH2827	RH2825 except myc-EMP47-TKKLL::LEU2	This study
RH2828	RH2825 except myc-EMP47-KTQLL::LEU2	This study
RH2829	RH2825 except myc-EMP47-QTKLL::LEU2	This study
RH2830	RH2825 except myc-EMP47-QTQLL::LEU2	This study
RH2831	RH2825 except myc-EMP47-RLAIIKTKLL::LEU2	This study
RH2833	RH2825 except myc-EMP47-TKKAA::LEU2	This study
RH2834	RH2825 except myc-EMP47-RQEII::LEU2	This study
RH3045	RH448 except emp47::LYS2	This study
RH3046	RH3045 except myc-glyco-EMP47::LEU2	This study
RH3047	RH3045 except myc-EMP47::LEU2	This study
RH3048	RH1491 except myc-EMP47::LEU2	This study
RH3049	RH1491 except myc-glyco-EMP47::LEU2	This study
RH3050	RH2825 except myc-EMP47-RTRLL::LEU2	This study
RH3051	RH2825 except myc-EMP47-KTKLLS4::LEU2	This study
RH3187	MATa, trp1, leu2, his4, ura3, bar1, ret1-1	This study
RH3193	MATa, trp1, leu2, his4, ura3, bar1	This study
RH3196	RH3187 except ste2::LEU, STE2EMP47PTAIL::URA3, myc-EMP47::TRP1, ret1-1	This study
RH3199	RH3193 except ste2::LEU, STE2EMP47PTAIL::URA3, myc-EMP47::TRP1	This study
RH3274	RH3187 except pep4::URA3	This study
RH3275	RH448 except emp47::LYS2, myc-EMP47-QTQLL::LEU2	This study
RSY918	MATa, mnn4	R. Scheckman
RSY919	MATα, mnn1, mnn2, ura3	R. Scheckman

HDEL/KDEL, can mediate retrieval of proteins from post-ER compartments. Analyzing sugar modifications and the subcellular distribution of chimaeric proteins in yeast, Townsley and Pelham (1994) and Gaynor et al. (1994) provided convincing evidence for retrieval, possibly from the Golgi cisterna that is functionally defined by the presence of Och1p. This glycosyltransferase adds the first α1,6 mannose in outer chain modification of N-linked oligosaccharides in yeast (Nakanishi-Shindo et al., 1994; Gaynor et al., 1994). The first hint towards the molecular machinery of that retrieval system came from the observation that a di-lysine motif can be recognized in vitro by coatomer (Cosson and Letourneur, 1994). The suggestion that COP I-coated vesicles may play a role in the itinerary of di-lysine proteins was strongly supported by a genetic screen in yeast. Mutants were identified that abrogated the ER-localization of an α-factor receptor with an engineered di-lysine motif. These mutants were shown to be affected in the genes coding for α-COP (*RET1*), β'-COP (*SEC27*), and γ-COP (*SEC21*) (Letourneur et al., 1994).

One protein of mammalian origin bearing a di-lysine signal is ERGIC-53 (Schindler et al., 1993). This protein is located in the ER-Golgi intermediate compartment (ERGIC)[1] at steady-state but can cycle between early compartments of the secretory pathway (Schweizer et al., 1988, 1990). ERGIC-53 and VIP36, a transmembrane protein isolated from secretory vesicles (Fiedler et al., 1994), were speculated to function as intracellular lectins based on sequence homology with leguminous lectins (Fiedler and Simons, 1994). Indeed ERGIC-53 has recently been

rediscovered as a major intracellular mannose-binding protein from a monocytic cell line (Arar et al., 1995).

In the present study we report the cloning and sequencing of a novel gene from Saccharomyces cerevisiae, EMP47, coding for a type I transmembrane protein with a di-lysine motif and with sequence homology to the ERGIC-53/VIP36 protein family. We characterized the distribution of Emp47p and showed that its steady-state Golgi localization requires its di-lysine motif, but is independent of α-COP, even though Emp47p cycles between the ER and the Golgi. We suggest models for Emp47p function based on its trafficking pattern.

Materials and Methods

Strains and Growth Conditions

Strains of Saccharomyces cerevisiae used in this study are listed in Table I. Unless otherwise mentioned, indicated strains were grown in complete medium (2% yeast extract, 2% peptone, 40 μg/ml uracil and adenine, and 2% glucose) to exponential phase (2–4 × 10^7 cells/ml) at 30°C. For cloning purposes Escherichia coli XL 1 blue (Stratagene, La Jolla, CA) was used if not otherwise indicated.

Cloning of EMP47

Degenerate oligonucleotides deduced from the NH$_2$-terminal sequence of p44 (NH$_2$-HPLGDTSDAGKL; Singer-Krüger et al., 1993) were used to identify a clone designated 61 (Birgit Singer-Krüger, 1993) from a yeast genomic library (Heitman et al., 1991). Clone 61 contained an insert of ~5.4 kb. The insert was mapped with respect to the site hybridizing with the degenerate oligonucleotides. A 2.3-kb EcoRI fragment was determined to comprise the whole gene. This fragment was subcloned into pBSK− (Stratagene, La Jolla, CA). Restriction fragments of the EcoRI-clone were sequenced using the dideoxynucleotide chain termination method (Sanger et al., 1977). Remaining gaps in the sequence were filled in by priming the sequencing reactions with specific oligonucleotides.

1. Abbreviations used in this paper: AL, anti-lumenal; AT, anti-tail; ERGIC, ER-Golgi intermediate compartment.

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Biocomputing

All sequence analysis was performed on a VAX-computer using the programs of the GCG-package (Genetics Computer Group, Madison, WI) and additional programs implemented by the Biozentrum Biocomputing Unit led by R. Doelz.

Construction of Deletion Disruption Mutants in EMP47

The 2.3-kB EcoRI fragment was subcloned into a pBSK− vector deleted for its single SalI site. The HincII fragment between nucleotides 469–1218 (numbering according to Fig. 1 *A*) was replaced with a 1.1-kb HindIII fragment blunt ended with Klenow enzyme and comprising *URA3*. Alternatively the NsiI fragment between nucleotides 106–1375 was deleted, the ends blunted with T4 polymerase and the 4.2-kb SalI fragment of pDP6 (Fleig et al., 1986) comprising *LYS2* was inserted. The resulting plasmid was cut with EcoRI and the digest transformed into RH1201, RH448, and RH732 using the LiAc-transformation method (Gietz et al., 1992) which was applied for all yeast transformations described in this work. Successful disruption was monitored by Western blotting with antiserum AL (see below).

Recombinant EMP47 Variants

Myc-tagging: the 2.3-kB EcoRI fragment was partially digested with NsiI, the opened sites were cut back to blunt ends and relegated. A clone with the NsiI site at 1375 destroyed was opened at the remaining NsiI site at nucleotide 106. Annealed oligonucleotides coding for the 9E10 epitope (Evan et al., 1985) were introduced, reconstituting the NsiI site at the 3' end of the epitope. The oligonucleotides introduced the sequence EQKLLSEEDLNEA at amino acid 9 of mature Emp47p. Introduction of an N-linked glycosylation site: the clone coding for the myc-tagged Emp47p was used to introduce two overlapping N-linked glycosylation consensus sites at position 49 and 50 (numbering of wild-type Emp47p as in Fig. 1 *A*) by PCR mutagenesis (Ho et al., 1989). The final PCR product was digested with DraIII and NsiI, and the corresponding fragment cloned back into the plasmid coding for the myc-tagged Emp47p. Mutants of the COOH-terminal cytoplasmic tail of Emp47p were also created by PCR mutagenesis. The SalI(1218)/BamHI(1567) fragment (nucleotide numbers refer to Fig. 1 *A*) of the final PCR product was exchanged for the corresponding fragment of the wild-type sequence. All inserted sequences introduced from oligonucleotides or PCR-mutagenesis were verified by sequencing. The recombinant *EMP47* genes were transplanted as EcoRI or EcoRI/BamHI fragments into either YIplac128 for integration at *leu2* or into YIplac204 for integration at *trp1* (Gietz and Sugino, 1988) after cutting the plasmids with BstXI.

Generation of Ste2p with the Cytosolic Tail of Emp47p: Ste2p-Emp47ptail

The plasmid pJR-320Bam-345Stop (Letourneur et al., 1994) was cut at the BamHI site corresponding to amino acid 320 of Ste2p. Annealed complementary oligonucleotides corresponding to the last 12 COOH-terminal amino acids of Emp47p and the stop codon were ligated into the BamHI site. The relevant region of the construct was sequenced. The construct was linearized with StuI for integration at the *ura3* locus. Recipient strains were *MATa* in which *STE2* had been deleted by disruption using plasmid pUSTE203 (Nakayama et al., 1988).

Antibodies

Two antisera against Emp47p-specific sequences were raised in rabbits. The first, denoted AT (for anti-tail) was generated against a peptide (Neosystem Laboratoire, Strasbourg, France) comprising the 12 COOH-terminal amino acids of Emp47p with an extra NH_2-terminal cysteine for coupling purposes. The peptide proved insoluble in aqueous solutions and was therefore injected into the rabbit as a PBS-suspension. IgGs of the resulting serum were purified on a protein A–Sepharose matrix (Pharmacia, Uppsala, Sweden). For further affinity purification of the IgGs a 6His-DHFR fusion protein with the last 39 amino acids of Emp47p (from the SalI site at 1218) was expressed in *E. coli* M15 from a construct made in pQE9 HDH (Kontron, Zürich, Switzerland). This construct was soluble only under strongly denaturing conditions and was therefore purified on a Ni^+-chelating matrix in buffer containing 8 M urea. It was coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) in the cou-

pling buffer recommended by the manufacturer supplemented with 6 M guanidinium/HCl. A glycine/HCl pH 2.5 eluate from the affinity-column was used in all cases where AT was used in this study. The second antiserum, called AL (for anti-lumenal) was raised against most of the lumenal domain of Emp47p. The gene fragment ranging from the XbaI(197) site to the SalI (1218) site (nucleotide numbering according to Fig. 1 *A*) was cloned behind a 5'-6His-tag in the vector pQE11 (Kontron, Zürich, Switzerland). The recombinant protein was expressed in *E. coli* M15 and purified on a Ni^+-matrix. The purified protein was used for immunization of a rabbit and later, after coupling the protein to CNBr-activated Sepharose 4B, for affinity purification of the serum. On Western blots, this antiserum recognized all recombinant variants of Emp47p described in this study. A glycine/HCl, pH 2.5, eluate from the affinity-column was used.

Antibodies against α1,3-linked mannose residues were generated according to Ballou (1970, 1976) using strain RSY918. The serum was absorbed on extract from RSY919 coupled to CNBr-activated Sepharose 4B. The peptide antibody against Ste2p was described in Zanolari et al. (1992).

The following antibodies were used at the recommended dilutions: (*A*) polyclonal: Kar2p (kindly provided by R. Schekman, University of California, Berkeley, CA), Och1p (kindly provided by Y. Jigami, National Chemical Laboratory for Industry, Tsukuba, Japan), Wbp1p (kindly provided by S. te Heesen, Eidgenössische Technische Hochschule, Zürich, Switzerland), alkaline phosphatase (kindly provided by D. Gallwitz, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany); (*B*) monoclonal: 13D11, directed against the 60-kD subunit of the vacuolar ATPase (kindly provided by T. Stevens, University of Oregon, Eugene, OR), 12CA5, directed against the HA-epitope (kindly provided by H. Rudolph, University of Stuttgart, Stuttgart, Germany), and 9E10, directed against the myc-epitope (kindly provided by R. Movva, Sandoz AG, Basel, Switzerland).

Indirect Immunofluorescence

Approximately 10^7 cells were sedimented for 1 h in a tabletop centrifuge and resuspended in fixative (PBS/3% paraformaldehyde/10% sorbitol). Prefixing the cells before spinning did not change the Emp47p pattern. After 1 h, cells were washed with PBS/10% sorbitol and the cell walls were digested for 1 h at 30°C (in 100 μl PBS/10% sorbitol with 0.14 μl β-mercaptoethanol) with an empirically determined amount of lyticase (Shen et al., 1991). Cells were washed with PBS/10% sorbitol and adsorbed to multiwell slides treated with poly-L-lysine. Quenching solution (PBS/10%sorbitol/1% BSA/1% Triton X-100) was added for 10 min at ambient temperature and then replaced by quenching solution containing the appropriate primary antibodies. The incubation at room temperature lasted for 1 h. The cells were then quickly washed five times with PBS/10% sorbitol. Fluorescently labeled secondary antibodies were added in quenching solution for 45 min at ambient temperature (Cy3-conjugated goat anti–rabbit IgG, Cy3-conjugated goat anti–mouse IgG; Jackson ImmunoResearch Laboratories, West Grove, PA; FITC-conjugated sheep anti–mouse IgG; Cappel Research Products, Durham, NC). Cells were then quickly rinsed five times, washed twice for 5 min, and three more times quickly with PBS/ 10% sorbitol. Embedding medium (vectashield; Vector Laboratories, Burlingame, CA) supplemented with 0.1 μg/ml DAPI was added and slides covered with cover slips. Cells were viewed in a fluorescence microscope and documented by conventional photography or by storage of a digital image obtained with a video camera.

