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Rer1p, a Retrieval Receptor for ER Membrane Proteins, Recognizes Transmembrane Domains in Multiple Modes

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The yeast Golgi membrane protein Rer1p is required for the retrieval of various endoplasmic reticulum (ER) membrane proteins such as Sec12p and Sec71p to the ER. We demonstrate here that the transmembrane domain (TMD) of Sec71p, a type-III membrane protein, contains an ER localization signal, which is required for physical recognition by Rer1p. The Sec71TMD-GFP fusion protein is efficiently retrieved to the ER by Rer1p. The structural feature of this TMD signal turns out to be the spatial location of polar residues flanking the highly hydrophobic core sequence but not the whole length of the TMD. On the Rer1p side, Tyr152 residue in the 4th TMD is important for the recognition of Sec12p but not Sec71p, suggesting that Rer1p interacts with its ligands at least in two modes. Sec71TMD-GFP expressed in the Δ*rer1* mutant cells is mislocalized from the ER to the lumen of vacuoles via the multivesicular body (MVB) sorting pathway. In this case, not only the presence of polar residues in the Sec71TMD but also the length of the TMD is critical for the MVB sorting. Thus, the Rer1p-dependent ER retrieval and the MVB sorting in late endosomes both watch polar residues in the TMD but in a different manner.

INTRODUCTION

The secretory pathway of eukaryotic cells consists of a series of discrete membrane-bounded organelles with distinct protein and lipid compositions. Each protein that functions in a particular organelle must have a specific signal for its localization. Transmembrane domains (TMDs) of membrane proteins have often been shown to contain important information for localization in the ER (Bonifacino *et al.,* 1990a, 1990b, 1991; Pedrazzini *et al.,* 1996, 2000; Sato *et al.,* 1996; Rayner and Pelham, 1997; Honsho *et al.,* 1998; Letourneur and Cosson, 1998; Massaad *et al.,* 1999) and the Golgi complex (Munro, 1995), for transport between the Golgi and endosomes (Lewis *et al.,* 2000) or the plasma membrane (Scheiffele *et al.,* 1997), for sorting between endosomes (Reggiori *et al.,* 2000; Zaliauskiene *et al.,* 2000), and for endocytosis (Zaliauskiene *et al.,* 2000). Despite such a variety of studies, molecular mechanisms of how these TMDs are recognized during sorting are still largely unknown.

A clue to understand such membrane protein sorting was obtained from our work on the Rer1p-dependent localization of ER membrane proteins. Rer1p is a Golgi-resident membrane protein of 188 amino acid residues containing four membrane-spanning domains and is required for the ER localization of the type-II membrane protein Sec12p (Nishikawa and Nakano, 1993; Boehm *et al.,* 1994; Sato *et al.,* 1995). The TMD of Sec12p contains an Rer1p-dependent retrieval signal from the Golgi to the ER (Sato *et al.,* 1996). Recently, we demonstrated that Rer1p physically recognizes the TMD of Sec12p and returns Sec12p to the ER via the COPI vesicles (Sato *et al.,* 1996, 1997, 2001). Besides Sec12p, Rer1p is required for localization of a variety of ER membrane proteins such as Sed4p, Mns1p, Sec71p, and Sec63p (Sato *et al.,* 1996, 1997; Massaad *et al.,* 1999). Sed4p (Hardwick *et al.,* 1992) and Mns1p (Camirand *et al.,* 1991) are type-II membrane proteins. Sec71p is type III (Feldheim *et al.,* 1993; Kurihara and Silver, 1993), and Sec63p spans the ER membrane three times (Rothblatt *et al.,* 1989; Sadler *et al.,* 1989). Sec71p and Sec63p form a multimeric complex required for the posttranslational translocation of newly synthesized secretory proteins (Deshaies *et al.,* 1991). Some mutant versions of Gas1p and Ste2p also show Rer1p-dependent ER localization (Letourneur and Cosson, 1998).

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Abbreviations used: CCD, charge coupled device; COP, coat protein; CPS, carboxypeptidase S; DSP, dithiobis(succinimidyl propionate); GFP, green fluorescent protein; HHR, highly hydrophobic region; MVB, multivesicular body; ORF, open reading frame; TMD, transmembrane domain.

Table 1. Yeast strains used in this study

Strain	Genotype
SNY9[a]	*MATα mfα1::ADE2 mfα2::TRP1 bar1::HIS3 ura3 leu2 trp1 his3 lys2 ade2*
SKY5[b]	*MATα rer1-2 mfα1::ADE2 mfα2::LEU2 bar1::HIS3 ura3 leu2 trp1 his3 lys2 ade2*
SKY7[b]	*MATα rer1::LEU2 mfα1::ADE2 mfα2::TRP1 bar1::HIS3 ura3 leu2 trp1 his3 lys2 ade2*
SKY27[c]	*MATα ret1-1 mfα1::ADE2 mfα2::TRP1 bar1::HIS3 ura3 leu2 trp1 his3 lys2 ade2*
SMY22-10B[d]	*MAT***a** *dap2::LEU2 mfα1::ADE2 bar1::HIS3 ura3 leu2 trp1 his3 his4 ade2*
BC180[e]	*MAT***a** *sst2-2 ura3 leu2 his3 ade2*
SKY75[f]	*MATα rer1::LEU2 mfα1::ADE2 mfα2::HIS3 bar1::HIS3 ura3-52 trp1-Δ901 his3-Δ200 lys2-801 suc2-Δ9*
SKY80[f]	*MATα Δvps27::HIS3 Δrer1::LEU2 ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-Δ63 his3-Δ200 leu2-Δ1*
SKY42-12D[f]	*MATα rer1::LEU2 pep4::ADE2 ura3 leu2 trp1 his*

Source or reference: [a] Nishikawa and Nakano, 1993; [b] Sato et al., 1995; [c] Sato et al., 1997; [d] Sato et al., 2001; [e] W. Courchesne; and [f] this study.

Another interesting example of membrane protein sorting is seen during the formation of lumenal small vesicles in late endosomes, which is known as multivesicular body (MVB) sorting (Odorizzi *et al.*, 1998). A key molecule in this sorting is the Golgi-membrane–localized ubiquitin ligase Tul1p, which ubiquitinates a subset of membrane proteins like Cps1p and Phm5p and by doing so makes them sorted into MVBs (Reggiori and Pelham, 2002). Polar residues in the TMD of these membrane proteins have been shown important to be ubiquitinated by Tul1p (Reggiori *et al.*, 2000, Reggiori and Pelham, 2002).

In the present study, we performed intensive analyses of the structural features of membrane protein recognition during the Rer1p-dependent ER retrieval using Sec71p as the representative ligand. We present evidence that the TMD of Sec71p not only contains a signal to bind to Rer1p but is also recognized by the MVB sorting mechanism in a different manner when it escapes from Rer1p.

MATERIALS AND METHODS

Yeast Strains and Culture Condition

Saccharomyces cerevisiae strains used are listed in Table 1. Cells were grown in the MVD medium [0.67% yeast nitrogen base without amino acids (Difco Laboratories Inc.), and 2% glucose] or MCD, which is MVD containing 0.5% casamino acids (Difco Laboratories Inc.), supplemented appropriately.

