Rer1p, a Retrieval Receptor for Endoplasmic Reticulum Membrane Proteins, Is Dynamically Localized to the Golgi Apparatus by Coatomer✪

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Abstract. Rer1p, a yeast Golgi membrane protein, is required for the retrieval of a set of endoplasmic reticulum (ER) membrane proteins. We present the first evidence that Rer1p directly interacts with the transmembrane domain (TMD) of Sec12p which contains a retrieval signal. A green fluorescent protein (GFP) fusion of Rer1p rapidly cycles between the Golgi and the ER. Either a lesion of coatomer or deletion of the COOH-terminal tail of Rer1p causes its mislocalization to the vacuole. The COOH-terminal Rer1p tail interacts in vitro with a coatomer complex containing α and γ subunits. These findings not only give the proof that Rer1p is a novel type of retrieval receptor recognizing the TMD in the Golgi but also indicate that coatomer actively regulates the function and localization of Rer1p.

Key words: retrieval • vesicle recycling • Golgi apparatus • coatomer • *Saccharomyces cerevisiae*

Introduction

ER proteins are strictly localized to the ER at the steady state by at least three retrieval mechanisms from the Golgi apparatus. Erd2p (Lewis and Pelham, 1990; Lewis et al., 1990; Semenza et al., 1990) and the coat protein (COP)[1] I complex (coatomer) (Cosson and Letourneur, 1994; Letourneur et al., 1994) directly bind to the COOH-terminal KDEL/HDEL signal (Munro and Pelham, 1987; Pelham, 1988) and the dilysine (KKXX) signal (Jackson et al., 1990) of ER proteins, respectively, and retrieve them from the Golgi to the ER by the COPI vesicles. Rer1p executes a very unique mechanism that is independent of either signal. Rer1p is a Golgi protein of 188 amino acid residues containing four transmembrane domains (TMDs) and is well conserved from yeast to human and plants (Boehm et al., 1994; Sato et al., 1995, 1999; Füllekrug et al., 1997). The *RER1* gene was identified initially by a mutation which mislocalized an ER membrane protein, Sec12p, to the trans-Golgi compartment (Nishikawa and Nakano, 1993). Further studies have revealed that not only Sec12p but also various ER membrane proteins, including Sed4p, Sec71p, Sec63p, and Mns1p, utilize the Rer1p-dependent retrieval mechanism (Sato et al., 1996, 1997; Massaad et al., 1999). These ER membrane proteins are not all in the same topology. For example, Sec12p is type II (Nakano et al., 1988; d'Enfert et al., 1991), Sec71p is type III (Feldheim et al., 1993; Kurihara and Silver, 1993), and Sec63p spans the membrane three times (Rothblatt et al., 1989; Sadler et al., 1989). Nevertheless, Rer1p recognizes these proteins and retrieves back to the ER (Sato et al., 1997). The Rer1p-dependent retrieval signals of Sec12p and Sec71p are present in the TMD (Sato et al., 1996; Sato, K., and A. Nakano, unpublished data).

Mutations of the α subunit of yeast coatomer result in mislocalization of Rer1p-dependent ER membrane proteins, suggesting that their retrieval by Rer1p is also fulfilled via the COPI vesicles (Boehm et al., 1997; Sato et al., 1997). Another piece of evidence supporting the link between COPI and Rer1p comes from a recent work on a yeast glycophosphatidylinositol (GPI)-anchored protein, Gas1p (Letourneur and Cosson, 1998). Gas1p is first synthesized as a precursor containing a TMD, and the GPI anchor is added after removal of the TMD. An invertase–Gas1p fusion protein, in which the TMD cleavage site was mutated, is localized to the ER in an Rer1p- and COPI-dependent manner. The TMD contains the determinant for the Rer1p-dependent retrieval. A mutant of the yeast α-factor receptor, Ste2p, is also retained in the ER in a similar fashion (Letourneur and Cosson, 1998).