Enzymatic Assays

Kex2p was detected according to the method of Cunningham and Wickner (1989) with the modifications of Singer-Krüger et al. (1993). GDPase was measured as described by Abeijon et al. (1989) and LeBel et al. (1978) with the modifications of Singer-Krüger et al. (1993).

Subcellular Fractionation by Velocity Sedimentation on Sucrose Density Gradients

The procedure was carried out with some modifications based on the protocol developed by Antebi and Fink (1992). Cells were spheroplasted in a volume of 2 ml as described previously (Schimmöller and Riezman, 1993) and then directly lysed by at least 10 passages through a 25-gauge needle after adding protease inhibitors to the final indicated concentrations: phenylmethylsulfonylfluoride (1 mM), leupeptin, aprotinin and pepstatin (5 μg/ml each). The lysate was cleared twice for 5 min at 500 *g* in 2-ml reaction tubes. The supernatant (~1 ml) was confirmed by microscopy to be

devoid of unbroken cells and loaded onto an 11-ml sucrose gradient made up from 1-ml steps of 18, 22, 26, 30, 34, 38 , 42, 46, 50, 54, and 60% (wt/vol) sucrose in 10 mM Hepes, pH 7.5, 1 mM MgCl₂. The gradients were spun for 2 h 20 min at 4°C in a TFT41.14 rotor (Kontron Instruments, Zürich, Switzerland) at 37,000 rpm. The gradient was then fractionated from the top into 12 equal fractions, the pellet being resuspended in the last fraction. Aliquots of the fractions were removed for enzymatic assays (see above) or resolved by SDS-PAGE, transferred to nitrocellulose and probed with the antibodies described above. Immunoreactivity was detected using the ECL kit (Amersham Int., Amersham, UK) and the ECL signal was quantitated by scanning films with a scanning densitometer (Molecular Dynamics, Sunnyvale, CA).

Radiolabeling

Cells were grown in SDYE (0.67% yeast nitrogen base without amino acids, 0.2% yeast extract, 2% glucose and the strain specific amino acid supplements). For the determination of Emp47p-turnover cells were grown in minimal medium supplemented with adenine and the amino acids His, Leu, Lys, Trp, Ura (Dulic et al., 1991). Radiolabeling with ³⁵S protein labeling mix EXPRE³⁵S³⁵S (Dupont, Germany) and subsequent chase was performed according to Horvath et al. (1994).

Immunoprecipitations

When performing immunoprecipitations from gradient fractions each fraction of ∼1 ml was mixed with 200 µl SDS-solution (5% SDS; 50 mM Tris-HCl, pH 8.0; 5 mM EDTA, pH 8.0) and samples were heated to 95°C for 10 min. The samples were centrifuged at 13,000 g for 10 min. 3 ml TET (100 mM Tris-HCl, pH 8.0; 5 mM EDTA, pH 8.0; 1% Triton X-100) and 300 µl 20% Triton X-100 were added to the samples. Emp47p was then precipitated by AT-antibody (45 min, room temperature) followed by the addition of protein A–Sepharose (Pharmacia, Uppsala, Sweden) and incubation on a rocking platform for 1 h. Precipitates were washed five times with TNET (=TET plus 100 mM NaCl) and twice with 20 mM Tris-HCl, pH 7.5. The Sepharose beads were then heated to 95°C for 10 min in 400 µl 1% SDS, 1 mM EDTA, pH 8.0; 10 mM Tris-HCl, pH 8.0. The supernatant was mixed with 1 ml TNET and 150 ml 20% Triton X-100, split in aliquots and precipitated as above with either AT-antibody or α1,3 mannose-specific antiserum. The second round precipitates were separated on 8% SDS-PAGE and the dried gels were quantitated on a PhosphoImager (Molecular Dynamics, Sunnyvale, CA).

For immunoprecipitations from whole cell extracts, cells were converted into spheroplasts in 150 µl and lysed by the addition of 15 µl 10% SDS and heating to 95°C for 10 min. For the determination of Emp47p-turnover duplicate aliquots of the labeling reaction were withdrawn after each time point and directly heated to 95°C for 5 min in the presence of 1% SDS, 50 mM Tris-HCl pH 8.0, 5 mM EDTA, 150 mM NaCl (final concentrations). Cells were then further disrupted with glass beads by shaking on an Eppendorf Mixer 5432 (Eppendorf, Hamburg, Germany) for 15 min. The samples were further processed for immunoprecipitation essentially as described above.

Redistribution Assay on Sucrose Density Gradients

Strain RH1491 was grown at 24°C to log phase. Cells were harvested and resuspended in fresh complete medium at ambient temperature at a density of 0.6 × 10⁹ cells/ml. Then 20 µg/ml cycloheximide was added. Within 5 min cells were split into aliquots of 0.6 × 10¹⁰ cells and transferred for 1 h to a shaking water bath set at either 24 or 35°C. After incubation, 10 mM NaN₃ and 10 mM NaF (final) were added for the subsequent processing for sucrose gradient analysis as described above. For the time course of Emp47p redistribution, aliquots of 1 × 10⁹ cells were taken per time point at the nonpermissive temperature of 37°C. To stop membrane traffic 10 mM NaN₃ and 10 mM NaF were added to each aliquot. The energy poisons were present throughout all subsequent processing steps as well.

Results

EMP47 Is a Novel Gene Coding for a Transmembrane Protein

In previous work, partially purified endosomal fractions were prepared from *Saccharomyces cerevisiae* cells and a major Triton X-114–soluble integral membrane protein in these fractions was named p44. NH₂-terminal sequencing of p44 yielded the sequence of the first 12 amino acids (Singer-Krüger et al., 1993). This sequence information was used to design degenerate oligonucleotides for the isolation of a genomic clone encoding p44. The clone was mapped and the sequence of a 2.3-kb EcoRI subfragment was determined (Fig. 1 *A*). An open reading frame of 1335 nucleotides is present on this fragment. 84 nucleotides downstream of the first ATG-initiation codon the open reading frame codes for the NH₂-terminal peptide of p44 (Fig. 1 *A*). Only a single amino acid difference at position 10 was noted between the NH₂-terminal peptide sequence (Gly) and the sequence derived from the DNA (Ser). The protein sequence upstream of the NH₂ terminus of the mature polypeptide is hydrophobic in nature (Fig. 1 *B*) and conforms with the rules for cleavable signal peptides (von Heijne, 1983). The mature polypeptide of 417 amino acids has one potential hydrophobic transmembrane domain (Fig. 1 *B*) ending only 12 amino acids before the COOH terminus. The presence of a cleaved signal peptide and the COOH-terminally located transmembrane domain imply that the protein adopts a type I-transmembrane orientation. This was verified by protease protection experiments (data not shown).

We named the gene *EMP47* because the mature polypeptide has a calculated molecular weight of 47 K. We deleted one copy of *EMP47* in the diploid strain RH1201 by replacing the internal HincII fragment (nucleotides 469–1218) with a 1.1-kb *URA3* fragment. The disruption was verified by Southern analysis. After sporulation and dissection all spores within a tetrad grew equally well at temperatures from 15 to 37°C (data not shown). The disruptants still coded for a hypothetical truncated protein of 129 amino acids. However, haploid strains in which *EMP47* was disrupted with *LYS2* (see below) leaving only nine amino acids of the mature Emp47p were also viable from 15 to 37°C (data not shown).

Emp47p Is Homologous to the ERGIC-53\VIP36 Class of Intracellular Lectins

Database searches with the Emp47p sequence did not yield any obviously homologous proteins. We noticed, however, a significant homology between Emp47p and ERGIC-53 (Schindler et al., 1993) when directly comparing the two sequences. ERGIC-53 is a human type I transmembrane protein that has been shown to cycle between the ER, ERGIC, and the *cis*-Golgi and was defined as a marker protein for the ER to Golgi intermediate compartment (Schweizer et al., 1988). Fiedler and Simons (1994) have found homology between ERGIC-53 and VIP36. VIP36 is a type I transmembrane protein described in epithelial cells to be present in the Golgi, on the apical and basolateral surface and in transport vesicles directed towards them (Fiedler et al., 1994). Pairwise alignments between all three proteins with the program BESTFIT yielded significant homology scores. The scores were always more than five standard deviations higher than the mean score for alignments of the randomized sequences (Table II). The pairwise alignment scores for ERGIC-53 versus VIP36 are clearly better than those for the alignments with Emp-

47p, indicating that Emp47p is a more distant member of the family. Multiple alignment (Fig. 1, *C* and *D*) underscores, however, the observation that all three proteins are related to each other. This is most notable in the first 250 amino acids of the mature proteins (Fig. 1 *C*). The first 250 amino acids of VIP36 and ERGIC-53 were previously reported to display some homology with leguminous lectins. It was suggested that VIP36 and ERGIC-53 may function as intracellular lectins (Fiedler et al., 1994; Fiedler and Simons, 1994). Indeed very recently Arar et al. (1995) have isolated a mannose binding protein from myelomonocytic cells that proved to be identical to ERGIC-53.

The tertiary structures of both leguminous lectins and human galectins are mainly organized into antiparallel β-sheets (Sharon, 1993; Lobsanov et al., 1993). Emp47p has the same structural feature predicted for its first 250 amino acids (Fig. 1 *B*). The sequence homology with ERGIC-53 and VIP36 and the similar secondary structure predictions both qualify Emp47p as a putative intracellular lectin. The region of Emp47p from near amino acid 250 to the start of the transmembrane domain is predicted to be mainly α-helical (Fig. 1 *B*). In particular there is a probability of 0.78 (PEPCOIL, Lupas et al., 1991) to form coiled coil structures in the region from amino acid 336 to 364. This region may form a spacer separating the putative lectin domain from the membrane.

The short cytoplasmic tails of ERGIC-53, VIP36, and Emp47p also seem to be related to each other both in length and sequence (Fig. 1 *D*). In particular we noted the presence of a consensus ER-localization sequence of the KXKXX type in Emp47p, corresponding to the KKXX-type motif in ERGIC-53 (Jackson et al., 1990, 1993; Schindler et al., 1993). Although the COOH-terminal KRXX in VIP36 does not fit the di-lysine consensus and VIP36 was not reported to be present in the ER, the cytoplasmic domain of VIP36 is nevertheless overall remarkably similar to those of Emp47p and ERGIC-53.

Emp47p Is Not an ER Protein but Colocalizes with a Golgi Marker

Three yeast proteins with a di-lysine ER-localization consensus sequence have been identified so far. The first was the type I transmembrane protein Wbp1p, a subunit of the *N*-oligosaccharyl-transferase complex in the ER (te Heesen et al., 1992, 1993). However, it neither depends on its KKXX sequence for proper function nor does it seem to escape from the ER if the di-lysine is mutated (Gaynor et al., 1994). The second is Vma21p, required for the assem-

bly of the vacuolar ATPase in the ER (Hill and Stevens, 1994). This protein was predicted to span the membrane twice with both NH_2 and COOH terminus in the ER-lumen. Vma21p depends on its di-lysine motif for retention in the ER. The third protein is Gaa1p, a polytopic transmembrane protein that has a COOH-terminal sequence KXKXX (Hamburger et al., 1995). Gaa1p is an essential protein that is required in the ER for the attachment of glycosylphosphatidylinositol onto proteins. Its localization to the ER was determined upon overproduction. Mutating the COOH-terminal sequence in the overproduced Gaa1p allowed partial escape of the mutant protein from the ER (Hamburger, 1994).

We wondered therefore whether the COOH-terminal KXKXX sequence of Emp47p would convey a steady-state ER localization to the protein. To localize the protein an antibody against a peptide comprising the whole cytoplasmic tail (AT-antibody) was raised in rabbits. By immunofluorescence we showed that the Emp47p staining pattern was clearly distinct from that of the ER, visualized in the same strain by anti-Kar2p antibody. The ER-marker Kar2p (Normington et al., 1989; Rose et al., 1989) displayed the typical ring-like staining around the nucleus and subplasmamembrane staining (Fig. 2 *A*, *right*). In contrast the Emp47p AT-antibody produced a punctate staining (Fig. 2 *B*, *left*). The fluorescent structures, typically between 4 and 10 per mother cell, are often of elongated shape and vary in size and fluorescence intensity. We did not notice any accumulation in any particular region of either the mother or the daughter cell. Strains deleted for EMP47 did not stain with the anti tail antibody (data not shown).