Plasmid Construction

Construction of *SEC12-MFα1* (*S12M*), *MFα1-SEC71-HA* (*MS71H*), and *MFα1-SEC63-myc* (*MS63F*) was described previously (Nishikawa and Nakano, 1993; Sato *et al.*, 1997). Chimeric genes, which consist of *MFα1*, *WBP1*, and *SEC71*, were constructed as follows. The 800-base DNA fragment, which contains the coding sequence of the C-terminal region of Wbp1p (154 amino acid residues) followed by the *WBP1* terminator, was synthesized by genomic PCR with WBP1-1 (5′-GGGTTCGAAACTAGTTATGACGAAGAGCCC-3′) and WBP1–2 (5′-GGGCTCGAGATGTTACAGGATGATAGGTGG-3′). This fragment was digested with *Bst*BI and *Xho*I and used to replace the *Bst*BI-*Xho*I region of *MS63L* containing the *SEC63* ORF on pBluescript II KS+ (Sato *et al.*, 1997), to produce pBluescript II KS+/*SEC63* promoter-*MFα1–WBP1-WBP1* terminator. Then, the *Nhe*I-digested DNA fragment encoding three copies of the HA epitope (YPYDVPDYA) was inserted into the *Spe*I site just after the *Bst*BI site between *MFα1* and *WBP1* to give pMWWWK. Mutations, which change the dilysine signal KKTN at the C termini of the MWWWK protein to SSTN, were introduced by PCR-mediated mutagenesis to make pMWWWS. To construct various chimeras of *MWWWS* with *SEC71*, *Bgl*II and *Hin*dIII sites were introduced adjacent to the TMD by PCR. Introduction of these sites did not change the amino acid sequence of MWWWS. DNA fragments encoding the lumenal, transmembrane, and cytoplasmic domains of Sec71p were synthesized by PCR and used to replace the corresponding regions of *MWWWS*, resulting in *M71WWS*, *MW71WS*, *MWW71*, and *M71W71*. These chimeric genes were subcloned into the single-copy plasmid pRS316 (Sikorski and Hieter, 1989). The plasmids for the mutational analysis of the Sec71pTMD were also constructed by PCR and subcloned into pRS316.

To insert the GFP peptide at the C-terminus of Sec71p, the *SEC71-B* construct was utilized, which contained *Bst*BI and *Nhe*I sites at the ends of the *SEC71* ORF (Sato *et al.*, 1997). The ORF of GFP in pEGFP-1 (Clontech Laboratories, Inc.) was synthesized by PCR with primers, EGFP-*Spe*I+ (5′-GACTAGTATGGTGAGCAAGGGCG-3′) and EGFP-*Spe*I- (5′-GACTAGTCTTGTACAGCTCGTCC-3′). The GFP fragment was digested with *Spe*I and inserted into the *Nhe*I site of pBluescript IIKS+/*SEC71-B*. The resulting *SEC71-GFP* fusion was subcloned into pRS316 and pSQ326. To construct the *SEC71TMD-GFP* fusion gene, the GFP ORF in pEGFP-1 was amplified by PCR with primers, GFP-*Nhe*I+ [5′-CTAGCTAGCATG-GTGAGCAAGGGCGAGGAGTTACTTGTACAGCTCCTC-3′] and EGFP-*Spe*I−. The *Nhe*I/*Spe*I-digested GFP fragment was inserted into the *Nhe*I site of pBluescript IIKS+/SEC71-B to make pKS/SEC71-GFP-*Nhe*I. DNA fragments encoding the Sec71p TMD or its mutants were synthesized by PCR using *MW71WS* or its mutants as a template and primers, SEC71TMD-*Cla*I+ [5′-CCATCGATGAGAC-GAAATCAATCTCCGTTTATACCCCA-3′] and SEC71TMD-*Nhe*I- [5′-CTAGCTAGCTTTTTTGGCCTGCTTCTTTCTGTAGCTTGA-3′]. The *Cla*I/*Nhe*I-digested *SEC71TMD* fragments were inserted into the *Bst*BI/*Nhe*I-digested pKS/SEC71-GFP-*Nhe*I to replace the *SEC71* ORF by *SEC71TM*D coding sequence. The *SEC71TMD-GFP* gene was subcloned into pRS306 and pRS316 (Sikorski and Hieter, 1989). pRS306 harboring *SEC71TMD-GFP* or its derivatives were cut with *Stu*I and introduced into wild-type (SNY9) and Δ*rer1* (SKY7) cells.

Construction of *GFP-RER1* was described previously (Sato *et al.*, 2001). Mutant forms of the *RER1* gene were constructed by PCR and subcloned into the *Bgl*II site of pSKY5 (Sato *et al.*, 2001). *RER1-3HA Y152L* mutant was also made by PCR-mediated mutagenesis using *RER1-3HA* as a template (Sato *et al.*, 1995).

Antibodies

Anti-Dap2p and anti-GFP polyclonal antibodies were provided by Y. Amaya of Niigata University and H. Abe of our laboratory, respectively. Monoclonal antibodies against the HA epitope, 12CA5 and 16B12, were purchased from Boehringer and Berkeley Antibody Company, respectively. Anti-HA polyclonal antibody (Y11) and

anti-GFP mAb were obtained from Santa-Cruz Biotechnology, Inc. (Santa Cruz, CA), Medical and Biological Laboratories Co. Ltd., and Clontech Laboratories, Inc., respectively.

Confocal Laser Microscopy

GFP fluorescence was visualized under an Olympus BX-60 fluorescence microscope equipped with a confocal laser scanner unit CSU10 (Yokogawa Electronic Corp.). Images were acquired by a high-resolution digital CCD camera (Hamamatsu Photonics, C4742–95), and processed by the IPLab software (Scanalytics Inc.).

Halo Assay

Halo assay was performed on MCD plates with a tester *MAT***a** *sst2* strain (BC180) as described previously (Nishikawa and Nakano, 1993; Hopkins *et al.*, 2000). The cells of BC180/pRS316 ($\sim 5 \times 10^5$/plate) were spread on MCD plates buffered at pH 3.5. For quantification of the secreted α factor, various amounts (1, 5, 10, 50 ng) of synthetic α-factor (Peptide Institute Inc.) were spotted on filter-paper disks placed on the *sst2* tester lawn. Measuring the radii of the halos and plotting them against the amount of α-factor established a standard profile of α-factor secretion (Hopkins *et al.*, 2000). Cells to be tested for α-factor production were spotted onto the BC180 plates and then incubated at 23°C for 48 h. According to the standard profile, eight or four independent transformants for each construct were examined to quantify the amount of α-factor secreted.

Cross-link Experiments

Cross-linking between Rer1p and its ligand was performed using the thiol-cleavable linker dithiobis (succinimidyl propionate; DSP) as described previously (Sato *et al.*, 2001). In a typical experiment, cells coexpressing Rer1-3HAp or Rer1Y152L-3HAp and Sec71-GFP or Sec71TMD-GFP were spheroplasted, lysed in 25 mM Na phosphate (pH 7.2), and incubated with 5 mM DSP at 20°C for 30 min. Reactions were terminated by the addition of 50 mM Tris HCl (pH 8.0), and then membranes were solubilized with 1% Triton X-100. After adjustment to 35 mM Tris-HCl (pH 8.0)/120 mM NaCl/2% SDS, the samples were heated to 75°C for 10 min and processed for immunoprecipitation with the anti-GFP polyclonal antibody and the anti-HA mAb (12CA5). The immunoprecipitates were treated with 5% β-mercaptoethanol to cleave DSP and then analyzed by immunoblotting with the anti-GFP monoclonal or polyclonal antibody, and the anti-HA monoclonal (16B12) or polyclonal (Y11) antibody. Interaction of Rer1p or Rer1Y152L-3HAp with DSD was also assessed as described previously (Sato *et al.*, 2001).