All of these observations suggested that the most likely role for Rer1p would be a receptor for the retrieval signals in the TMDs. However, such a mechanism recognizing a signal in the lipid bilayer had no precedent and awaited biochemical demonstration. Here, we will present the first evidence for the physical interaction of Rer1p with the TMD of Sec12p and with the coatomer. We will also show that Rer1p performs a very dynamic behavior in living yeast cells which is essential for its function and localization.

✪The online version of this article contains supplemental material.

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[1]*Abbreviations used in this paper:* CCD, charge-coupled device; COP, coat protein; DSP, dithiobis (succinimidyl propionate); GFP, green fluorescent protein; GST, glutathione *S*-transferase; HA, hemagglutinin; MVB, multivesicular body; TMD, transmembrane domain.

Table I. Yeast Strains Used in This Study

Strain	Genotype	Source
SKY43	*MAT***a** *pep4::ADE2 ura3-52 leu2-3,112 trp1-289 his3 his4 suc gal2*	This study
SKY60-13A	*MAT***a** *ret1-1 sec16-2 ura3 trp1 leu2 his3*	This study
SMY22-10B	*MAT***a** *dap2::LEU2 mfα1::ADE2 bar1::HIS3 ura3 leu2 trp1 his3 his4 ade2*	This study
MBY3-15A	*MATα sec13-1 ura3-52 leu2-3, 112 trp1-289 his3 his4*	M. Bernstein and R. Schekman*
MBY4-1A	*MAT***a** *sec16-2 ura3-52 trp1-289 his3 his4*	M. Bernstein and R. Schekman*
MBY12-6D	*MATα sec18-1 ura3-52 leu2-3, 112 trp1-289 his3 his4*	M. Bernstein and R. Schekman*
MBY6-4D	*MATα sec21-1 ura3-52 leu2-3, 112 trp1-289 his3 his4*	M. Bernstein and R. Schekman*
RSY1315	*MATα ret1-1 ura3 leu2 trp1*	R. Schekman*
RSY1318	*MATα ret1-3 ura3-52 leu2-3, 112 lys2-801*	R. Schekman*
RSY770	*MATα sec27-1 ura3-52 leu2-3, 112 trp1*	R. Schekman*
EGY101	*MAT***a** *ret1-1 ura3 leu2 his3 trp1 suc2Δ9*	F. Letourneur‡
EGY103	*MAT***a** *sec21-2 ura3 leu2 his3 lys2 suc2Δ9*	F. Letourneur‡
SEY6210	*MATα ura3-52 leu2-3,112 trp1-D901 his3-D200 lys2-801 suc2-Δ9*	S. Emr§
SEY6211	*MAT***a** *ura3-52 leu2-3,112 trp1-D901 his3-D200 ade2-101 suc2-Δ9*	S. Emr§
SEY2102	*MATα ura3-52 leu2-3,112 his4-519 gal2 suc2-Δ9*	S. Emr§
YPH500	*MATα ura3-52 lys2-801a ade2-101oc trp1-Δ63 his3-Δ200 leu2-Δ1*	Sikorski and Hieter, 1989
SKY64	*MAT***a** *ste2::LEU2 ura3-52 leu2-3,112 trp1-D901 his3-D200 ade2-101 suc2Δ9*	This study
SKY65	*MAT***a** *sec21-2 ste2::LEU2 ura3 leu2 his3 lys2 suc2Δ9*	This study
ANY21	*MAT***a** *ura3-52 leu2-3,112 trp1-289 his3 his4 suc gal2*	Nakano et al., 1988

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Materials and Methods

Yeast Strains and Culture Condition

Saccharomyces cerevisiae strains used are listed in Table I. The *STE2*-deleted strains were constructed as described previously (Letourneur et al., 1994). Cells were grown in MVD medium (0.67% yeast nitrogen base without amino acids [Difco Laboratories, Inc.], and 2% glucose) or MCD medium, which is MVD containing 0.5% casamino acids (Difco Laboratories, Inc.), supplemented appropriately.