The steady-state Emp47p immunofluorescence pattern is most reminiscent of that reported for Golgi proteins in yeast, such as the peripheral protein Sec7p and the transmembrane endoprotease Kex2p (Franzusoff et al., 1991; Redding et al., 1991). Another protein with a Golgi-like distribution in yeast is Pmr1p (Antebi and Fink, 1992). In a double immunofluorescence experiment we demonstrated that Emp47p and HA-Pmr1p (tagged with the influenza hemagglutin-epitope, Antebi and Fink, 1992) exhibit 99 and 89% colocalization, respectively (Fig. 2 *B*, *legend*).

Emp47p Comigrates with Golgi Proteins during Subcellular Fractionation

We used a second independent method to determine the steady-state localization of Emp47p in relation to other subcellular markers. Spheroplasts of cells transformed with an episomal plasmid expressing HA-Pmr1p were broken and the supernatant of a 500 *g*-spin was fractionated by sedimentation on a sucrose gradient (similar to Antebi and Fink, 1992). The gradient fractions were analyzed by the appropriate enzymatic assays or by immunoblotting. The results are shown in Fig. 3. In accordance with the immunofluorescence data (Fig. 2 *B*) Emp47p cofractionated with HA-Pmr1p. Both migrated together in the middle of the gradient. Emp47p separated almost completely from the ER marker Wbp1p (te Heesen et al., 1991), which occupied the bottom fractions of the gradient. The vacuoles hardly entered the gradient and also separated from Emp47p (Vacuolar ATPase, 60-kD subunit; and alkaline

Table II. Quality Scores of Pairwise Alignments Performed with the Program BESTFIT

	Emp47p		VIP36	
	Complete	aa 1-250	Complete	aa 1-250
ERGIC-53	166.5 (8.6)	98.1 (7.4)	178.5 (21.7)	159.5 (25.6)
VIP36	118.8 (5.5)	100.8 (7.8)		

For each sequence comparison a mean random quality score and its standard deviation was obtained by performing 100 alignments with one sequence randomized (using default parameters). They were used to calculate the values given in parentheses: quality score of actual alignment minus mean score of random alignments divided by standard deviation of random alignments. *aa1–250*, comparison of the first 250 amino acids of the respective sequences; *complete*, comparison with the whole sequences.

A

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-488  GAATTCCCCTCAGGGCCCTCGGAAACTGTCACCCCTTCGTCTTCATCTTCCTGCACTGCCTTCAGGTTGTCCATACCGGCTTCTATACCA  -399

-398  AACAACTGCCTCCCATGACTGACTTCAAATTGCATTCTTGACGCAAATAATTGAATGAACAAGTCGCATTGCTCACAACCATGGTATAAT  -309

-308  CTATCAAAAAGCACATCTAGACCTGAGACAAGGTCTTCAGACCAAAAATAGTCGCAAAATGATAGCATGCTCGCTTGCAGGTGTACAACA  -219

-218  TCTATTACAATTCAACGGAATATTATTCCTGTTTCATAATATTTCAATTTCCATCTATTAATGTCATTACGTCTTCGTATCACTATACAC  -129

-128  GAGTAGAGAAACAGTATGAACTCTTAAGAATGTTTTTGATCTTTTAATAGAAGCCTTAATTGGTATTGAATAATTAGAATTATACCAATA  -39

-38   AAATTAACAAATAGTTAGCATCAGGGAATCTGTATTTTATGATGATGTTAATTACTATGAAAAGTACAGTACTGTTGAGTGTTTTTACCG  51
                                              M  M  M  L  I  T  M  K  S  T  V  L  L  S  V  F  T  V  -11

52    TCTTAGCGACATGGGCTGGATTGCTAGAAGCTCATCCATTGGGTGACACTTCAGATGCATCCAAATTAAGCTCAGACTACTCGCTCCCTG  141
-10    L  A  T  W  A  G  L  L  E  A  H  P  L  G  D  T  S  D  A  S  K  L  S  S  D  Y  S  L  P  D    20

142   ATCTCATTAATACACGTAAAGTGCCCAATAACTGGCAAACTGGAGAACAAGCTAGTCTAGAGGAAGGGAGAATTGTATTGACTTCTAATC  231
21     L  I  N  T  R  K  V  P  N  N  W  Q  T  G  E  Q  A  S  L  E  E  G  R  I  V  L  T  S  N  Q    50

232   AAAATTCCAAGGGGTTCACTTTGGTTGAAGCAAGGATTCGATTTGAAGGATTCTTTTACTATGGAGTGGACATTTAGGAGTGTTGGTTATT  321
51     N  S  K  G  S  L  W  L  K  Q  G  F  D  L  K  D  S  F  T  M  E  W  T  F  R  S  V  G  Y  S    80

322   CTGGCCAAACCGACGGTGGCATATCATTTTGGTTTGTTCAAGATTCTAACATACCACGCGATAAGCAGTTATACAATGGGCCAGTGAACT  411
81     G  Q  T  D  G  G  I  S  F  W  F  V  Q  D  S  N  I  P  R  D  K  Q  L  Y  N  G  P  V  N  Y   110

412   ATGATGGTTTACAATTATTAGTGGATAACAATGGTCCATTGGGCCCAACACTTCGTGGTCAACTAAATGATGGTCAAAAGCCTGTAGATA  501
111    D  G  L  Q  L  L  V  D  N  N  G  P  L  G  P  T  L  R  G  Q  L  N  D  G  Q  K  P  V  D  K   140

502   AGACCAAAATCTATGATCAGAGTTTTGCATCTTGTTTGATGGGTTATCAGGATTCCTCCGTTCCTTCCACGATCAGAGTAACTTATGATT  591
141    T  K  I  Y  D  Q  S  F  A  S  C  L  M  G  Y  Q  D  S  S  V  P  S  T  I  R  V  T  Y  D  L   170

592   TGGAAGACGACAACTTATTAAAGGTTCAGGTGGACAATAAAGTCTGTTTCCAAACTAGGAAGGTTCGTTTTCCCTCTGGGTCTTACCGTA  681
171    E  D  D  N  L  L  K  V  Q  V  D  N  K  V  C  F  Q  T  R  K  V  R  F  P  S  G  S  Y  R  I   200

682   TTGGTGTCACTGCTCAAAATGGAGCAGTGAATAATAATGCAGAGTCTTTTGAAATATTCAAAATGCAATTTTTTAATGGAGTGATTGAAG  771
201    G  V  T  A  Q  N  G  A  V  N  N  N  A  E  S  F  E  I  F  K  M  Q  F  F  N  G  V  I  E  D   230

772   ATTCTTTGATCCCTAATGTGAATGCAATGGGTCAGCCAAAACTGATCACCAAATACATTGACCAACAAACCGGCAAAGAAAAATTGATTG  861
231    S  L  I  P  N  V  N  A  M  G  Q  P  K  L  I  T  K  Y  I  D  Q  Q  T  G  K  E  K  L  I  E   260

862   AAAAAACAGCATTTGACGCTGACAAAGACAAAATTACAAACTATGAATTATATAAGAAACTGGATAGAGTTGAAGGTAAAATTCTTGCGA  951
261    K  T  A  F  D  A  D  K  D  K  I  T  N  Y  E  L  Y  K  K  L  D  R  V  E  G  K  I  L  A  N   290

952   ACGATATCAATGCTTTAGAAACAAAGCTAAATGATGTCATTAAGGTCCAACAAGAGCTATTATCATTCATGACTACGATAACTAAACAGC  1041
291    D  I  N  A  L  E  T  K  L  N  D  V  I  K  V  Q  Q  E  L  L  S  F  M  T  T  I  T  K  Q  L   320

1042  TCTCTTCTAAGCCACCAGCTAATAATGAAAAGGGAACGTCCACCGATGATGCAATCGCTGAGGATAAAGAAAATTTCAAAGACTTCTTAT  1131
321    S  S  K  P  P  A  N  N  E  K  G  T  S  T  D  D  A  I  A  E  D  K  E  N  F  K  D  F  L  S   350

1132  CAATCAATCAGAAATTGGAGAAAGTCCTGGTTGAACAAGAAAAGTATAGGGAAGCTACCAAACGTCATGGACAAGATGGTCCTCAGGTCG  1221
351    I  N  Q  K  L  E  K  V  L  V  E  Q  E  K  Y  R  E  A  T  K  R  H  G  Q  D  G  P  Q  V  D   380

1222  ACGAAATTGCCAGAAAACTAATGATTTGGTTACTTCCATTGATTTTCATCATGTTGGTTATGGCATATTACACATTCAGAATCAGACAAG  1311
381    E  I  A  R  K  L  M  I  W  L  L  P  L  I  F  I  M  L  V  M  A  Y  Y  T  F  R  I  R  Q  E   410

1312  AGATCATAAAGACCAAACTGCTATGATTTTGGATTATTCCTTCCACTCAAACCAATTGTGCACATGCATTCCCTATATATGTATATATAT  1401
411    I  I  K  T  K  L  L  *

1402  GTAACAATACATATTTTATAAAATAACGAACGATAGTTTATATTACTACTTCAACAATGGTTTATTTCTTATTGGAGTGTTCCCTACATT  1491

1492  ATGTTTTGCTTCTTTCAATTATTCATAGCTACTTCGTTGCTGAAGTTAGCCAGGTTGTCCGGTTTAGCGGCGACCGGATCCGTCGCCACC  1581

1582  ACTGGATTAGAATTTGTGTTCTTTACTTCAGTGTCCAGCTTAACAGCCGTTTTATCCCCAGCGACATTTTGTGGTACTTTGTTCACATGA  1671

1672  GAATAAATGTATGGAGGTTTAAAATCCTTAATGTTACAATTTCCTAGGAAGTTTGGGTTCGGTACCCTCTTTGTTGTTATTGTTTCATCC  1761

1762  GTATTCGTTATTTCGTTAAAAATTTGTAGTAACACTCGTTTGCGCCAACACATCTTTCAAATATTTATTTTTGAATTC  1838
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Figure 1. Structure of Emp47p. (*A*) Nucleotide sequence and derived amino acid sequence of *EMP47.* Numbering of nucleotides begins at the start of the open reading frame. Numbering of amino acids begins at the first amino acid of the mature protein as determined by direct NH₂-terminal protein sequencing (*dashed line*). The putative transmembrane-domain is underlined. These sequence data are available from GenBank/EMBL/DDBJ under accession number X87622. (*B*) Biophysical parameters of Emp47p as determined with the program PEPTIDESTRUCTURE and PEPCOIL (*GCG*). Numbering starts with first amino acid translated from the open reading frame. Hydrophobicity calculated according to Kyte-Doolittle (1982), Turns (indicates β-turns), α-helices and β-sheet predictions according to Garnier et al. (1978), coiled-coil probability according to Lupas et al. (1991). (*C*) Multiple alignment of the first 250 amino acids of the mature polypeptides of ERGIC-53, VIP36 (putative NH₂ terminus according to Fiedler et al., 1994) and Emp47p. The alignment was produced with PILEUP (*GCG*) using a gap weight of 2.4 and a gap length weight of 0.1 and plotted with the program PRETTYPLOT (*GCG*) using the default parameters. Homologous residues according to PILEUP are boxed. (*D*) Multiple alignment of

B
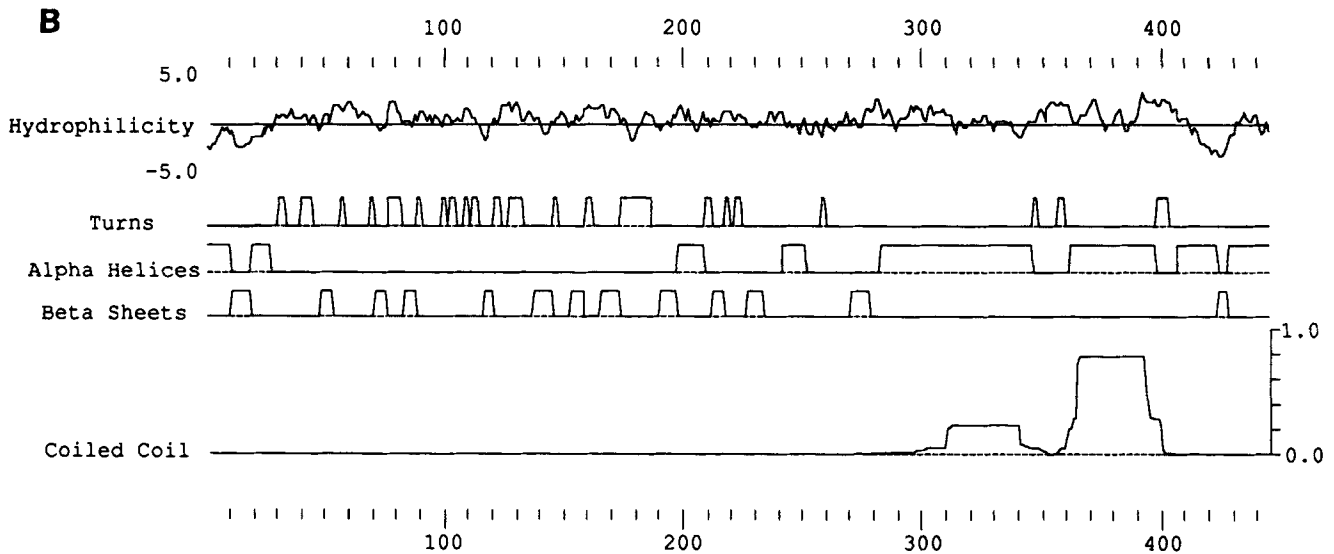
C

Emp47p	H P L G D T S D A S - - - - K L S S D Y S L - - P D L I N T R K V P N N W Q T G E Q A S L E E G R I	44
Ergic53	D G V G G D P A V A L P H R R F E Y K Y S F K G P H L V Q S D G T V P F W A H A G N A I P S S D Q I	50
VIP36	D I T D G N S E - - - - - - H L K R E H S L I K P Y Q G V G S S S M P L W D F Q G S T I L T S Q Y V	44