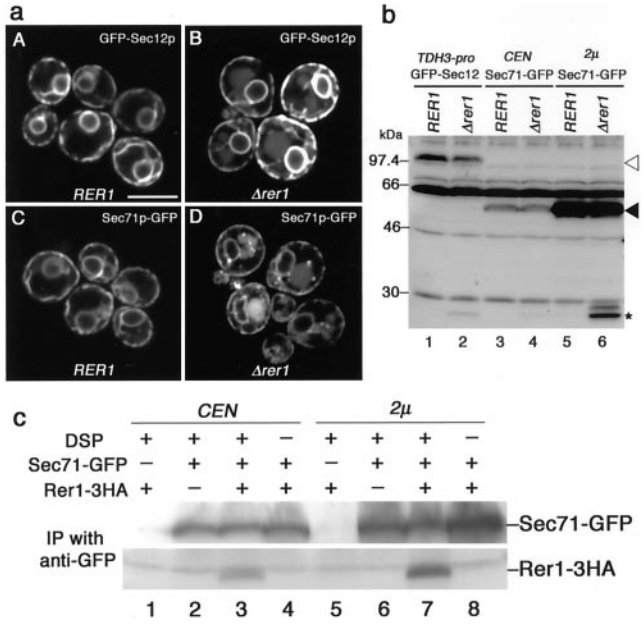

Figure 1. Rer1p-dependent ER localization of GFP-Sec12p and Sec71p-GFP. (a) Subcellular localization of GFP-Sec12p and Sec71p-GFP. The wild-type (SNY9) and Δ*rer1* (SKY7) cells were transformed with a single-copy plasmid containing *GFP-SEC12* (*TDH3* promoter) or *SEC71-GFP* (*SEC71* promoter) and subjected to confocal laser scanning microscopy. Bright ring-shaped structures are nuclear envelopes, a part of the ER, and fuzzy large organelles seen in the Δ*rer1* mutant are vacuoles. (b) Immunoblotting analysis of GFP-Sec12p and Sec71p-GFP. Wild-type (SNY9) and Δ*rer1* (SKY7) cells expressing GFP-Sec12p under the *TDH3* promoter or Sec71p-GFP under the own promoter on a single-copy (*CEN*) or multicopy (2 μ) plasmid were grown at 20°C. Cell extracts were subjected to immunoblotting with the anti-GFP antibody. Open arrowhead, GFP-Sec12p; closed arrowhead, Sec71p-GFP; asterisk, degradation product. (c) Physical interaction between Rer1p and Sec71p. Cell lysates of the Δ*rer1* Δ*pep4* strain (SKY42) expressing Rer1-3HAp on a multicopy plasmid and Sec71p-GFP on a single-copy (*CEN*) or multicopy (2 μ) plasmid were subjected to chemical cross-linking with DSP. The immunoprecipitates with the anti-GFP antibody were examined by immunoblotting with anti-GFP and anti-HA antibodies.

RESULTS

GFP-Sec12p and Sec71p-GFP Are Localized to the ER by the Rer1p-dependent Retrieval

The Rer1p-dependent ER localization of Sec12p and Sec71p that we showed previously (Sato *et al.*, 1997) was confirmed by a more straightforward microscopic approach. GFP was fused to the NH_2-terminus of Sec12p and to the COOH-terminus of Sec71p. GFP-Sec12p and Sec71p-GFP complemented Δ*sec12* and Δ*sec71* mutants, respectively, indicating that they are fully functional (our unpublished results). These fusions were expressed in the wild-type and Δ*rer1* cells and examined by confocal laser scanning microscopy (Figure 1a). As expected, GFP-Sec12p and Sec71p-GFP were almost exclusively localized to the ER in the wild-type cells. The fluorescence shown in panels A and C visualizes the nuclear envelope and peripheral ER, typical for the yeast ER. In the Δ*rer1* cells, in contrast, some portion of GFP-Sec12p and Sec71p-GFP were mislocalized to the vacuole (Figure 1a, B and D). The presence of fluorescence signals in the vacuolar lumen rather than the vacuolar membrane is noteworthy because the GFP moiety of these fusions is expected to face the cytoplasm. It is reminiscent of carboxypeptidase S (CPS), which is transported to the vacuole via the multivesicular body (MVB) sorting pathway (Odorizzi *et al.*, 1998). The GFP-CPS fusion protein is internalized into the lumen of late endosomes via small vesicles and eventually ends up in the vacuolar lumen. Indeed in the immunoblotting analysis (Figure 1b), GFP-Sec12p (open arrowhead) and Sec71-GFP (closed arrowhead) remained intact in the wild-type cells, but part of them were processed to a 27-kDa species (asterisk, perhaps cleaved GFP) in the Δ*rer1* cells. This degradation is due to vacuolar proteases, because it was not observed in Δ*rer1 pep4* mutant cells. This problem is pursued later.

Next, we performed chemical cross-linking experiments using a thiol-cleavable linker DSP to prove physical interac-

tion between Rer1p and Sec71p. Lysates of the Δ*rer1* Δ*pep4* cells expressing Rer1-3HAp and Sec71p-GFP were prepared and let react with DSP. Cross-linked products were immunoprecipitated with the anti-GFP antibody, treated with β-mercaptoethanol to cleave the linker, and subjected to immunoblotting with anti-GFP and anti-HA antibodies. As shown in Figure 1c, Rer1-3HAp was reproducibly and dose-dependently cross-linked to Sec71p-GFP (lanes 3 and 7). This provides biochemical evidence that Sec71p is also a ligand of Rer1p, although its sequence and topology are completely different from Sec12p.

The ER Retrieval Signal of Sec71p Is Confined in the TMD

To identify and localize the Rer1p-dependent ER localization signal of Sec71p, we planned to construct chimeric proteins between Sec71p and an appropriate passenger protein that is innocent for its destination, as we did for the study of Sec12p (Sato *et al.*, 1996). For this purpose, we first made a fusion protein between the α-mating factor precursor (Mfα1p) and the COOH-terminal half of Wbp1p (277–430 residues; see the left panel in Figure 2). Wbp1p is a type-I ER membrane protein, which functions as a component of the yeast *N*-oligosaccharyltransferase complex, and carries the KKXX signal at its COOH-terminus (te Heesen *et al.*, 1991, 1992, 1993). The 3HA epitope was inserted between Mfα1p and Wbp1p. This chimeric protein named MWWWK was expressed in the wild-type, Δ*rer1*, and a coatomer mutant *ret1-1* cells. The *ret1* mutant is known to be defective in the Golgi-to-ER retrieval of KKXX-harboring proteins including Wbp1p (Letourneur *et al.*, 1994). The transformants were then examined for the secretion of α-factor using the halo assay (Nishikawa and Nakano, 1993; right panel, Figure 2). The halo, a growth inhibition zone around an α cell colony on the plate, indicates the area where the tester **a** cells spread as lawn is G_0-arrested because of α-factor secreted from the colony. The diameter of the halo is proportional to the logarithm of the amount of α-factor and thus provides a quantitative measure of the secreted amount of α-factor. Because the processing of Mfα1p to yield mature α-factor takes place in the *trans*-Golgi, a large halo produced by Mfα1 chimera is an indication of its transport to the late Golgi. In the case of Wbp1p and Sec71p fusion proteins, ER localization is expected to lead to a small halo, whereas mistargeting to later compartments will give a large halo.

As shown in Figure 2, MWWWK, which is localized to the ER by virtue of the KKXX motif, produced only a small amount of α-factor in the wild-type and Δ*rer1* mutant cells. The *ret1-1* mutant made a large halo because it is defective in the KKXX protein retrieval. When the lysine residues in the KKXX motif were mutated to serines, the chimera produced a large halo even in the wild type (MWWWS). Now we performed a swapping experiment between MWWWS and Sec71p. Among several combinations we tested, only the MW71WS chimera, which contains the TMD from Sec71p, showed marked reduction of α-factor secretion (arrowhead). This effect was abolished in Δ*rer1*. Thus, as was the case for Sec12p, the TMD region of Sec71p appears to contain the Rer1p-dependent ER retrieval signal.