Plasmid Construction

DSD mutants were constructed by PCR-mediated mutagenesis as described previously (Sato et al., 1996). NQ-L and SY-L mutants were made by the replacement of Asn/Gln and Ser/Tyr residues in the TMD by two leucines, respectively (corresponding to N358L Q370L and S366L Y367L in Sec12p). In the +2L mutant, two leucines were inserted between Leu and Ser (L365 and S366 in Sec12p) in the TMD. LeuX19 is the mutant whose TMD was completely replaced by 19 leucines. The ORF of green fluorescent protein (GFP) in pEGFP-1 (CLONTECH Laboratories, Inc.) was amplified by PCR with primers: 5′-CGGGATCCATGGTGAG-CAAGGGCG-3′ and 5′-GAAGATCTCTTGTACAGCTCGTCC-3′. The obtained fragment was digested with BamHI and BglII and inserted between the *TDH3* promoter and the *CMK1* terminator on a single-copy plasmid (pTU1) with the *URA3* marker (Sato et al., 1999), resulting in pSKY5. The ORF of *RER1* or its derivatives was inserted into the BglII site of pSKY5. To construct *STE2* derivatives, we first replaced the *TDH3* promoter of pTU1 by the PCR-synthesized *STE2* promoter. A DNA fragment encoding the hemagglutinin (HA) epitope (MYPYDVPDYARS) and the PCR-amplified *STE2* ORF were sequentially inserted between the *STE2* promoter and the *CMK1* terminator. Similarly, an HA–Ste2-Rer1p chimera was constructed by ligating HA, a COOH-terminal truncated form of Ste2p (297 residues), and the COOH-terminal tail of Rer1p (28 residues) and placed between the *STE2* promoter and *CMK1* terminator.

Antibodies

Anti-Dap2p and anti-GFP polyclonal antibodies were provided by Y. Amaya (Niigata University, Niigata, Japan) and H. Abe (RIKEN), respectively. Rabbit anticoatomer and anti-Sec21p polyclonal antibodies were gifts from R. Duden (University of Cambridge, Cambridge, UK). The 12CA5 and 16B12 monoclonal antibodies against the HA epitope were purchased from Boehringer and Berkeley Antibody Company, respectively. Polyclonal antibodies against HA (Y11) and the myc epitope were obtained from Santa Cruz Biotechnology, Inc. and Medical & Biological Laboratories Co. Ltd., 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.) and a thermocontrol stage (Tokai Hit Co.). Images were acquired by a high-resolution digital charge-coupled device (CCD) camera (C4742-95; Hamamatsu Photonics) and processed by the IPLab software (Scanalytics).

Immunofluorescence Microscopy

Indirect immunofluorescence was performed as described previously (Sato et al., 1995) except that fixed cells were permeabilized with PBS, 1% Triton X-100, 10% sorbitol, 1% BSA. Staining of the HA-tagged Ste2p, HA–Ste2-Rer1p, and myc-Emp47 was performed by the use of the 16B12 monoclonal antibody and an anti-myc polyclonal antibody. These antibodies were decorated by the Alexa 488–conjugated goat anti–mouse antibody or the Alexa 568–conjugated goat anti–rabbit antibody (Molecular Probes).