Emp47p	V L T S N Q N S K - G S L W L K Q G F D L K D S F T M E W T F R - - S V G Y S G Q T D G G I S F W F	91
Ergic53	R V A P S L K S Q R G S V W T K T K A A F E N - W E V E V T F R V - - T G R G R I G A D G L A I W Y	97
VIP36	R L T P D E R S K E G S I W N H Q P C F L K D - W E M H V H F K V H G T G K K N L H G D G I A L W Y	93

Emp47p	V Q D S N I P R D K Q L Y N G P V N Y D G L Q L L V D - - - - - - N N G P L G P T L R G Q L N D G Q	135
Ergic53	A E N Q G L E - - G P V F G S A D L W N G V G I F F D T F D N D G K K N N - - P A I V I I G N N G Q	143
VIP36	T R D R L V P - - G P V F G S K D N F H G L A I F L D T Y P N D E T T E R V F P Y I S V M V N N G S	141

Emp47p	K P V D K T K I Y D - Q S F A S C L M G Y Q D S S V P S T I R V T Y D - - - - - - L E D D N L L K V	178
Ergic53	I H Y D H Q N D G A S Q A L A S C Q R D F R N K P Y P V R A K I T Y Y Q N T L T V M I N N G F T P D	193
VIP36	L S Y D H S K D G R W T E L A G C T A D F R N R D H D T F L A V R Y S R G R L T V M T D L E - - - D	188

Emp47p	Q V D N K V C F Q T R K V R F P S G S Y R I G V T A Q N G A V N N N A E S F E I F K M Q F F N G V I	228
Ergic53	K N D Y E F C A K V E N M I I P A Q G H - F G I S A A T G G L A D D H D V L S F L T F Q L T E P G K	242
VIP36	K N E W K N C I D I T G V R L P T G Y Y - F G A S A G T G D L S D N H D I I S M K L F Q L - - - - M	233

Emp47p	E D S L I P N V N A M G Q P K L I T K Y I D	250
Ergic53	E P P - T P D K E - - - - - - - - - - - - -	250
VIP36	V E H - T P D E E N I D W T K I E P - - - -	250

D

Emp47p	K L M I W L L P L I F I M L V M A Y Y T F R I R Q E I I K T K L L	417
Ergic53	T V H F I I F V V V Q T V L F I G Y I M Y R S Q Q E A A A K K F F	480
VIP36	L L L L C A L L G I I V C A V V G A V V F Q K R Q E - R N K R F Y	312

the last 33 amino acids of ERGIC-53 and Emp47 and the last 32 amino acids of VIP36. The alignment was produced with PILEUP (*GCG*) using a gap weight of 2.5 and a gap length weight of 0.1 and plotted with the program PRETTYPLOT (*GCG*) using the default parameters. Homologous residues according to PILEUP are boxed. The arrow indicates the putative border between the transmembrane domains and the COOH-terminal cytoplasmic tails.

phosphatase, data not shown). Functionally the *cis*-most Golgi marker identified thus far is Och1p (Nakanishi-Shindo et al., 1994; Gaynor et al., 1994). Och1p consistently migrated in a very sharp peak displaced by at least one fraction from Emp47p. The overlap with other as-

sayed Golgi markers was best for GDPase (Abeijon et al., 1989). The majority of GDPase is located *cis* of the *trans*-most Golgi marker Kex2p and may be in all Golgi subcompartments that contain mannosyltransferases (Cunningham and Wickner, 1989; Bowser and Novick, 1991;
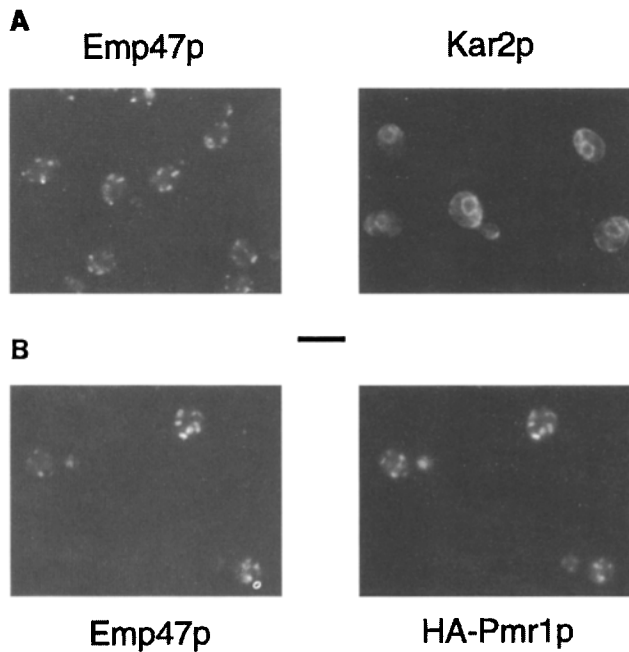
A

Emp47p   Kar2p

B

Emp47p   HA-Pmr1p

Figure 2. Emp47p localization. (*A*) Emp47p staining is different from Kar2p staining representing ER. Fixed and permeabilized RH732 cells were incubated with Emp47p anti-tail antibody (*left*) or anti-Kar2p antibody (*right*). Primary antibodies were visualized using Cy3-conjugated goat–anti rabbit IgG. (*B*) Emp47p and HA-Pmr1 colocalize. RH448 cells transformed with pl161, coding for HA-Pmr1p (Antebi and Fink, 1992) were fixed, permeabilized, and then simultaneously incubated with 12CA5 (directed against the HA-Epitope of HA-Pmr1) and AT-antibody. 12CA5 was visualized with FITC-conjugated rabbit anti–mouse IgG and the anti-tail antibody with Cy3-conjugated goat anti–rabbit IgG. Because pl161 is a multicopy-plasmid strong variance in HA-Pmr1p expression levels from cell to cell were seen. 57 cells with an HA-Pmr1-signal comparable to the one seen in the figure were used for quantitation of the colocalization. In these cells a total of 224 structures positive for both markers were counted. Two structures were positive for Emp47p only and 29 structures were positive for HA-Pmr1p only. This amounts to a colocalization of 99% between Emp47p and HA-Pmr1p and of 89% between HA-Pmr1p and Emp47p. Bar, 6 μm.

Graham et al., 1994). Kex2p also migrated in the middle of the gradient, but its profile was different from that of Emp47p.

Emp47p Has Access to the α1,3 Mannosyltransferase Containing Golgi Subcompartment

Emp47p does not have any putative N-linked glycosylation sites. We wanted however to use the analysis of outer chain glycosylation as a further tool for collecting evidence about the localization of Emp47p. Therefore, we introduced two overlapping N-linked glycosylation consensus sites (NXT/S) at position 49 and 50 of the mature protein. This was achieved by mutating amino acids Q_{50} to N and N_{51} to T, creating the sequence NNTS. The mutant Emp47p was also tagged with a c-myc epitope immediately after the signal-peptidase cleavage site at amino acid 9. We integrated *myc-glyco-EMP47* into the genome of a haploid strain in which *EMP47* was deleted (nucleotides 106–1375 replaced with *LYS2*, see Materials and Methods). In a

pulse-chase experiment, we radiolabeled cells with [^{35}S]methionine and cysteine for 5 min at 30°C and then chased for 0 min or 30 min at the same temperature. Cell extracts were prepared, first precipitated with the AT-antibody and reprecipitated with either the same antibody or a polyclonal antibody against α1,3-linked mannose. The precipitates of the second round of precipitation were then analyzed by SDS-PAGE followed by autoradiography (Fig. 4 *A*). An isogenic strain expressing myc-tagged Emp47p was treated the same way in a parallel experiment. After the 5-min pulse the AT-antibody precipitated one sharp band ∼49 K corresponding to myc-Emp47p (the weak extra band at ∼62 K is not related to Emp47p as it is also present in a delete strain, data not shown). Myc-Emp47p was stable during the chase period. 76% of the signal precipitated by the AT-antibody immediately after the pulse was recovered after the 30-min chase (quantification by PhosphoImager). In the case of the myc-glyco-Emp47p a prominent band of slightly higher molecular mass was precipitated, probably corresponding to the core glycosylated protein. In addition the autoradiogram revealed a broad smear of radioactivity ranging in apparent molecular mass up to >100 kD. This is typical of glycoproteins with extensive α1,6-, α1,2-, and α1,3-linked mannose outer chain modifications. 54% of the total myc-glyco-Emp47p had received outer chain modifications after the pulse and 89% after the chase. The core-glycosylated myc-glyco-Emp47p had largely disappeared after the chase (only 16% remained) while the high molecular smear slightly increased in intensity (106% of the 0 min chase value). After the chase we further noted the appearance of a new band at slightly lower mobility than that of the core-glycosylated myc-glyco-Emp47p.

In yeast outer chain modifications of N-linked glycans have been allocated to functionally successive steps corresponding to different Golgi cisternae. Only recently it was found that the first α1,6-linked mannose is added to the core oligosaccharide by Och1p (Nakanishi-Shindo et al., 1994). Och1p also operationally defines a new *cis*-Golgi compartment (Gaynor et al., 1994). It is separated from the elongating α1,6 mannosyltransferase and the α1,3 mannosyltransferase that had previously been defined as *cis* and *medial*-Golgi markers, respectively (Graham and Emr, 1991). To study whether Emp47p can be modified by the α1,3 mannosyltransferase we precipitated the newly synthesized myc-glyco-Emp47p with the anti-α1,3 serum. As can be seen in Fig. 4, immediately after the pulse, 63% of the high molecular mass smear that was precipitated with the AT-antibody could be precipitated by the anti α1,3 serum as well. After 30 min of chase the intensity of the anti α1,3 mannose precipitate reached 95% of the intensity of the total smear. Thus almost complete α1,3 mannose modification of the N-linked carbohydrates was observed within 30 min. As expected myc-Emp47p was not precipitated by the anti α1,3 serum. This also provided an internal control for the complete inactivation of the AT-antibody from the first round of precipitation. The experiment supports the view that Emp47p rapidly reaches a *medial*-Golgi compartment as defined by the α1,3 mannosyltransferase.