To corroborate this conclusion, we went on to construct a GFP chimera, which was fused to a small peptide fragment (35 residues) containing Sec71TMD (Figure 3a). This fusion

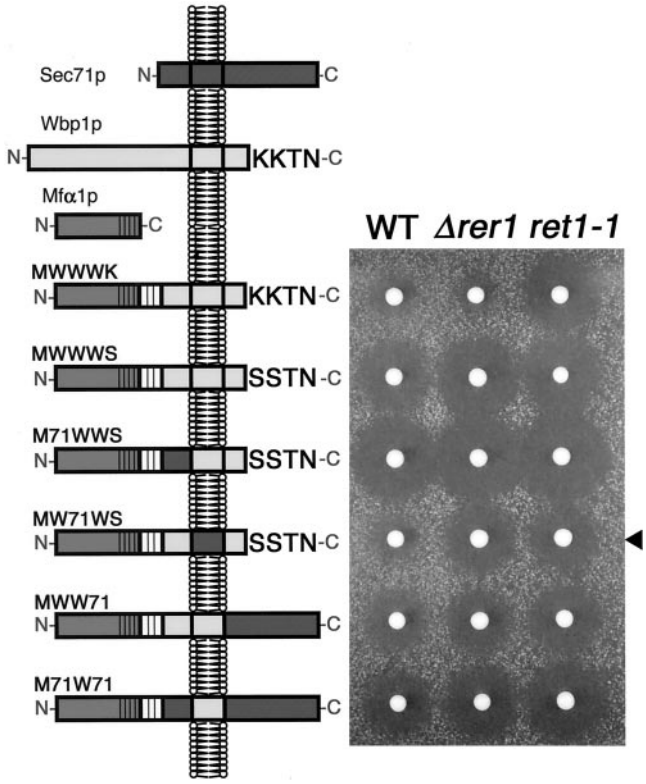

Figure 2. The TMD of Sec71p contains a signal for ER localization. Various chimeras between Mfα1p, Wbp1p, and Sec71p were constructed as depicted on the left and expressed in the wild-type (SNY9), Δ*rer1* (SKY7), and *ret1-1* (SKY27) cells. The transformants were examined for α-factor secretion by the halo assay. Note that a smaller halo indicates less secretion of α-factor, in other words, good ER localization of the chimeric protein. MW71WS (arrowhead) shows good ER localization in the wild-type but not in the Δ*rer1* or *ret1-1* mutant.

protein (Sec71TMD-GFP) was introduced into the wild-type and Δ*rer1* cells and observed under the confocal laser scanning microscope (Figure 3a, A–D). In the wild-type cells, Sec71TMD-GFP was localized to the ER at steady state (Figure 3a, A), indicating that the TMD of Sec71p is sufficient for the insertion into the ER membrane and the ER residence. The ER localization was almost completely gone in Δ*rer1*, indicating that it is clearly Rer1p dependent (Figure 3a, C). It should be noted that the GFP fluorescence was in the lumen of vacuoles in panel C, again suggesting the MVB sorting.

We further asked whether Rer1p physically interacts with the Sec71TMD in this GFP construct (Figure 3b). In the presence of DSP, the cross-linking between Rer1-3HAp and the Sec71TMD-GFP was reproducibly detected (lane 5). These results demonstrate that the TMD of Sec71p does indeed function as the Rer1p-dependent ER localization signal.

To pursue the possibility that the mislocalized Sec71TMD-GFP (Figure 3a, C) was targeted to the vacuolar lumen via the MVB sorting pathway, we expressed this fusion in the Δ*rer1* Δ*vps27* double-mutant cells. Vps27p belongs to the class E Vps proteins and is required for

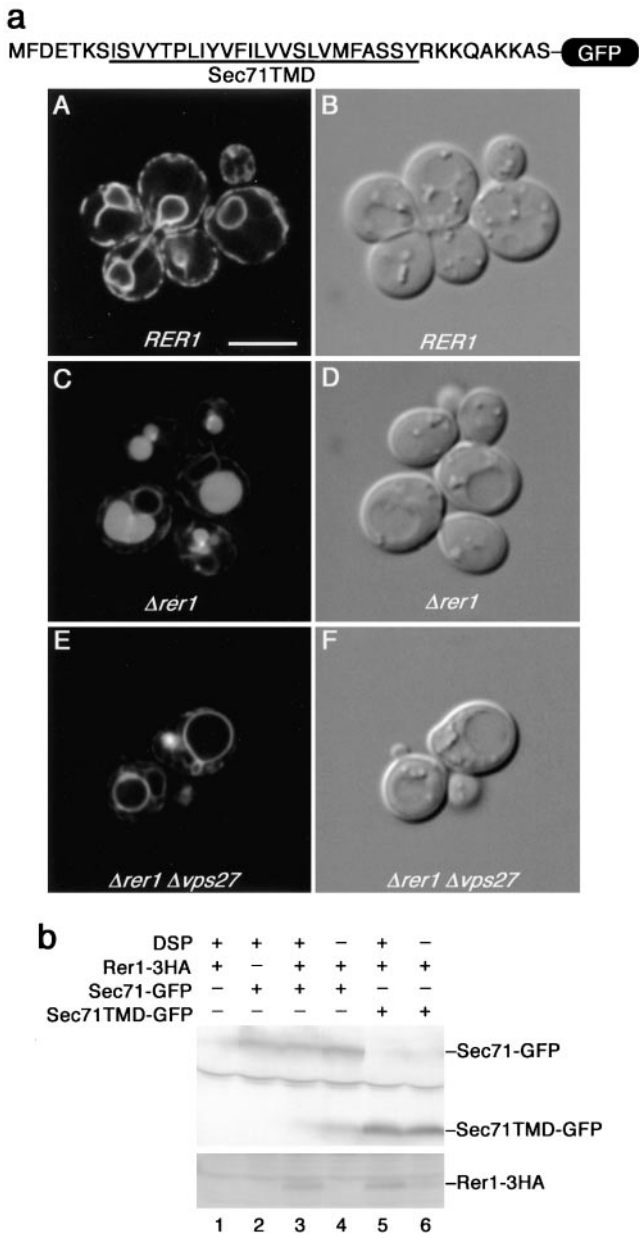

Figure 3. The TMD of Sec71p is sufficient for interaction with Rer1p and for ER localization. (a) The Sec71TMD and its neighboring residues (35 amino acids) were fused to the NH_2-terminus of GFP. The TMD region is underlined. This Sec71TMD-GFP fusion was expressed in the wild-type (SNY9), Δ*rer1* (SKY7), and Δ*rer1* Δ*vps27* (SKY80) cells and examined by confocal laser scanning microscopy. Fluorescence (A, C, and E) and Nomarski (B, D, and F) images are shown. (b) Physical interaction between Rer1p and the Sec71TMD. Lysates were prepared from Δ*rer1* Δ*pep4* cells (SKY42) coexpressing Rer1-3HAp on a multicopy plasmid and Sec71-GFP or Sec71TMD-GFP on a single-copy plasmid and incubated with or without DSP. The immunoprecipitates with the anti-GFP antibody were subjected to immunoblotting with anti-GFP and anti-HA antibodies.

MVB sorting. In the Δ*vps27* mutant, GFP-CPS is not transported to the vacuolar lumen but localized to the aberrant endosome-like structure (the class E compartment) and the vacuolar membrane (Odorizzi *et al.,* 1998). As shown in Figure 3a, panel E, it was also the case for Sec71TMD-GFP. The mislocalized chimera in Δ*rer1* could not reach the lumen of vacuoles when the Δ*vps27* mutation was combined.