In Vitro Binding of the COPI Subunits to the COOH-terminal Cytoplasmic Tail of Rer1p

In vitro coatomer binding assay was performed as described by Cosson and Letourneur (1994). Oligonucleotide fragments encoding the full-length COOH-terminal region of Rer1p (28 residues: M160RRQIQ. . . . . SHSSN188-c) or its mutant versions were inserted into the bacterial expression vector pGEX4T-1. The expressed glutathione *S*-transferase (GST) fusions were purified from *Escherichia coli* lysates by the use of the GSTrap column (Amersham Pharmacia Biotech). After desalting, the purified protein (250 μg) was immobilized on glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). To prepare yeast cytosol, spheroplasts of the wild-type strain or COPI mutants were lysed on ice in Hepes-Triton buffer (50 mM Hepes, pH 7.4, 90 mM KCl, 0.5% Triton X-100) containing protease inhibitors (1.8 mg/ml iodoacetamide, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 100 μg/ml PMSF). After centrifugation at 20,000 *g* for 15 min, the supernatant was incubated twice with the untreated glutathione-Sepharose beads at 4°C for 1 h. The glutathione-Sepharose beads coupled with the GST fusions were incubated with this cytosol at 4°C for 2 h. The beads were washed three times with Hepes-Triton buffer and once with 50 mM Hepes (pH 7.4). Bound proteins were eluted with the SDS sampling buffer and analyzed by SDS-PAGE and immunoblotting with anticoatomer and anti-Sec21p antibodies.

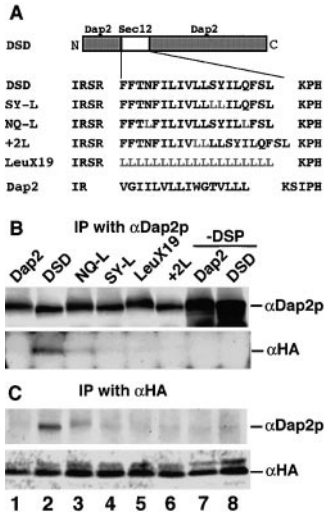

Figure 1. Physical interaction between Rer1p and DSD. Amino acid sequences of the junction regions of DSD and its derivatives are shown in A. *Δdap2* cells (SMY22-10B) expressing Rer1-3HAp on a multicopy plasmid and Dap2p, DSD, or DSD mutants (NQ-L, SY-L, Leu × 19, and +2L) on another multicopy plasmid under the *TDH3* promoter were spheroplasted, lysed with 25 mM sodium phosphate (pH 7.2), and further incubated with 5 mM DSP at 20°C for 20 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 the adjustment to 35 mM Tris-HCl (pH 8.0), 120 mM NaCl, and 2% SDS, the samples were heated at 75°C for 10 min and processed for immunoprecipitation with the anti-Dap2p (B) and anti-HA (C) antibodies. The immunoprecipitates were treated with 50 mM DTT to cleave DSP and then analyzed by immunoblotting with anti-Dap2 polyclonal antibody (B and C), and anti-HA monoclonal (B) and polyclonal (C) antibodies. SMY22-10B cells expressing Rer1-3HAp and Dap2p or DSD were also subjected to the same procedures (C, lanes 7 and 8) as controls in the absence of DSP.