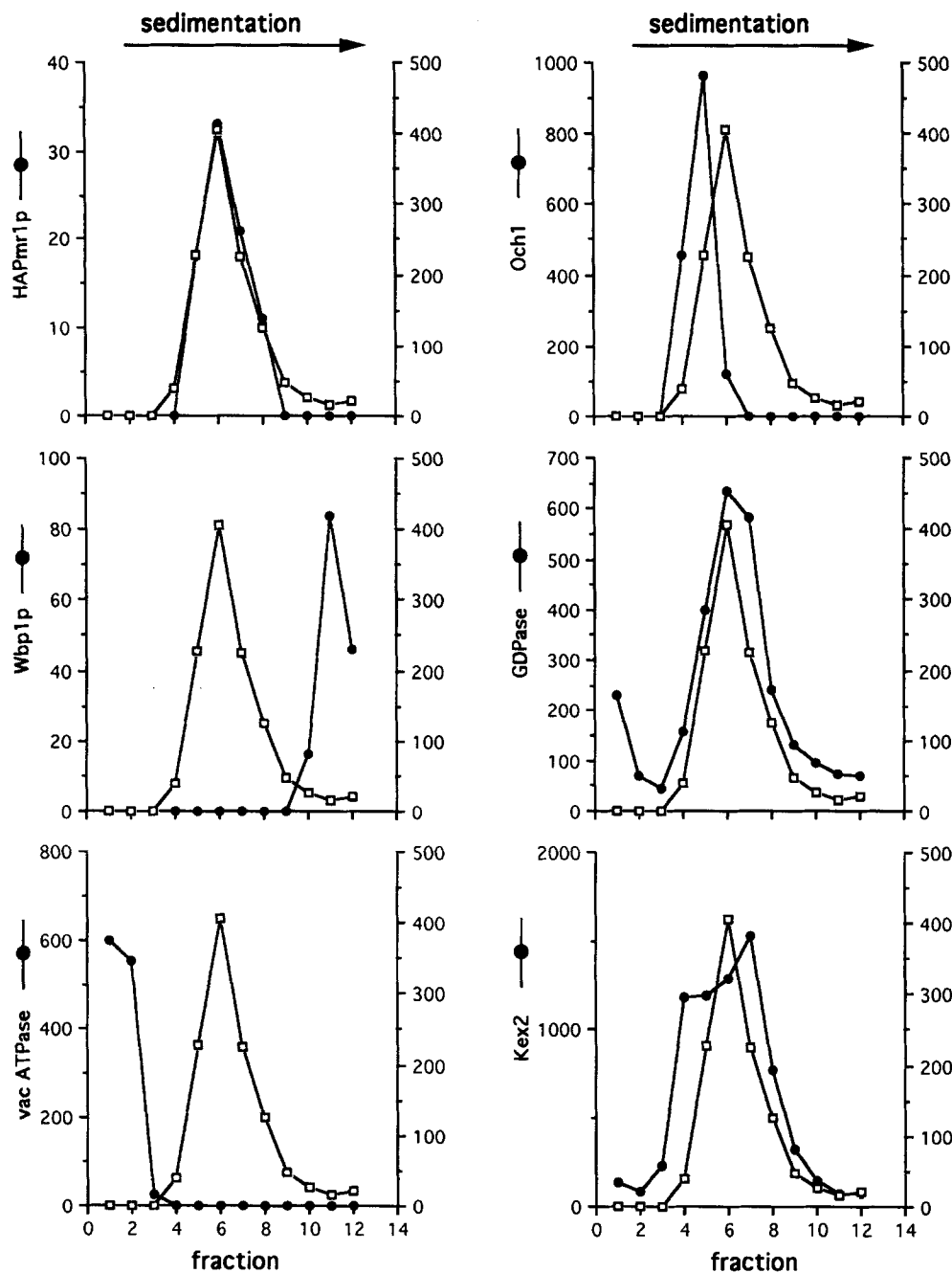Visualization of myc-glyco-Emp47p by immunofluorescence (Fig. 4 *B, top*) revealed a pattern similar to that of

Figure 3. Migration of organelle-specific markers in sucrose density gradients. RH448 cells transformed with pl161 were grown overnight at 30°C in minimal glucose medium supplemented with leucine, histidine, and lysine (20 μg/ml each) to 0.5 × 10^7 cells/ml. Cells were harvested and resuspended in complete medium for 2 h at 30°C. Cells (2 × 10^9) were converted to spheroplasts and lysed. The lysate, cleared by two 500 *g* spins, was loaded on top of the sucrose gradient and centrifuged as described in Materials and Methods. The arrow indicates the direction of sedimentation. Emp47p, HA-Pmr1p, Wbp1p, Och1p, and the 60-kD subunit of the vacuolar ATPase were Western blotted and detected with ECL and quantified on a densitometer (arbitrary units). Kex2p and GDPase were detected with enzymatic assays (see Materials and Methods, arbitrary units).

Emp47p (compare Figs. 2 *A* and 5, *top*), indicating that introducing the tags did not cause a mislocalization of the protein. This was confirmed by coexpressing HA-Pmr1p and performing double-immunofluorescence (Fig. 4 *B*, *middle* and *bottom*). Quantification (see Fig. 4 *B*, legend) yielded a colocalization between myc-glyco-Emp47p and HA-Pmr1p of 98%.

Steady-state Golgi Localization Depends on COOH-terminal KXKXX Sequence

We next determined whether the COOH-terminal KTKLL sequence plays a role in the steady-state localization of Emp47p. For that purpose we created a set of site-directed mutations affecting the consensus features of di-lysine–

based signals. All constructs were tagged with a myc-epitope at amino acid 9 of the mature protein because our AT-antibody was not expected to recognize the mutated tails. As it is possible that Emp47p forms higher order complexes, we integrated the mutant genes into the genome of a haploid *pep4* strain in which *EMP47* was deleted (nucleotides 106–1375 replaced with *LYS2*, see Materials and Methods). The *pep4* mutation, which alleviates the problem of vacuolar proteolysis, was used because we had preliminary indications that some tail-mutant proteins were rapidly degraded.

The immunofluorescence analysis of some key constructs is shown in Fig. 5, while the results with the other constructs are summarized in Table III. The c-myc tagged but otherwise unchanged Emp47p yielded the wild-type
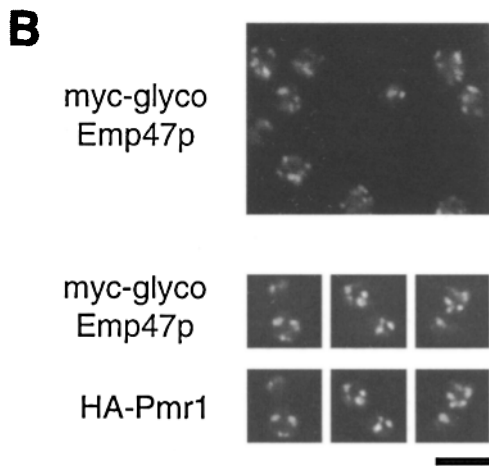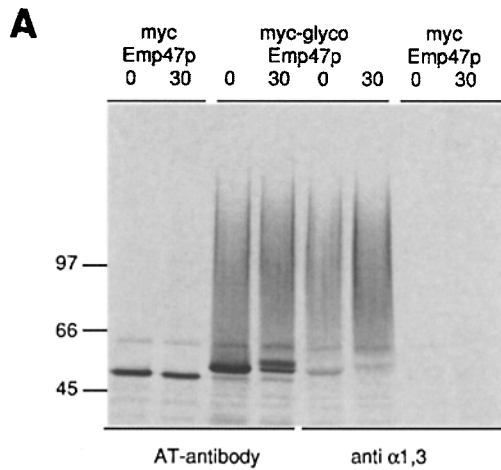
A

B

myc-glyco
Emp47p

myc-glyco
Emp47p

HA-Pmr1

Figure 4. Glycosylation and localization of an Emp47p variant. (*A*) Cultures of strains RH3046 (myc-glyco-Emp47p) and RH-3047 (myc-Emp47p) were grown at 24°C, radiolabeled for 5 min at 30°C, and then chased at the same temperature for 0 min or 30 min as indicated. Cell extracts were prepared and precipitated first with AT-antibody. Precipitates were reprecipitated with either AT-antibody or with antiserum against α1,3 linked mannose as denoted. The final precipitates were separated by 8% SDS-PAGE (migration of molecular weight standards of 45, 66, and 97 K is indicated) and the gels were analyzed by exposure to film and PhosphoImager-plates. (*B*) (*Top*) Immunofluorescence labeling of myc-glyco-Emp47p in RH3046 using 9E10 anti-myc antibody; (*middle* and *bottom*) double-immunofluorescence on RH3046 transformed with pl161, stained for myc-glyco-Emp47p with AT-antibody (*middle*) and HA-Pmr1p with HA-antibody (*bottom*). The images of these cells were recorded with a video equipment, adjusted to similar background and brightness with the shadow/highlight tool of the image editor PSP (JASC Inc., Minnetonka, MN) and printed on a 600 dpi printer. 20 cells with an HA-Pmr1–signal comparable to the one seen the figure were used for quantitation of the colocalization. In these cells a total of 81 structures positive for both markers were counted. Two structures were positive for Emp47p only and one structure was positive for HA-Pmr1p only, yielding a colocalization of 98% between Emp47p and HA-Pmr1p and of 99% between HA-Pmr1p and Emp47p. Bar, 6 μm.

punctate pattern (Fig. 5, *top*). Moving the lysine at position -5 to position -4 did not change the pattern, demonstrating the equivalence of KXKXX and KKXX in this case (Fig. 5, *TKKLL*). Changing lysine -3 to glutamine
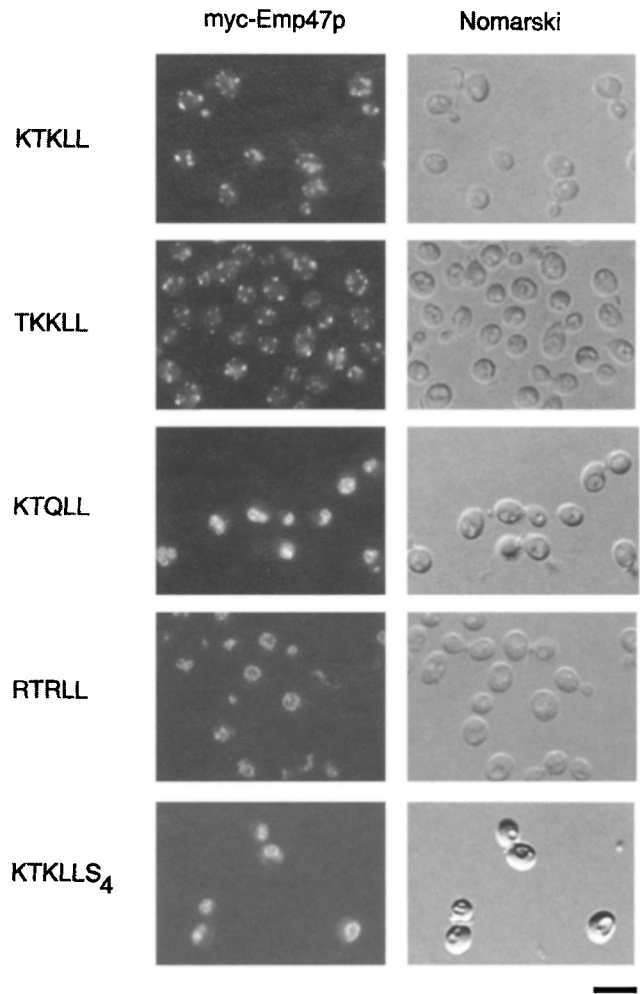

Figure 5. Influence of the COOH-terminal KXKXX motif of Emp47p on its intracellular steady-state localization. Constructs encoding myc-tagged Emp47p with a wild-type or mutated dilysine motif were expressed in RH2825 (deleted for *EMP47*). Each construct is denoted by its last five COOH-terminal amino acids. KTKLLS$_4$ indicates the construct in which the wild-type sequence was tailed with four serines. The left panels show the immunofluorescence patterns detected with the 9E10 antibody directed against the myc-epitope. The right panels show the corresponding Nomarski image with the vacuoles appearing as indentations. Bar, 6 μm.

however led to a complete redistribution of the mutant protein to the vacuolar membrane. This is obvious from comparing the immunofluorescence with the corresponding Nomarski image (Fig. 5, *KTQLL*). As summarized in Table III, changing lysine -5 or both lysines also resulted in vacuolar staining. The same was true for the deletion of the KTKLL sequence. Like for other di-lysine motifs, arginines replacing the lysines resulted in loss of function and the mutant Emp47p was found in the vacuole (Fig. 5, *RTRLL*). Furthermore, the sequence KTKLL displayed a position dependence. Adding four serines at the COOH-terminus of Emp47p also resulted in a vacuolar staining pattern (Fig. 5, *KTKLLS$_4$*). Thus the KTKLL sequence in Emp47 displays all the hallmarks of a di-lysine ER-localization motif. The Emp47p-tail is also sufficient to confer ER-localization to the plasma membrane protein Ste2p

COOH-terminal sequence	IF pattern	
	Punctate	Vacuolar
wt: ..RIRQEIIKTKLL	+	
..RIRQEIIQTKLL		+
..RIRQEIIQTQLL		+
..RIRQEII		+
..RIRQEIIKTKAA	+	
..RIRLAIIKTKLL	+	

Site-directed mutations are indicated in bold type. For comparison the wild-type sequence of the cytoplasmic tail of Emp47p (*wt*) is given as well.

upon transplantation (see below). Therefore Emp47p is the first known protein in which a functional di-lysine motif is essential for a Golgi localization.

Mutating two other features that are conserved in the cytoplasmic tails of Emp47p, ERGIC-53, and VIP36 (Fig. 1 *C*) did not change the wild-type distribution. Those mutations (Table III) are the exchange of the two COOH-terminal leucines (consensus: large hydrophobic) with alanine and the exchange of the strictly conserved hydrophilic/charged amino acids QE for LA (hydrophobic/neutral).