Spatial Location of Polar Residues in the Sec71TMD Is Important for the Recognition by Rer1p

Having established that the Sec71TMD as well as the Sec12TMD contain Rer1p-dependent ER localization signals, we decided to determine the nature of this type of signal. By comparison of these two TMDs, we noticed a feature of hydrophobicity, which peaks in the middle of the TMD. In the Sec71TMD, there is a cluster of very hydrophobic amino acid residues VFILVV (see Figure 4, box). We will hereafter refer to this sequence as the highly hydrophobic region (HHR). We introduced a variety of mutations in this HHR and its flanking residues and examined their localization in the wild-type and *rer1* cells by the halo assay (Figure 4). Expression of these mutants was comparable to that of the wild-type version as confirmed by immunoblotting (our unpublished results). We picked up eight independent transformants for each construct and quantified the amount of the secreted α-factor by measuring the radii of halos formed around them (Hopkins *et al.,* 2000). To minimize the possible effect of ER retention caused by other mechanisms such as the ER quality control (Ellgaard *et al.,* 1999), the Rer1p-dependent ER localization of each mutant protein was assessed by the ratio of the secreted α-factor from the wild-type cells to that from the Δ*rer1* cells (WT/Δ*rer1*). Smaller numbers indicate better Rer1p-dependent ER localization. First, we introduced a polar residue into the HHR (LS) to examine whether the hydrophobicity of this region is critical for the Rer1p-dependent ER localization. This mutation did not significantly affect the Rer1p dependency. On the contrary, the replacement of the flanking polar residues (Y and S, asterisked on the top) by alanine or leucine (YL, YA, SL, and SA) resulted in marked increase of the α-factor secretion. Replacement to other polar residues (YS, YQ, and SQ) had more modest effects, suggesting that the presence of polar residues in these positions is very important for the recognition by Rer1p. We also inserted increasing numbers of leucine residues into the HHR and examined their effects. Interestingly, all the insertion mutants (1L, 2L, 3L, and 4L) lost the ability to remain in the ER. Deletion of the leucine from the HHR (ΔL) also caused a defect. It is not the whole length of the TMD that is important, because the 2LΔLV mutant, which had two leucines inserted in the HHR but downstream LV was deleted to keep the length of the TMD constant, was still defective in ER localization. These results indicate that the spatial distribution of the two polar residues that flank the HHR is the very important determinant for the Rer1p recognition.

To visually confirm the effects of these mutations in the Sec71TMD, we constructed the same mutants in the Sec71TMD-GFP construct and observed under the confocal laser scanning microscope (Figures 5 and 6). Consistent with the results of the halo assay, the LS mutation in the HHR showed virtually no defect and complete ER localization in

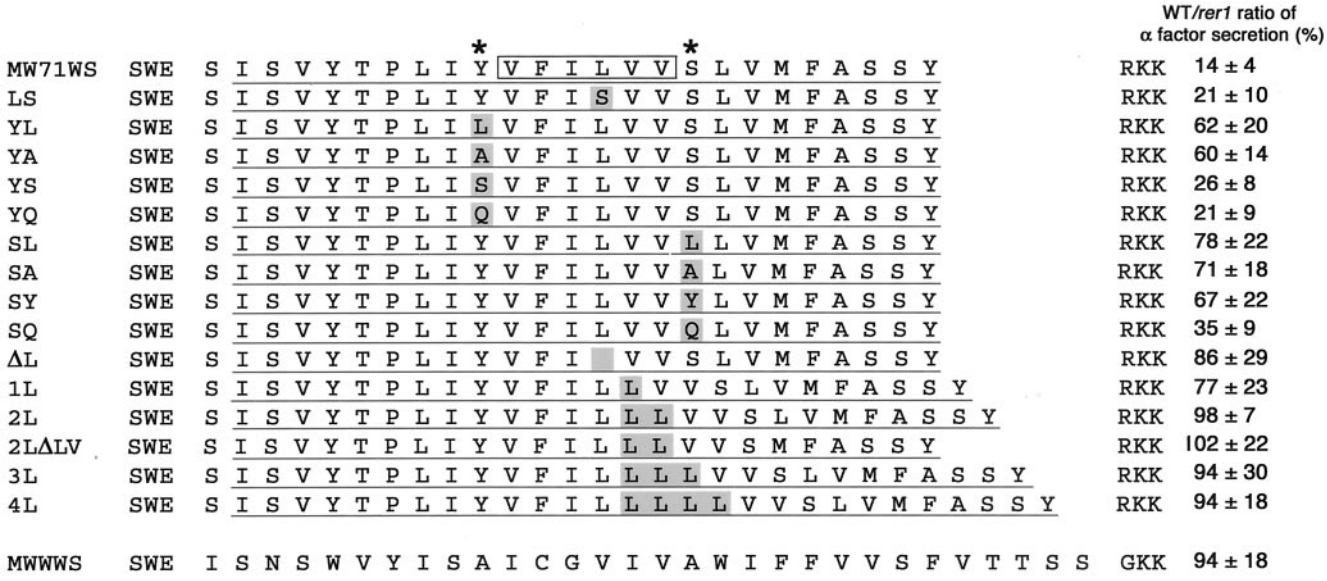

Figure 4. Mutational analysis of the Sec71TMD. In the MW71WS chimera of Figure 2, a variety of mutations were introduced into the TMD region. Putative TMDs are underlined. In the control (top), the highly hydrophobic region (HHR) and the flanking polar residues are marked with a box and asterisks, respectively. Sequences of the mutants constructed are shown below. These constructs were expressed in the wild-type (SNY9) and Δ*rer1* (SKY7) cells. Eight independent transformants were subjected to the halo assay and the secreted α-factor was quantified as described in MATERIALS AND METHODS. The ratio of the amount of secreted α-factor from the wild-type cells to that from the Δ*rer1* cells are shown on the right. Smaller numbers indicate better Rer1p-dependent ER localization.

the Rer1p-dependent manner (Figure 5b). The mutations of the flanking polar residues (YL and SL) caused mislocalization of the GFP fusion to the vacuole. The GFP signals milocalized to vacuoles in the cases of YL and SL mutants are not completely lumenal, regardless of wild type or Δ*rer1*. Significant portion is on the vacuolar membranes. This suggests that these polar residues are also involved in the MVB sorting late in the pathway. As shown in Figure 6b, all insertion mutants (1L, 2L, 3L, and 4L) were mislocalized to vacuoles in wild-type or Δ*rer1* cells, but the localization patterns in vacuoles were different. The GFP fluorescence of the 1L mutant was observed both on the membrane and in the lumen, but those of longer insertion mutants (2L–4L) were mostly on the vacuolar membrane. On the contrary, the 2LΔLV mutant, which shows a defect in the Rer1p-dependent ER localization, appears to be well sorted into the MVB pathway, suggesting that the length of TMD is important for MVB sorting. These results are similar to the previous findings about the MVB sorting of type-II membrane proteins and C-terminal tail-anchored proteins such as SNARE proteins (Reggiori *et al.,* 2000). It appears that the Rer1p-dependent Golgi-to-ER retrieval and the MVB sorting in late endosomes both watch polar residues in the TMD, but the latter mechanism is more sensitive to the whole length of the TMD than the former.

Two Different Modes of Ligand recognition by Rer1p

As a complementary approach to understanding the molecular mechanism of ligand-receptor interaction, we looked at the structural feature of Rer1p. Naturally, candidate residues of Rer1p that are involved in the recognition of the polar residues in Sec71TMD (as well as Sec12TMD) would be also present in the TMDs of Rer1p. Rer1p has four TMDs, which are unusually rich in polar residues (Figure 7a). We chose 10 noncharged polar residues in or near the TMDs (numbered in the diagram of Figure 7a), replaced each of them by leucine and examined their properties. First, the mutants in the context of GFP-Rer1p (Sato *et al.,* 2001) were expressed in Δ*rer1* cells and observed by confocal microscopy. As shown in Figure 7b, Y68L, Q77L, T128L, S138L, and Y152L mutants stayed mostly in the Golgi at steady state. S131L, S135L, and T160L mutants gave mixed patterns of ER and Golgi. The T83L mutant was mislocalized to vacuoles, whereas the N76L mutant showed both Golgi and vacuolar localization.