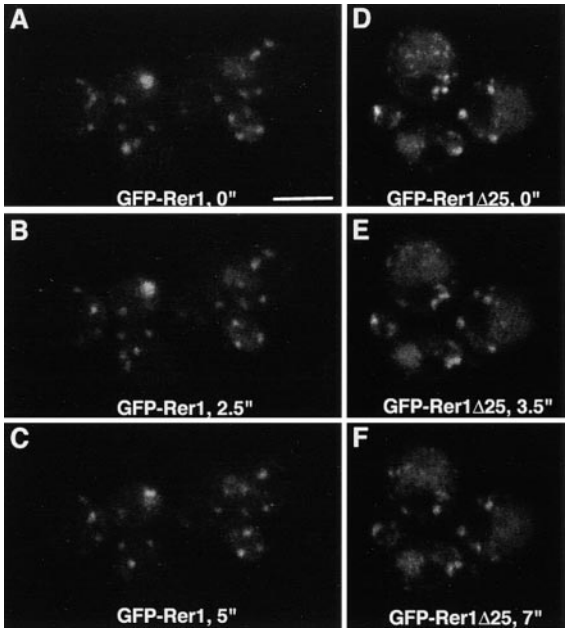

Figure 2. Real-time movements of GFP-Rer1p and GFP-Rer1Δ25p in living cells. Wild-type cells expressing GFP-Rer1p (A–C) and *Δpep4* cells expressing GFP-Rer1Δ25p (D–F) were grown to the early log phase at 20°C, and images were collected by real-time confocal fluorescence microscopy. Frames are taken at the indicated times (in seconds). Videos are available at http://www.jcb.org/cgi/content/full/152/5/935/DC1.

Online Supplemental Material

Video 1 and 2 (available at http://www.jcb.org/cgi/content/full/152/5/935/DC1) further depict Fig. 2. Images of wild-type cells expressing GFP-Rer1p (Video 1) and *Δpep4* cells expressing GFP-Rer1Δ25p (Video 2) were captured at the video rate (30 frames/s) by an Olympus BX-60 fluorescence microscope equipped with a confocal laser scanner unit CSU10 in combination with an image intensifier (VS4-1845; Video Scope) and a high-speed CCD camera (CCD-300T-RC; Dage-MTI) and processed by the IPLab software (Scanalytics).

Results

Physical Interaction of Rer1p with Sec12p TMD

We have shown in our previous paper (Sato et al., 1996) that Sec12p contains two signals for ER localization: one in the cytoplasmic domain for static retention and the other in the TMD for dynamic retrieval. The mechanism of dynamic retrieval depends on Rer1p. Sec71p also contains an Rer1p-dependent retrieval signal in its TMD (Sato, K., and A. Nakano, unpublished data). The TMD of Sec12p competes with Sec71p for the recognition by Rer1p (Sato et al., 1997). All these facts led us to the presumption that Rer1p directly binds to a structural motif in the TMDs of these membrane proteins.

After a long struggle to prove the physical interaction between Rer1p and the TMD of Sec12p, we decided to use a chimeric protein between Sec12p and Dap2p. Dap2p, a type II vacuolar membrane protein, has been used as a passenger protein to determine the ER localization signals of Sec12p (Sato et al., 1996). DSD, a chimeric protein comprised of the lumenal and cytoplasmic domains from Dap2p and the TMD from Sec12p, is almost completely localized to the ER by the Rer1p-dependent retrieval (Fig. 1 A). Chemical cross-linking experiments using a thiol-cleavable linker dithiobis (succinimidyl propionate) (DSP) were performed with the cell lysate prepared from a yeast strain overexpressing both Rer1-3HAp and DSD. Cells expressing Rer1-3HAp and Dap2p were used as a negative control. Cells were lysed and allowed to react with DSP. After the immunoprecipitation with either anti-Dap2p or anti-HA antibody, DSP was cleaved with 50 mM DTT, and the products were subjected to immunoblotting with anti-Dap2p and anti-HA antibodies. As shown in Fig. 1 (B and C), DSD and Rer1-3HAp were reproducibly coimmunoprecipitated by either anti-Dap2p or anti-HA antibody (lane 2). Such coprecipitation was not observed with the Dap2p control (Fig. 1, B and C, lane 1). Mutants of DSD, which have amino acid replacements in the TMD, and thus show less Rer1p dependency (NQ-L, SY-L, +2L, and LeuX19; Fig. 1 A) (Sato et al., 1996; Sato, M., unpublished data), were not efficiently coimmunoprecipitated with Rer1-3HAp by the anti-Dap2p antibody and vice versa (Fig. 1, B and C, lanes 3–6), indicating that Rer1p indeed recognizes the polar residues in the TMD of Sec12p.

GFP-Rer1p Actively Cycles between the Golgi and the ER

To examine the dynamic behavior of Rer1p in terms of localization, we took a morphological approach. GFP was fused to the NH_2 terminus of Rer1p (GFP-Rer1p). GFP-Rer1p complemented *rer1-2* and its cis-Golgi localization was indistinguishable from that of Emp47p (Schröder-Köhne et al., 1998) by double staining (data not shown).