Emp47p Can Recycle to the ER

Di-lysine ER-localization motifs can bring about a steady-state ER-localization by recycling (see Introduction and Discussion). To test whether Emp47p could also follow a recycling pathway to the ER we made use of the *sec12* mutant. In *sec12* secretion is blocked before the budding of transport vesicles from the ER at nonpermissive temperature (Nakano et al., 1988; Barlowe and Schekman, 1993). If recycling still occurs under these conditions and if Emp47p follows this pathway it should be possible to redistribute Emp47p to the ER, thus depleting the Golgi pool at the same time.
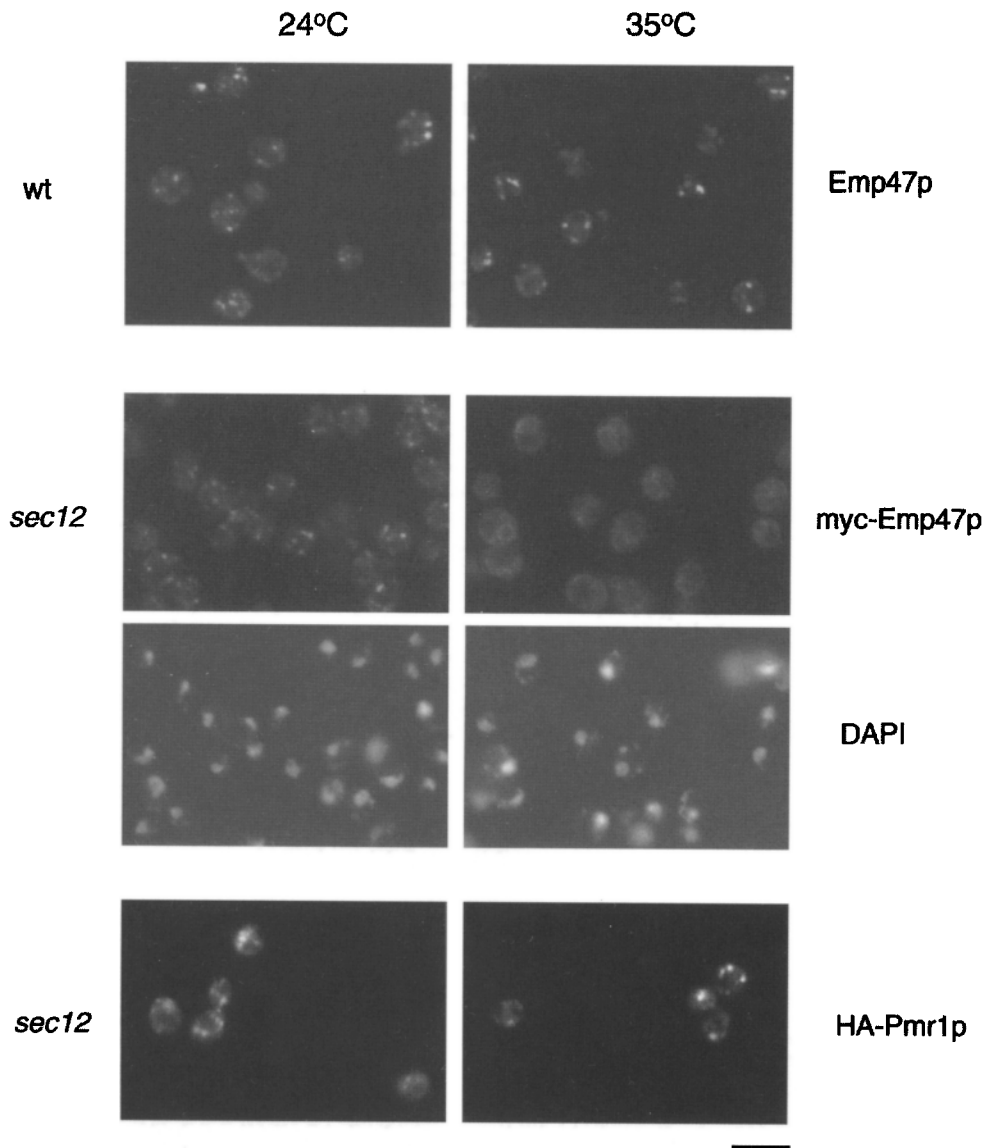

Figure 6. Indirect immunofluorescence analysis of the dynamics of intracellular distribution of Emp47p and Pmr1p in *sec12* cells. *sec12* cells expressing either *myc-EMP47* integrated at the *leu2* locus (*sec12, middle*) or *HA-PMR1* from the 2μ-plasmid pl161 (*bottom*) were incubated for 1 h in the presence of 20 μg/ml cycloheximide at either permissive (24°C) or nonpermissive temperature (35°C). The wild-type strain RH448 (*wt, top*) was treated the same way in parallel. Emp47p in RH448 was detected with AT-antibody. The images of the latter cells were recorded with a video equipment and printed on a 600 dpi printer. myc-Emp47p was detected with 9E10 antibody. HA-Pmr1p was detected with 12CA5. Bar, 6 μm.

Wild type or *sec12* cells (in some cases transformed to express a particular marker-protein) were grown at the permissive temperature (24°C). Cycloheximide was added to avoid a contribution of newly synthesized Emp47p. Then aliquots were put at either 24 or 35°C (nonpermissive) for 1 h. Cells were then fixed and processed for immunofluorescence. Fig. 6 shows that wild-type cells still displayed a punctate pattern after incubation at both temperatures (*top*). *sec12* cells expressing myc-Emp47p in addition to the endogenous Emp47p also retained a punctate pattern after incubation at permissive temperature (Fig. 6, *sec12*, 24°C). At restrictive temperature, however, the immunofluorescence pattern in *sec12* cells changed qualitatively. The punctate structures disappeared and a ring around the nucleus (compare corresponding DAPI stain) and some staining in the periphery of the cell became visible. This pattern is equivalent to ER-staining in yeast as exemplified by the Kar2p-immunofluorescence in Fig. 2 *A*. The comparatively low intensity of the Emp47p immunofluorescence signal after recycling was most likely due to the distribution of the antigen over a much larger surface area.

The change in the immunofluorescence pattern of Emp47p is consistent with the interpretation that Emp47p redistributed from the Golgi to the ER upon imposing the *sec12* block. Importantly, Pmr1p, with which Emp47p colocalizes under normal growth conditions, does not qualitatively change its punctate appearance in immunofluorescence when the *sec12* block is imposed (Fig. 6, *bottom*). Therefore, the redistribution seen for Emp47p was not simply due to a change of morphology of the Emp47p compartment nor to a general merging of the Golgi with the ER.

We also applied the sucrose gradient analysis to monitor the changes in intracellular distribution of Emp47p after a *sec12* block. *sec12* cells were incubated in the presence of cycloheximide at permissive or nonpermissive temperature. They were then spheroplasted and lysed. The cleared lysates were fractionated on sucrose-gradients as described in Materials and Methods. The immunoblot-quantification of the gradient fractions is shown in Fig. 7 *A*. It provides quantitative evidence for the recycling of Emp47p that is complementary to the immunofluorescence analysis. 1 h after imposing the *sec12* block, Emp47p was redistributed from its steady-state position in the middle of the gradient to the bottom of the gradient (Fig. 7 *A*, *top*), where the ER marker Wbp1p migrated under both *sec12* permissive and nonpermissive conditions (Fig. 7 *A*, *middle*). The *cis*-most Golgi marker, Och1p, did not change its migration properties after the *sec12* block (Fig. 7 *A*, *bottom*). This is similar to what we had observed for the HA-Pmr1p localization in the immunofluorescence experiment (Fig. 6, *bottom*) and provides additional evidence that the Golgi apparatus is still present. A quantification of the kinetics of the redistribution process is shown in Fig. 7 *B*. We added NaF and NaN₃ at various times after shift to the *sec12* nonpermissive temperature (37°C). One cell aliquot was kept at the permissive temperature (24°C) throughout the duration of the experiment. Golgi and ER fractions were then separated on sucrose gradients as above. Significant redistribution of Emp47p took place only at 37°C and it was time and energy dependent with a half time of retrograde transport of ~30 min.
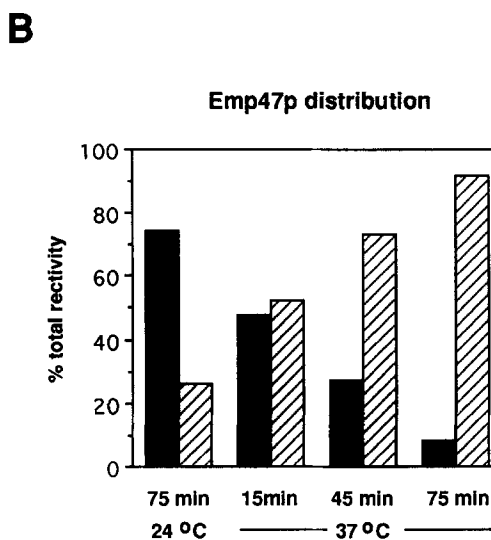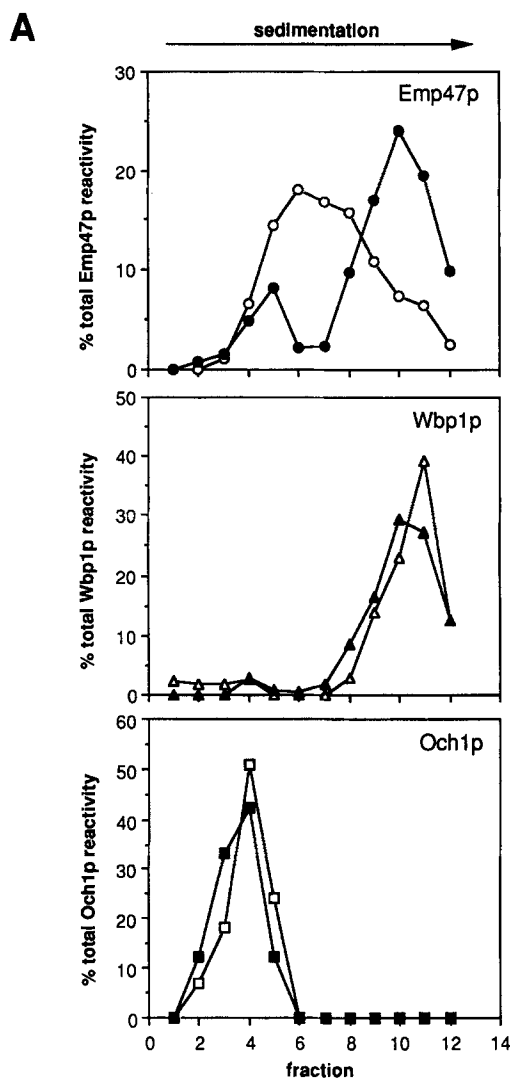

Figure 7. Dynamic intracellular redistribution of Emp47p in *sec12* cells observed by velocity sedimentation on sucrose gradients. RH1491 (*sec12*) cells were incubated for the indicated times at either permissive (24°C) or nonpermissive (35 or 37°C) temperature in the presence of 20 μg/ml cycloheximide before spheroplasting and homogenization. Lysates cleared by a spin at

α1,3 Mannose-modified Emp47p Can Recycle to the ER

The myc-glyco-Emp47p provided a tool to address the question from which Golgi subcompartment recycling can occur. We radiolabeled *sec12* cells stably expressing the myc-glyco-Emp47p. Labeling was performed at 24°C for 15 min followed by a 10-min chase at the same temperature. We then added cycloheximide and incubated aliquots at either 24 or 35°C. Cells were spheroplasted, broken, and then the lysate was spun at 500 g. Supernatants of that spin were separated on sucrose gradients as described in Materials and Methods. The gradient fractions were first immunoprecipitated with AT-antibody. The precipitates were then split into aliquots and reprecipitated with either AT-antibody or with antiserum against α1,3-linked mannose. Fig. 8 shows the PhosphoImager quantification of the endogenous Emp47p and the high molecular weight "smear" precipitated by the antiserum against α1,3 linked mannose. It can be seen that both the nonglycosylated Emp47p and the α1,3 mannose modified Emp47p variant qualitatively behaved in the same way. At the permissive temperature for *sec12* the majority of the radioactivity was found in the middle of the gradient. After shift to 35°C for 1 h the Golgi-signal decreased in size and the signal from the ER fractions increased. Therefore we conclude that myc-glyco-Emp47 can recycle from an α-1,3 mannosyltransferase containing Golgi compartment to the ER.