Next, the GFP-Rer1p mutants were coexpressed with either Sec12p-Mfα1p or Mfα1p-Sec71p in the Δ*rer1* cells, and their retrieval activities were tested by the halo assay (Figure 8, Table 2). The abilities to complement Δ*rer1* (smaller halo means better complementation) varied from mutant to mutant, and in general, mutants normally residing in the Golgi seemed to have better chances to function than others. The one exception, Y152L, appeared to recognize Mfα1-Sec71p very well (small halo in Figure 8b), but not Sec12-Mfα1p (large halo in Figure 8a). This result suggests the possibility that Tyr152 residue is differently involved in the recognition of Sec12p and Sec71p. So we further examined whether the Y152L mutant had indeed difference in the interaction with these two ligands. Figure 8, panels c and d, show the results of cross-linking experiments. Dap2p or dipeptidyl aminopeptidase B, a type-II vacuolar membrane protein (Suarez Rendueles and Wolf, 1987), has been used as a passenger

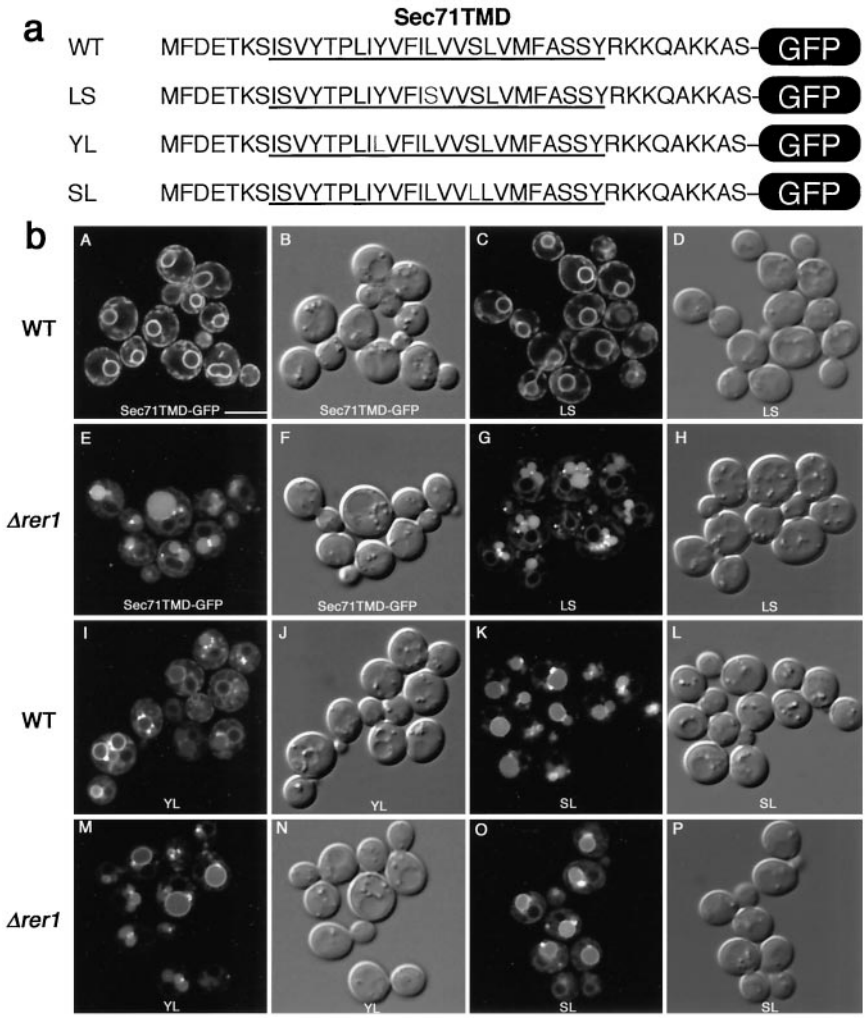

Figure 5. Localization of GFP-fused Sec71TMD mutants. (a) Amino acid sequences of the TMD mutants. Predicted TMD regions are underlined. (b) Effects of mutations of the polar residues. LS, YL, and SL mutants were expressed in the wild-type (SNY9) and Δ*rer1* (SKY7) cells and examined by confocal laser scanning microscopy. Fluorescence (left panel in the pair) and Nomarski (right panel in the pair) images are shown. Bar, 5 μm.

protein to determine the ER localization signals of Sec12p (Sato *et al.,* 1996). DSD is a chimeric protein comprised of the lumenal and cytoplasmic domains from Dap2p and the TMD from Sec12p and has shown to be almost completely localized to the ER by the Rer1p-dependent retrieval (Sato *et al.,* 1996). As shown in Figure 8c, the Sec12TMD in the DSD chimera (see Sato *et al.,* 1996) was recognized by and cross-linked with the wild-type Rer1p (IP with anti-Dap2 and immunoblot with anti-HA and vice versa). However, the efficiency of cross-link was much reduced when Rer1p Y152L was used (compare lanes 5 and 7). On the other hand, cross-linking between Sec71TMD and Rer1p (Figure 8d) was almost equally efficient for the wild-type Rer1p and the Y152L mutant (lanes 3 and 5). Thus the effect of the Y152L mutation was clearly different for Sec12p and Sec71p, suggesting distinct modes of interaction for Rer1p in the recognition of ligands with opposite topologies.

DISCUSSION

The question as to how membrane proteins are sorted from each other during dynamic membrane traffic is one of the central issues of organelle identification. Interactions through TMDs have been thought to be the key for mutual recognition but its molecular details remained elusive. In the present study, we have shown that the presence of polar residues in the TMD and the whole length of the TMD are used as independent cues of such recognition.

ER Localization of Membrane Proteins

In addition to the COOH-terminal KDEL/HDEL (Munro and Pelham, 1987; Pelham, 1988) and KKXX (Jackson *et al.,* 1990) signals, TMDs have been known to serve as ER localization determinants (Pedrazzini *et al.,* 1996; Sato *et al.,* 1996; Rayner and Pelham, 1997; Honsho *et al.,* 1998). Rer1p is a sorting receptor in the Golgi apparatus to retrieve a set of ER membrane proteins from Golgi to ER (Nishikawa and Nakano, 1993; Sato *et al.,* 1997; Massaad *et al.,* 1999). It physically recognizes the TMDs of Sec12p (Sato *et al.,* 2001) and Sec71p (this study; Figures 1-3) and thus provides an ideal system to understand the mechanism of membrane protein sorting.