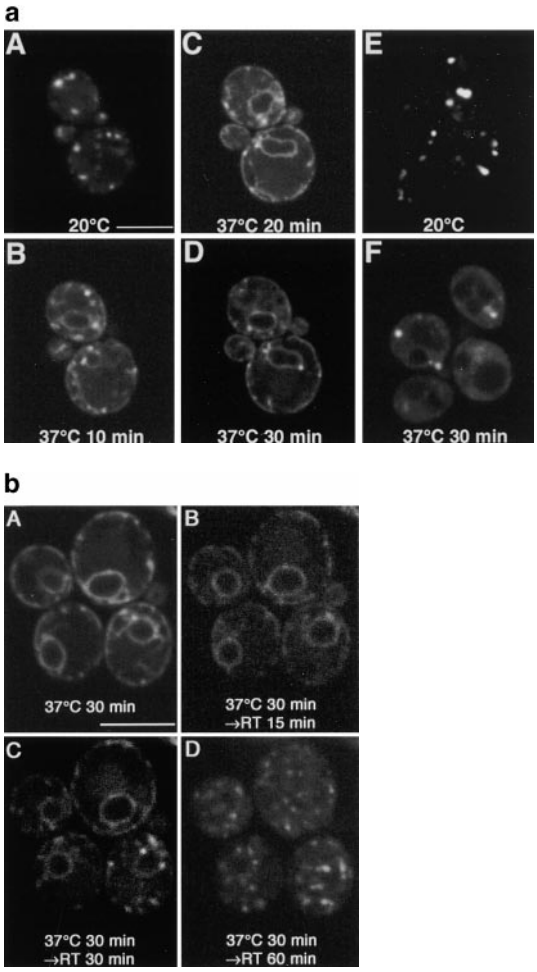

Figure 3. Dynamic behavior of GFP-Rer1p in *sec* mutants. (a) *sec13-1* (panels A–D) and *sec18-1* (panels E–F) cells expressing GFP-Rer1p were first observed at 20°C (panels A and E) by confocal fluorescence microscopy. The temperature of the microscope stage was then raised to and kept at 37°C for the indicated times (panels B–D and F). The same cells (panels A–F) are shown. (b) Reversibility of the relocalization of GFP-Rer1p to the ER in *sec13-1* cells. *sec13-1* cells expressing GFP-Rer1p were incubated at 37°C for 30 min (panel A) and then shifted to room temperature (RT) and observed for the indicated times (panels B–D). Bars, 5 μm.

Considering the advantage of GFP whose fluorescence is observable in living cells, we have developed a very rapid confocal laser scanning system which enables the video rate (30 frames/s) acquisition of images. The observation of cells expressing GFP-Rer1p under this microscope system shows real-time movement of bright punctate structures of the Golgi (Fig. 2, A–C; Video 1) like the case of GFP-Sed5p (Wooding and Pelham, 1998). These GFP-Rer1p–labeled structures moved rapidly and randomly. Interestingly, some structures appear to interact with each other.

If Rer1p is recycling between the Golgi and the ER, ER-to-Golgi anterograde transport would be required to ensure its steady-state localization in the Golgi. To test this possibility, GFP-Rer1p was expressed in the *sec13* mutant cells which have a temperature-sensitive defect in the COPII vesicle formation from the ER (Kaiser and Schekman, 1990). As shown in Fig. 3 a, GFP-Rer1p showed normal Golgi localization at 20°C (panel A), but when the temperature was shifted to 37°C, the fluorescence signal rapidly changed its pattern (panels B–D). The staining of the nuclear envelope and cell periphery seen at 37°C is a typical ER pattern of yeast. This relocalization is reversible. When the temperature is returned to the room temperature, GFP-Rer1p exhibits the Golgi pattern again (Fig. 3 b). Similar relocalization from the Golgi to the ER was also observed in *sec16-2* (see Fig. 6 c) and *sec23-1* cells at 37°C which are also defective in budding of the COPII vesicles from the ER.