Steady-state Localization of Emp47p Is Unchanged in a ret 1-1 Mutant Cell

Letourneur et al. (1994) isolated a mutant in α-COP (*ret1-1*) that was deficient in the intracellular retention of an α-factor receptor (Ste2p) chimera bearing the cytosolic, COOH-terminal di-lysine motif of Wbp1p. To determine whether the di-lysine motif of Emp47p would also be recognized by α-COP and whether Emp47p would depend on α-COP for its steady-state Golgi-localization, we fused the 12–amino acid tail of Emp47p to Ste2p and expressed the construct together with myc-Emp47p in *MATa* cells that were deleted for the endogenous *STE2* and were either wild type or *ret1-1*. The cells were grown at 30°C, conditions under which *ret1-1* is deficient for the retention of Ste2p-Wbp1ptail (Letourneur et al., 1994). We then examined the intracellular distribution of myc-Emp47p and Ste2p-Emp47ptail by immunofluorescence. The results are

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500 g were loaded onto the sucrose gradients and spun as described in Materials and Methods. The sucrose gradient fractions were separated by SDS-PAGE and immune blotted with AL-antibody, anti-Wbp1p, and anti-Och1p. Immunoreactivity was detected with ECL and the signals quantified by densitometry. (*A*) Gradient profiles after 1 h incubation at permissive and nonpermissive temperatures. The arrow indicates the direction of sedimentation. (*Top*) Emp47p (*open circles*, 24°C; *filled circles*, 35°C), (*middle*) Wbp1p (*open triangles*, 24°C; *filled triangles*, 35°C), (*bottom*) Och1p (*open squares*, 24°C; *filled squares*, 35°C). (*B*) Time-course of Emp47p redistribution. Gradient profiles of incubations at 24°C for 75 min and 37°C for 15, 45, and 75 min were immune blotted for Emp47p using the AL-antibody. The ECL-signals from fractions 1–8 were summed up as Golgi reactivity (*filled bars*) and the cumulated signals from fractions 9–12 are referred to as ER reactivity (*hatched bars*).
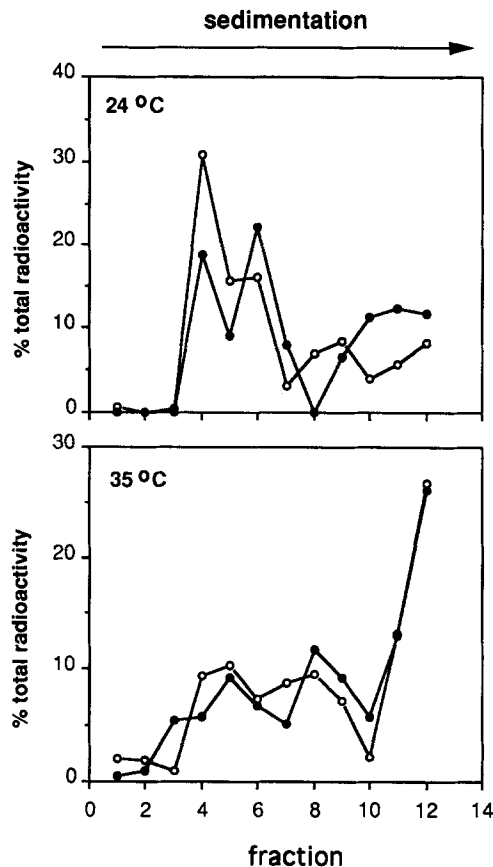

sedimentation

Figure 8. Dynamic intracellular redistribution of Emp47p and α1,3 mannose modified myc-glyco-Emp47p in *sec12* cells. RH3049 (*sec12, myc-glyco-EMP47*) were radiolabeled at 24°C for 15 min and chased for 10 min. Cells were transferred into medium containing 20 μg/ml cycloheximide and aliquots incubated for 1 h at either 24°C (permissive) or 35°C (nonpermissive). Cells were then converted into spheroplasts and lysed. After clearing the lysates at 500 g the supernatants were loaded onto sucrose gradients and spun as described in Materials and Methods. Gradient fractions were precipitated with AT-antibody. Precipitates were then reprecipitated either with AT-antibody or with a serum specific for α1,3-linked mannose. Second round precipitates were separated on SDS-PAGE and exposed to phosphoimager plates. The radioactive signals from each fraction were quantified and plotted (arrow indicates direction of sedimentation). (*Top*) Gradient distribution of Emp47p (*open circles*) and α1,3 linked mannose modified myc-glyco-Emp47p (*closed circles*) after incubation at 24°C; (*bottom*) distribution of same after incubation at 35°C.

shown in Fig. 9 *A*. Clearly Ste2p-Emp47ptail was retained in the ER in *RET1* cells (compare DAPI staining of the nucleus). In *ret1-1* mutant cells, however, the staining around the nucleus was abolished, and staining at the perimeter of the cells was observed. This is the pattern also observed for the wild type plasma membrane protein Ste2p in the parental strains (Fig. 9 *B*). Thus the cytoplasmic tail of Emp47p controlled the intracellular distribution of the Ste2p fusion protein in very much the same way as the Wbp1p-tail (Letourneur et al., 1994). Surprisingly, Emp47p distribution was apparently unaffected by the *ret1-1* mutation (Fig. 9 *A*, *top*). This held true when we visualized Emp47p by immunofluorescence in *ret1-1 pep4Δ*

A

RET1
ste2ΔSTE2EMP47TAIL
MYC-EMP47

ret1-1
ste2ΔSTE2EMP47TAIL
MYC-EMP47

myc-
Emp47p

Ste2-
Emp47ptail

DAPI

B   RET1   ret1-1

Ste2p

C

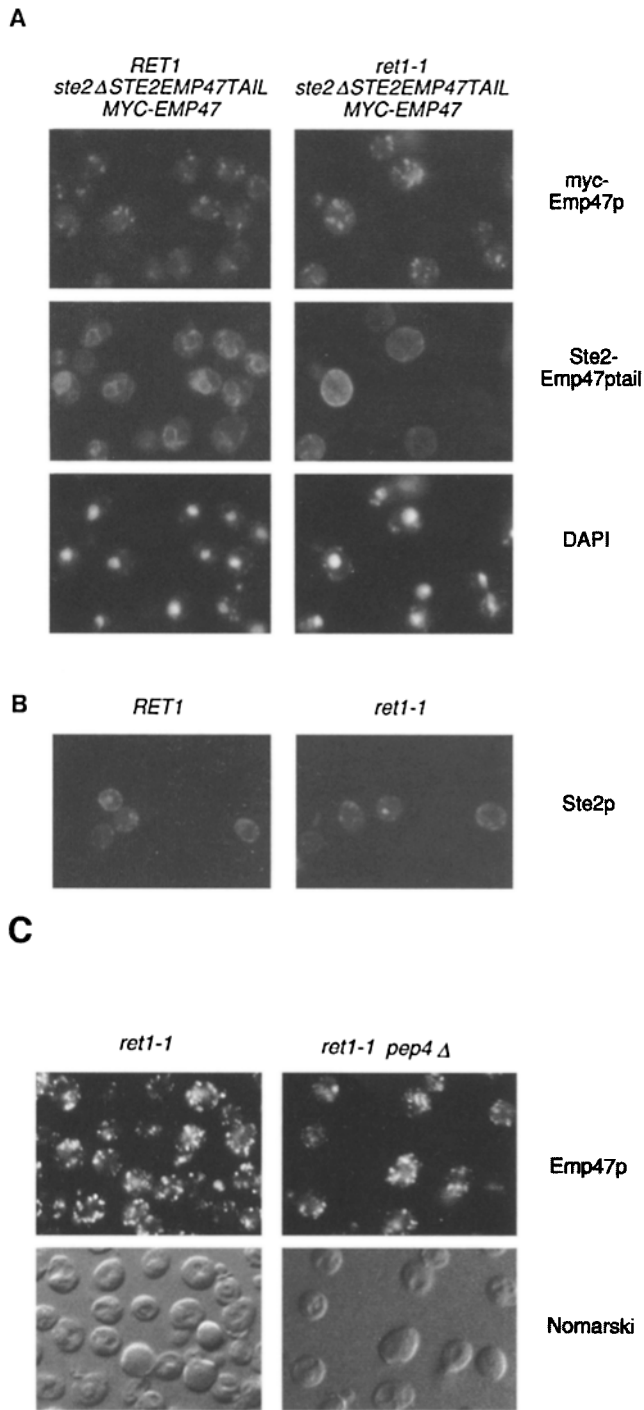ret1-1   ret1-1 pep4 Δ

Emp47p

Nomarski

Figure 9. Influence of *RET1* on the localization of Emp47p and Ste2pEmp47ptail. *RET1* (RH3193) and *ret1-1* (RH3187) cells were deleted for *STE2* and then *STE2EMP47TAIL* and *MYC-EMP47* were integrated into the genome resulting in RH3199 and RH3196 respectively (see Materials and Methods). These recombinant strains, the corresponding parental strains, as well as a *ret1-1 pep4Δ* strain (RH3274) were grown at 30°C and then processed for immunofluorescence. (*A*) Cells of the strains RH3199 and RH3169 were double labeled with 9E10 recognizing myc-Emp47p (*top*), and with a Ste2p peptide-antibody, recognizing Ste2p-Emp47ptail (*middle*). Secondary antibodies were FITC-conjugated anti–mouse and Cy3-conjugated anti–rabbit. The bottom panels show corresponding DNA-staining with DAPI. (*B*) Cells of the the parental *RET1* and *ret1-1* strains (RH3193 and

Table IV. The Influence of RET1, PEP4 and the Di-lysine Signal on the Turnover of Emp47p

Strain	Relevant genotype	Half-time for degradation (hrs)
RH3193	*RET1, PEP4, EMP47*	5.1
RH3187	*ret1-1, PEP4, EMP47*	5.0
RH3274	*ret1-1, pep4Δ, EMP47*	5.0
RH3275	*RET1, PEP4, emp47Δ, myc-EMP47-QTQLL*	0.8
RH2830	*RET1, pep4Δ, emp47Δ, myc-EMP47-QTQLL*	5.0

After a 15-min pulse with ^{35}S protein-labeling mix at 30°C at least five chase time points were taken in duplicate for each strain, covering 60 min for RH3275 and 4.5–6 h for the other strains. Cell extracts were prepared (see Materials and Methods) and precipitated with AT-serum, which also recognises the QTQLL mutant tail. Average values of each timepoint were obtained by PhosphoImager-analysis. The values were fitted with a linear equation from which the half-time was determined (rounded to the nearest tenth of an hour). Correlation coefficients of the optimal curve-fits were in the range of $R = 0.928$ to $R = 0.995$.

(RH3274) cells (Fig. 9 *C*). There was no apparent accumulation of wild-type Emp47p in the vacuole of *ret1-1 pep4Δ* cells, while mutant Emp47p with an impaired di-lysine motif could be visualized in the vacuole of *pep4Δ* cells (Fig. 5, Table III). In addition, the turnover rates of Emp47p were equally low ($t_{1/2}$ = 5 h) in RET1 and *ret1-1* strains (RH3193 and RH3187; Table IV). In contrast, mutant Emp47p with a QTQLL-tail, which escapes to the vacuole (table III), was degraded much more rapidly ($t_{1/2}$ = 0.8 h; RH 3275; Table IV). Therefore we should have detected even a modest increase in leakage of wild-type Emp47p to the vacuole due to the *ret1-1* mutation. This conclusion is supported by the finding that the rate-limiting step in turnover of wild-type Emp47p seems to be its slow access to the vacuole, because the turnover-rate of wild-type Emp47p in *PEP4* strains was the same as that for both wild-type and mutant Emp47p in *pep4Δ* strains ($t_{1/2}$ = 5 h; RH3193, RH3187, RH3274, RH2830; Table IV).

Discussion

The Steady-state Localization of Emp47p

We have cloned and sequenced a gene coding for a novel type I transmembrane protein, Emp47p. This protein has sequence homology to ERGIC-53 and to VIP36, a newly defined class of putative intracellular lectins (Fiedler and Simons, 1994; Arar et al., 1995). Emp47p carries a COOH-terminal di-lysine motif typically found on resident ER-membrane proteins, but is nevertheless found in the Golgi under steady-state conditions. Golgi localization was demonstrated by a combination of subcellular fractionation on sucrose gradients and immunofluorescence that showed that Emp47p is not localized to the ER, to the plasma membrane, nor the vacuole. On the other hand, Emp47p colocalized with Pmr1p and overlapped with GDPase, two presumed Golgi markers.

RH3187) were labeled with α Ste2p peptide-antibody followed by Cy3-conjugated secondary antibody to visualize Ste2p. (*C*) Cells of the *ret1-1* and *ret1-1 pep4Δ* strains (RH3187 and RH3274) were labeled with AT-antibody followed by Cy3-conjugated secondary antibody to visualize Emp47p. Bottom panels show corresponding Nomarski images visualizing the vacuoles.

Although recent progress has been made in the ultrastructural characterization of the Golgi in *Saccharomyces* (Preuss et al., 1992), analysis of the subcompartmentalization of this organelle still rests on the biochemical analysis of Golgi-specific processing events and the characterization of some proteins involved in these events. The framework was established by looking at the successive glycosylation and proteolytic processing of proteins on the biosynthetic route (Graham and Emr, 1991; Gaynor et al., 1994) and can be summarized as follows. The first and perhaps *cis*-most Golgi-specific modification is the addition of an α-1,6 mannose by Och1p (Nakanishi-Shindo et al., 1994), followed by elongation with additional α-1,6 mannose residues, followed by addition of α-1,3 mannose residues, followed by the proteolytic cleavage by Kex2p in perhaps the *trans*-most Golgi compartment. This would define four Golgi subcompartments. One would predict that GDPase, which is required for import of GDP mannose into the Golgi lumen, would be present in the first three Golgi subcompartments. This is consistent with its fractionation pattern and overlap with Och1p. Other Golgi markers have been placed partially within this framework by means of subcellular fractionation and/or immunofluorescence (Abeijon et al., 1989; Bowser and Novick, 1991; Cleves et al., 1991; Cunningham and Wickner, 1989; Franzusoff et al., 1991; Segev et al., 1988; Stearns et al., 1990).