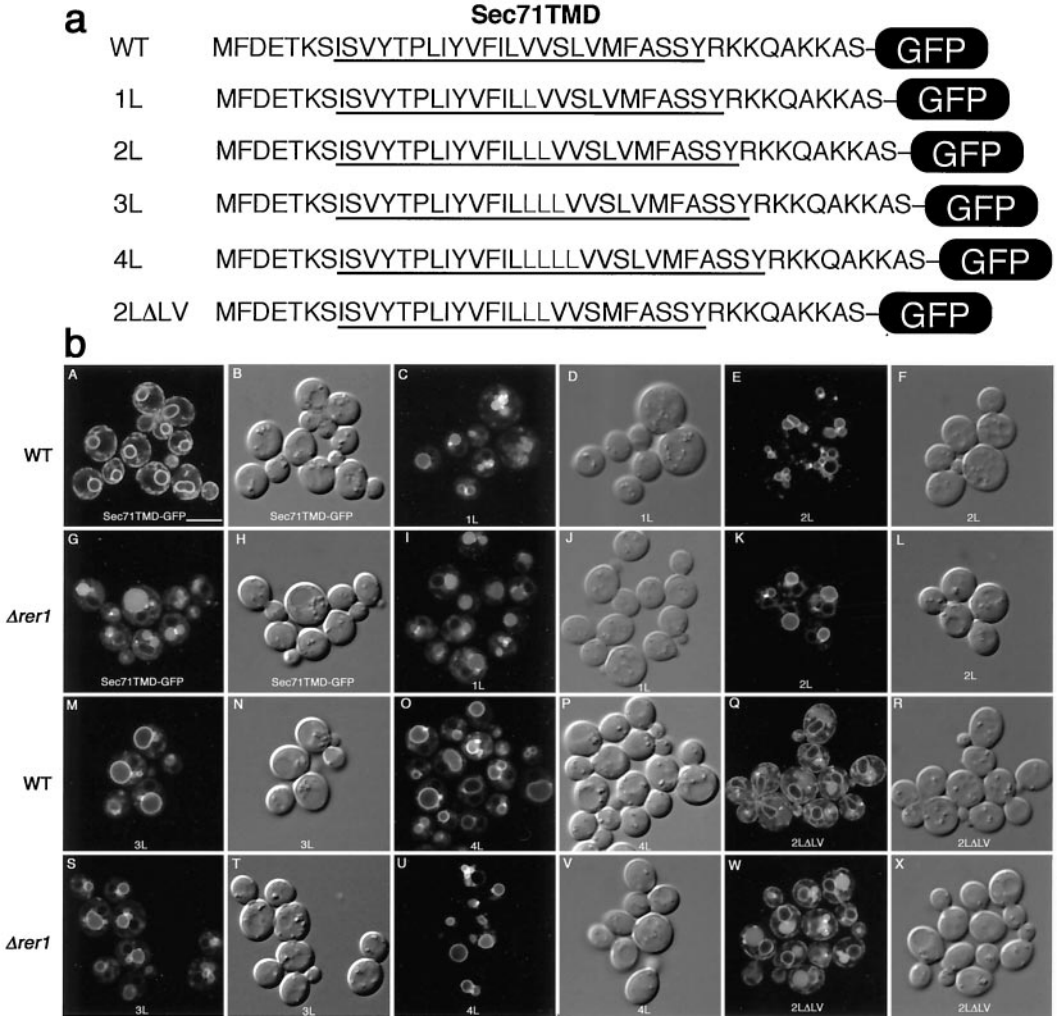

Figure 6. Effects of the length of the TMD on the localization of Sec71TMD-GFP. Control and leucine insertion mutants (1L, 2L, 3L, 4L, and 2LΔLV; a) were expressed in the wild-type (SNY9) and Δ*rer1* (SKY7) cells and observed by confocal microscopy (b). Fluorescence (left panel in the pair) and Nomarski (right panel in the pair) images are shown. Bar, 5 μm.

Our detailed mutational analysis of the Sec71TMD demonstrated that the essential feature to be recognized by Rer1p is the presence of two polar amino acid residues (Y and S) that flank the very hydrophobic cluster VFILVV (HHR; Figures 4–6). The distance of these polar residues is important because insertion or deletion of leucine residues into and from the HHR almost completely abolished the ability to act as the retrieval signal. A very similar motif is seen in the TMD of Sec12p. In this case, two sets of polar residues TN and SY stand on the edges of the HHR, FILIVLL in this case, which motif is also proved essential for the Rer1p-dependent ER localization (Sato *et al.*, 1996, 2001). It should be reminded that Sec12p and Sec71p are both transmembrane proteins with a single TMD, but in the opposite topology. Nevertheless, the structural feature that polar residues flank the HHR is common. The length of the HHR is a little different: seven residues for Sec12p and six for Sec71p. These numbers correspond to about two turns if α-helix conformation is assumed, but considering their opposite directions, the binding modes cannot be the same.

Another possible feature of TMDs that could be recognized during sorting is the length. For Sec71p to be recognized by Rer1p for ER retrieval, the TMD length itself appears not to be important. The key experiment was the use of the 2LΔLV mutant, in which two leucines were inserted into the HHR while downstream LV was removed to keep the length constant (Figure 4). This mutant form was not recognized by Rer1p any more, indicating that it is the distance of the two polar residues but not the whole length of the TMD that Rer1p is watching. A small part of Sec71TMD-GFP 2LΔLV still localizes to the ER even in the Δ*rer1* mutant (Figure 6b, panel w), suggesting that there are Rer1p-independent ER localization mechanism(s) as well (Rayner and Pelham, 1997).

Coming back to the difference between Sec12p and Sec71p, a very important insight into the binding modes of

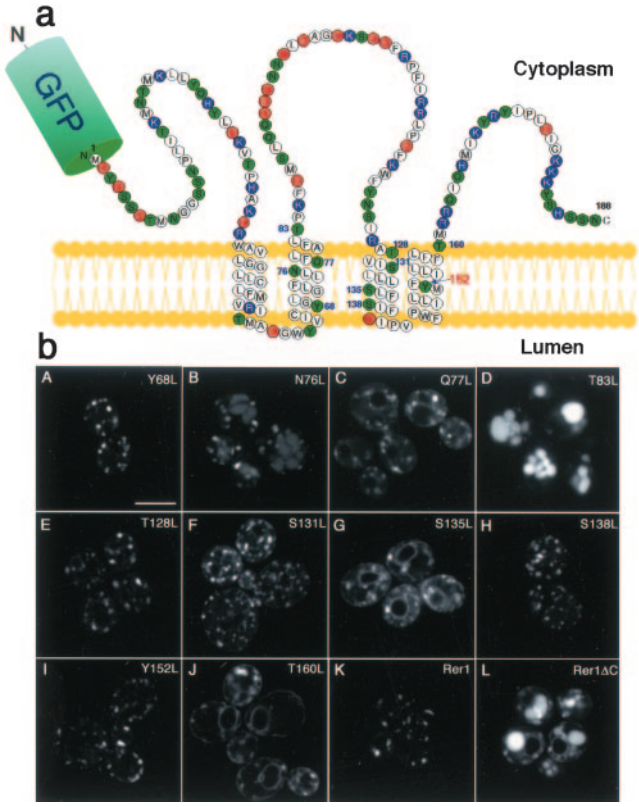

Figure 7. Mutational analysis on the Rer1p side. (a) Schematic structure of Rer1p. Polar residues are colored: red, acidic; blue, basic; green, noncharged. Ten noncharged polar amino acid residues in or near the four TMDs (shown numbered) were selected and mutated. (b) Subcellular localization of Rer1p mutants. The polar residues marked in panel a were individually mutated to leucine in the GFP-Rer1p construct and were analyzed for localization in Δ*rer1* cells (SKY7) by confocal microscopy. Rer1ΔC is the truncation mutant that lacks the COOH-terminal 25 residues. Bar, 5 μm.

Rer1p was provided by the mutational analysis of the TMDs of Rer1p (Figures 7 and 8). Among the mutants we constructed, Y152L showed a very interesting behavior. The Y152L mutant Rer1p had a problem in the recognition of Sec12p but not Sec71p. This selective defect for Sec12p was further confirmed by a cross-linking experiment. Thus the Tyr152 residue in the fourth TMD of Rer1p is important for the binding mode of Sec12p but not for that of Sec71p. This residue is completely conserved among the known members of the Rer1p family (Sato *et al.*, 1999). Presumably, in order to accommodate a variety of ER membrane proteins for the Rer1p-dependent retrieval system, Rer1p would be capable of taking several different modes of binding for different ligands. The ultimate proof of this hypothesis will await structural analysis of the receptor-ligand complex, which will be a very challenging but fruitful project in the future.

Sorting of Membrane Proteins into Multivesicular Bodies

In the process of analyzing the structural requirements of the Sec71TMD to be retrieved by Rer1p, we found that the motif of Sec71TMD could also be recognized by the MVB sorting mechanism. In the Δ*rer1* mutant cells, Sec71p is not efficiently localized to the ER and the majority is missorted to the lumen of vacuoles (Figures 1 and 3). This was indeed due to the MVB sorting because the knockout of *VPS27*, a gene required in this pathway, abolished targeting of Sec71p-GFP to the vacuolar lumen.