When GFP-Rer1p was expressed in *sec18-1*, a mutant defective in the fusion of COPI and COPII vesicles with target membranes (Graham and Emr, 1991), the change of pattern is quite different. As shown in Fig. 3 a, panels E and F, the intensity and the number of bright dots of the Golgi decreased at 37°C, but the relocalization to the ER did not take place and instead cytoplasmic scattering signals showed up. These signals were very rapidly moving around when observed with an image intensifier and a high-speed CCD camera, and thus presumably represent retrograde COPI vesicles.

These results indicate that GFP-Rer1p is indeed rapidly recycling between the Golgi and the ER in a COPII- and Sec18p-dependent fashion.

COOH-terminal Tail of Rer1p Is Essential for Its Function and Localization

Rer1p and its orthologues in other organisms are well conserved in structure and in fact animal and plant *RER1* genes complement yeast *rer1* mutants (Füllekrug et al., 1997; Sato et al., 1999). We realized that the best-conserved part of the Rer1 family is located in the COOH-terminal tail which is predicted to be cytoplasmic. Furthermore, a new mutant allele of *RER1* which we recently isolated (*rer1-4*) had a missense mutation G179D in the tail. These suggest that the COOH-terminal tail region of Rer1p is important for its function. Indeed, a fusion construct of Rer1p which we made by hooking GFP to the COOH terminus did not complement *rer1* and stained the vacuole. Deletion of the COOH-terminal 25 amino acid residues also led to a functionless protein. These observations tempted us to pursue the role of the Rer1p tail in more detail.

We constructed a mutant version of GFP-Rer1p which lacks the COOH-terminal 25 residues (GFP-Rer1Δ25p). This GFP fusion did not complement the Sec12p-missorting phenotype of *rer1*. Wild-type and *Δpep4* cells expressing GFP-Rer1Δ25p were observed by confocal laser scanning microscopy (Fig. 4 a). Major fluorescent signals were detected in the vacuole, suggesting that the COOH-terminal 25 residues had the information for correct localization to the Golgi. The staining of vacuolar lumen rather than vacuolar membranes was surprising, however, because the GFP moiety of GFP-Rer1Δ25p is expected to face the cytoplasm. This is reminiscent of the behavior of carboxypeptidase S, which is transported to the vacuole via the multivesicular body (MVB)-mediated sorting pathway (Odorizzi et al., 1998). Interestingly, in the *Δpep4* cells in which vacuolar proteases are mostly inactive due to the lack of proteinase A (Jones, 1984), punctate fluorescent signals of GFP-Rer1Δ25p move around very rapidly in the vacuolar lumen (Fig. 2, D–F; Video 2). Immunoblotting analysis (Fig. 4 b) reveals that GFP-Rer1p remains intact (49 kD) in both wild-type and *Δpep4* cells (lanes 1 and 2)