A more precise Golgi localization of Emp47p can be inferred from the virtually complete colocalization with the Ca²⁺ ATPase, Pmr1p, both in sucrose density gradient fractionation and in immunofluorescence, and from the glycosylation pattern of the Emp47p variant. In a *pmr1Δ* strain Golgi-specific sugar modifications of invertase are abolished (Abeijon et al., 1993) and heterologous proteins are secreted in a core-glycosylated form. The growth lesion of *ypt1-1* is alleviated. Ypt1p is a peripheral Golgi GTPase that was shown to be required for ER to Golgi and early intra-Golgi transport steps (reviewed by Segev, 1994). Furthermore *pmr1Δ* interacts with several *sec* alleles (Rudolph et al., 1989; Antebi and Fink, 1992). It will be interesting to see if one of the functions of Pmr1p is to provide Ca²⁺ for the putative lectin-activity of Emp47p (see below).

Antebi and Fink (1992) found that on sucrose density gradients Pmr1p migrated in a profile widely overlapping though not congruent with the *trans*-most Golgi-marker Kex2p, a result we confirmed in the present study. In immunofluorescence studies on Pmr1p only a modest colocalization with Kex2p (27%) was observed. An even lower degree of colocalization was found with Sec7p (17%), a peripheral Golgi-membrane protein which itself colocalizes to a large extent with Kex2p (reviewed by Franzusoff, 1994). Thus Pmr1p and Emp47p are at least not extensively associated with the *trans*-most Golgi subcompartment.

Additional information as to which Golgi subcompartments Emp47p is accessible, comes from studies of an N-glycosylated version of the protein. We could show that its glycans are largely modified by α1,3 linked mannose within 5 min. This rapid acquisition of α-1,3 mannose linkages corresponds to the time required for similar modifications on carboxypeptidase Y and α-factor, suggesting that Emp47p is not retained for any significant amount of time

in the ER (Graham and Emr, 1991; Klionsky et al., 1988). The access of tagged Emp47p to the α1,3 mannosyltransferase compartment did not reflect aberrant targeting due to N-glycosylation by three criteria. First, the steady-state localization of the tagged Emp47p was not changed compared to the WT-protein as judged by colocalization with HA-Pmr1p. Second, we noted a similar stability of Emp47p and its glycosylated version. This argues against the possibility that we visualized the glycosylated Emp47p in transit to the vacuole, where mutant Emp47p with an impaired localization signal is targeted (see below). Third, we obtained evidence for a similar recycling to the ER of wild type Emp47p and its glycosylated version (see below). In conclusion, from the subcellular fractionation and the glycan-analysis the steady-state localization of Emp47p must be after the Och1p subcompartment (*cis*-most Golgi) and before the Kex2p (*trans*-most Golgi) subcompartment.

The Di-lysine Signal Is Required for the Steady-state Localization of Emp47p

As mentioned before, functional di-lysine signals have been demonstrated for proteins located at the ER at steady-state. ERGIC-53 is the only nonchimeric protein with a di-lysine motif that is known to reside partially downstream of the ER at steady-state, in the ER-Golgi intermediate compartment, and to a minor extent in the *cis*-Golgi (Schweizer et al., 1988). Emp47p is the first protein that is dependent on a di-lysine signal for its steady-state distribution in the Golgi. This adds the di-lysine motif to signals involved in Golgi protein localization (Machamer, 1993; Nilsson and Warren, 1994; Wilsbach and Payne, 1993).

The change of any conserved feature within the di-lysine motif abolished the normal Golgi localization of Emp47p. Mutant Emp47p accumulated in the vacuole of *pep4* strains and was rapidly degraded in strains not deficient in vacuolar proteolysis (data not shown). We have not investigated the itinerary of the mutant Emp47p to the vacuole, but it is likely to occur by the proposed default pathway (Roberts et al., 1992; Gaynor et al., 1994). The di-lysine motif of Emp47p complied with all the tests developed so far for the identification of functional ER-localization signals (Jackson et al., 1990; Nilsson et al., 1989). The lysine at position −5 can be moved to −4 without apparent effect, the lysines can not be replaced by arginine, and the lysines have to be in the right position with respect to the COOH-terminus of the protein. Furthermore the cytoplasmic tail of Emp47p is sufficient to confer ER-localization to Ste2p, which normally resides at the plasma membrane. What features of Emp47p could then determine a post-ER localization?

The transmembrane domain of Emp47p has one peculiarity in that it contains a proline at position 392 (Fig. 1 *A*). Proline residues have been implicated in structural and dynamic aspects of transmembrane domains and their interactions (Williams and Deber, 1991). Mutating proline 392 to leucine in Emp47p did not change the apparent steady-state distribution of the protein (data not shown). The lumenal domain could also contribute to the steady-state distribution of Emp47p in a post-ER compartment. If the lumenal domain has lectin function it is conceivable

that the putative interaction with glycans would provide a targeting signal (see below).

Recycling of Emp47p

To date, di-lysine motifs have been found to function in ER localization of proteins. The underlying mechanism, retrieval from a post-ER compartment, was indirectly deduced from the presence of Golgi-modified forms of these proteins in the ER (Gaynor et al., 1994; Jackson et al., 1993; Townsley and Pelham, 1994). For the first time we were able to directly investigate retrograde transport of a di-lysine protein from the Golgi complex to the ER. Furthermore, we examined a protein that was not overexpressed nor chimeric. To study the kinetics and quantity of recycling we developed an assay making use of the *sec12* mutation. Sec12p is required for the formation of ER-derived, COP II-coated transport vesicles (Barlowe et al., 1994). Thus, in the absence of Sec12p, transport out of the ER was abolished and material that recycled to the ER accumulated there. We found recycling to be almost quantitative within 1 h. This is a long time compared to the rate of secretion, transport to the vacuole (see above) and the forward transport of Emp47p itself. However, it should be noted that the cells were incubated in the presence of a protein synthesis inhibitor and forward transport was blocked during the recycling assay. We were surprised that the redistribution of Emp47p to the ER was almost complete. This indicates that either there are no factors that have to be reexported to the Golgi to sustain multiple rounds of retrograde transport or that a *SEC12*-independent forward transport pathway exists that enables the reexport of these hypothetical components. In any event, it is clear from our results that the retrograde pathway is independent of *SEC12*, suggesting that COP-II–coated vesicles are not required for this step. This novel transport assay should allow us to investigate the in vivo role of other gene products in retrograde traffic.

The appearance of Golgi-modified di-lysine proteins in the ER has been well documented (Gaynor et al., 1994; Jackson et al., 1993; Townsley and Pelham, 1994). There is less information however about the Golgi subcompartment(s) from which recycling of di-lysine proteins can occur. Gaynor et al. (1994) demonstrated for an invertase-Wbp1p fusion protein that it did not receive sugar modifications beyond the addition of the first $\alpha 1,6$ mannose by Och1p. Therefore, the *cis*-most Golgi subcompartment seems to be the salvage compartment for this particular hybrid protein. We present evidence that Emp47p with $\alpha 1,3$ mannose-modified glycans redistributed on gradients from a Golgi-position to an ER-position. The redistribution was as extensive as that of the wild-type Emp47p under the same conditions. This suggests that Emp47p can recycle to the ER from the $\alpha 1,3$ mannosyltransferase compartment or beyond. It remains to be shown whether this recycling is a one step process or occurs step by step backwards through the secretory pathway.

We have no evidence that Emp47p which returned to the ER can be reexported to the Golgi. Yet our data about the trafficking of Emp47p and the knowledge about the transport of other di-lysine proteins suggest that their distribution in the cell reflects a steady-state equilibrium between forward and retrograde transport. The steady-state position of different di-lysine proteins must therefore depend upon additional targeting information. For some proteins such targeting information can exist in the fine-tuning of the di-lysine signal by flanking amino acids (Jackson et al., 1990, 1993; Gaynor et al., 1994). In the case of Emp47p forward transport information is likely to reside in the lumenal domain, as we could not find any other determinants in the tail, and the tail itself was sufficient to confer ER-localization to Ste2p.

The Role of COP I

Di-lysines signals have been shown to bind coatomer in vitro (Cosson and Letourneur, 1994). Even more importantly, several subunits of yeast coatomer are required for the intracellular retention of a di-lysine–tagged Ste2p (Letourneur et al., 1994). Letourneur et al. therefore discussed the possibility that coatomer may function in retrograde transport of di-lysine proteins. Emp47p could be an important tool to test the potential involvement of coatomer in recycling. Our results showed that *ret1-1* cells, mutated in α-COP, were not able to correctly localize the Ste2p-Emp47ptail to the ER. However, the Golgi localization of Emp47p itself was unaffected in the mutant. It could be that other alleles of *ret1* would affect Emp47p localization, but we consider this unlikely. First, the di-lysine motif of Emp47p is definitely required for its Golgi localization. Second, coatomer in the *ret1-1* cells has lost the ability to bind di-lysine motifs in vitro (Letourneur et al., 1994). It could be that retrograde transport from *cis*-Golgi to the ER is blocked in *ret1-1* cells, but that this transport step is not required for Emp47p localization. Alternatively, it could be that retrograde transport from Golgi compartments distal to the *cis*-Golgi does not require *RET1*, but a different coat. By analogy to the clathrin adaptor complexes (Keen, 1993) perhaps different, compartment-specific coatomers exist that recognize similar signals. *ret1-1* could affect a *cis*-Golgi/ER coatomer. Emp47p would reach later Golgi subcompartments due to forward targeting and therefore only interact with a *medial*-Golgi–specific coatomer. We attempted to clarify this issue using our retrograde transport assay in a *sec12/ret1-1* double mutant. Unfortunately the double mutants did not yield reproducible results (data not shown) perhaps due to indirect effects of the simultaneous presence of the two mutations in the cells.

The Lectin-like Domain

Emp47p shows significant homology in its lumenal domain to two mammalian proteins located in the secretory pathway, ERGIC-53 and VIP36, the former of which has been shown to have lectin activity (Arar et al., 1995). There is another sugar-binding, membrane protein of the secretory pathway that does not display any sequence homology with the aforementioned lectins, the ER protein calnexin. It retains incompletely folded glycoproteins by recognition of $Glc_1Man_9GlcNAc_2$-oligosaccharides (Ou et al., 1993; Ware et al., 1995). In the case of ERGIC-53, VIP36 and Emp47p it is impossible to predict substrate specificity from the sequence homologies. Sugar-specificities would

probably be different, given the divergent intracellular distributions and sources of the proteins.

Although Emp47p is not essential, a putative function in glycoprotein-processing and/or sorting could manifest itself in differences in glycosylation-patterns or kinetics of transport of glycoproteins. We have looked at the biosynthesis of three glycoproteins: invertase, carboxypeptidase Y, and the glycosylphosphatidylinositol-anchored protein Gas1p. All three were apparently processed normally and transported with wild-type kinetics in the *emp47* mutant cells (data not shown). The effect of Emp47p could however be restricted to particular glycoproteins or involve nonprotein substrates. Therefore, based on the steady-state and dynamic localization of Emp47p and the glycosylation events that occur in yeast we propose three principal scenarios for a lectin-function of Emp47p. First, Emp47p could function in the Golgi as a membrane bound, compartment-specific cofactor for glycosylation. Due to the Emp47p steady-state distribution beyond the Och1p-compartment it would be involved in late Golgi-specific glycosylation events. These could be α1,6 mannose elongation, α1,2/α1,3 mannose addition, phosphomannose addition, or mannosylsphingolipid biosynthesis (Herscovics and Orlean, 1993; Puoti et al., 1991). Second, Emp47p could be a receptor for the retrieval of certain glycoproteins, similar to the HDEL/KDEL-receptor, which retrieves HDEL/KDEL proteins from the Golgi to the ER (Lewis and Pelham, 1992). This model is compatible with both the observed steady-state and dynamic localization of Emp47p. Third, Emp47p could be a true chaperon. It would help to sort and concentrate newly synthesized glycoproteins with ER-modifications and accompany them on their way to and through the Golgi, until modifications are complete. After that, it would release the substrate and return to the ER to bind a new one. Evidence for sorting and concentration of glycoproteins at the ER has been obtained (Balch et al., 1994; Mizuno and Singer, 1993) and one protein possibly involved in this process, Emp24p (Schimmöller et al., 1995), has been identified. It will be interesting to test these three models for Emp47p function.

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