Sec71p is a type-III membrane protein, of which N-terminus is facing the ER lumen and the C termini is cytoplasmic (Feldheim *et al.*, 1993, Kurihara and Silver, 1993). In the case of type-II transmembrane proteins such as SNARE proteins, the presence of a polar residue in the TMD at the position of third or fifth from the cytoplasmic border and the whole length of TMD are both crucial for the MVB targeting (Reggiori and Pelham, 2000). The mutational analysis of Sec71TMD-GFP revealed that the polar residues essential for the Golgi-to-ER retrieval by Rer1p are also important for the MVB sorting. When these residues were mutated, fluorescence from the limiting membrane of vacuoles became clearer, indicating that these mutants were less efficiently sequestrated into lumenal vesicles (Figure 5). The distance between these two polar residues first appeared to be again important because leucine insertion/deletion mutants in the HHR were also defective in efficient MVB sorting. However, in the case of the 2LΔLV mutant, the mutant Sec71TMD-GFP was mostly missorted to the vacuole due to the disability to be recognized by Rer1p as discussed above, but was still targeted to the vacuolar lumen. In other words, this mutant TMD is not a good ligand for Rer1p but is recognized by the MVB-sorting mechanism. Because the 2LΔLV mutant contains the two polar residues in a different arrangement from the wild type but has the same length of TMD, these results imply that the MVB-sorting machinery senses the length of the type-III TMD strictly but not the positions of polar residues very rigorously unlike the cases of type-II membrane proteins and C-terminal tail-anchored proteins. These observations suggest that a similar but different mechanism is responsible for the MVB sorting of membrane proteins.

A Concept of Sorting Chaperones

The exposure of polar amino acid residues in the lipid bilayer would be a dangerous event. The fact that the two independent sorting mechanisms, Rer1p-dependent ER retrieval and the MVB sorting in late endosomes, recognize polar residues as the cue may indicate that these processes are developed to avoid deleterious effects of polar TMDs (see also Reggiori and Pelham, 2002). In fact, the MVB sorting leads to the dead end in the vacuole, i.e., destruction, and the ER retrieval could result in the quality control in the ER.

For the case of Rer1p, one of the biggest questions that remain to be answered in the future is how it releases the ligand in the ER. The ligands of Rer1p so far identified appear to have characteristics to form oligomers in their normal status. Sec71p and Sec63p are known to be in the posttranslational translocon complex and Sec12p may well be in oligomers in the ER. It could be the monomeric form of these molecules that are recognized by Rer1p in the Golgi. Higher affinity with its native partner(s) would explain the force to dissociate the ligand from Rer1p in the ER. If this hypothesis is correct, the role of Rer1p in the Golgi would be not only as the receptor to catch mistransported proteins but

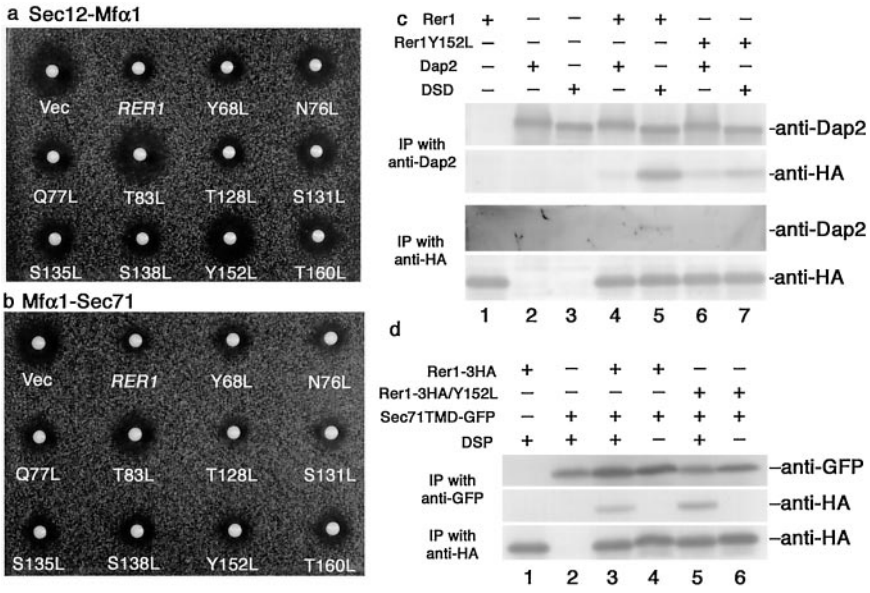

Figure 8. Differential modes of interaction between Rer1p and its ligands. The Rer1pY152L mutant can recognize the Sec71TMD but not the Sec12TMD. (a and b) GFP-Rer1p and its TMD mutants complement Δ*rer1* to different degrees. The Δ*rer1* cells (SKY75) expressing either Sec12p-Mfα1p (a) or Mfα1p-Sec71p (b) were transformed with *GFP-RER1* or its mutants. The transformants were examined for the α-factor secretion by the halo assay. Note that the Y152L mutant forms a large halo from Sec12-Mfα1p but not from Mfα1-Sec71p. (c) Reduced interaction between Rer1Y152L and the Sec12TMD. The Δ*dap2* cells (SMY22–10B) coexpressing Rer1-3HAp or Rer1Y152L-3HAp and Dap2p or DSD (Dap2-Sec12-Dap2 chimera) were subjected to cross-linking with DSP. The immunoprecipitates with anti-Dap2p or anti-HA antibodies were further analyzed by immunoblotting again with anti-Dap2p and anti-HA antibodies. (d) Rer1Y152L is normal for the interaction with the Sec71 TMD. Lysates were prepared from the Δ*rer1* Δ*pep4* cells (SKY42) coexpressing Sec71TMD-GFP and Rer1-3HAp or Rer1Y152L-3HA and incubated with or without DSP. The immunoprecipitates with the anti-GFP antibody were subjected to immunoblotting with anti-GFP or anti-HA antibodies.

also as a chaperone to conceal polar residues exposed in the lipid bilayer. Analysis of their oligomerization states will be very important to address this problem. In this context, it is interesting that some membrane proteins are recognized by Rer1p only when the TMD was mutated. Such examples can be seen for invertase-Gas1p and Ste2p (Letourneur and Cosson, 1998). Whether the same scenario also applies to these cases would be a curious question.

In mammalian cells, unassembled chains of the T-cell antigen receptor (TCR) complex are known to be often localized to the ER and subsequently degraded by the ER-associated degradation (Klausner and Sitia, 1990). In the case of the TCR α chain, two positively charged amino acid residues in the TMD are critical for retention and ER degradation (Bonifacino *et al.,* 1990a). Introduction of a polar residue in the TMD of the Tac antigen has also been shown to convert its destination from the plasma membrane to the ER and degradation (Bonifacino *et al.,* 1991). These observations have been regarded as good examples of the quality control in the ER but for us they appear to point to the putative role of Rer1p-dependent retrieval in these processes. Whether the retrieval by Rer1p indeed plays a role in the ER quality control can be tested in yeast and is now being pursued.

Table 2. Complementation activities of the Rer1TMD mutants for mislocalization of Sec12p-Mfα1p and Mfα1p-Sec71p in *rer1*

	Sec12p-Mfα1p	Mfα1p-Sec71p
Vector	100 ± 0	100 ± 0
RER1	24 ± 3	14 ± 4
Y68L	32 ± 9	50 ± 18
N76L	48 ± 9	25 ± 3
Q77L	34 ± 8	16 ± 5
T83L	90 ± 11	74 ± 14
T128L	33 ± 7	21 ± 6
S131L	45 ± 5	49 ± 13
S135L	37 ± 4	18 ± 4
S138L	30 ± 3	16 ± 5
Y152L	56 ± 6	27 ± 6
T160L	41 ± 9	49 ± 13

Δ*rer1* cells (SKY75) expressing Sec12p-Mfα1p and *rer1-2* cells (SKY5) expressing Mfα1p-Sec71p were transformed with vector alone, *GFP-RER1,* and the *GFP-RER1* TMD mutants indicated. Four independent transformants were subjected to the halo assay, and the secreted α-factor was quantified. The amounts of secreted α-factor are shown as proportions relative to that secreted from the Δ*rer1* cells carrying vector alone (100%). Smaller numbers indicate better ER localization.

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