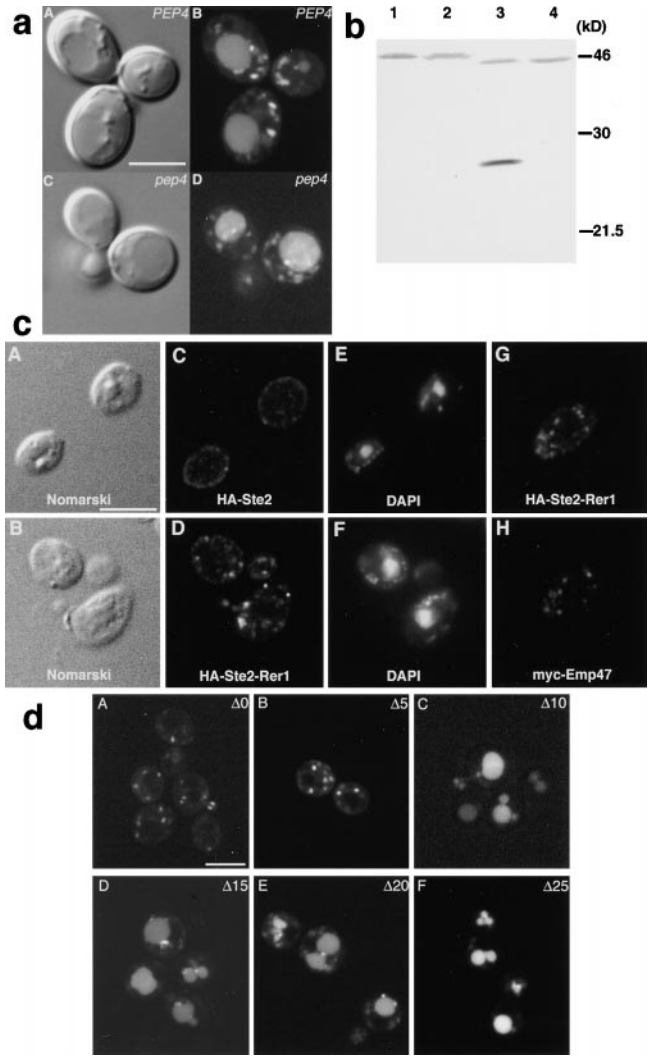

Figure 4. COOH-terminal tail of Rer1p is important for the Golgi localization. (a) Wild-type (ANY21; panels A and B) and *Δpep4* (SKY43; panels C and D) cells expressing GFP-Rer1Δ25p were observed by confocal laser microscopy. Nomarski (panels A and C) and fluorescence (panels B and D) images are shown. (b) Immunoblotting analysis of GFP-Rer1p and GFP-Rer1Δ25p. Wild-type (ANY21; lanes 1 and 3) and *Δpep4* (SKY43; lanes 2 and 4) cells expressing GFP-Rer1p (lanes 1 and 2) or GFP-Rer1Δ25p (lanes 3 and 4) under the *TDH3* promoter were grown at 23°C. Cell extracts (50 μg) were separated by SDS-PAGE and subjected to immunoblotting with the anti-GFP antibody. (c) Immunofluorescence staining of HA-Ste2p and HA–Ste2-Rer1p. Wild-type cells (SEY6211) expressing HA-Ste2p (panels A, C, and E) or HA–Ste2-Rer1p (panels B, D, and F) were grown at 30°C and subjected to immunofluorescence microscopy with the anti-HA monoclonal antibody (16B12). Panels C and D show Alexa 488 fluorescence corresponding to HA-Ste2p and HA–Ste2-Rer1p, respectively. Nomarski images (panels A and B) and DAPI images (panels E and F) are also shown. Panels G and H show double staining of HA–Ste2-Rer1p (G) and myc-Emp47p (H) in the SEY6211 cells expressing these two proteins. (d) Deletion analysis on the COOH-terminal region of GFP-Rer1p. Wild-type cells (ANY21) expressing GFP-Rer1p (Δ0) or its deletion mutants (Δ5, Δ10, Δ15, Δ20, and Δ25) were observed for GFP fluorescence. Bars, 5 μm.

but GFP-Rer1Δ25p (46 kD) is processed to the 27-kD species in a *PEP4*-dependent manner (lanes 3 and 4). Similar results were obtained when GFP fusions were expressed under the authentic *RER1* promoter (not shown). These observations suggest that GFP-Rer1Δ25p is targeted to the vacuole via the MVB pathway, and the very mobile structures in the vacuolar lumen of *Δpep4* cells are undegraded internal membranes of MVB.

If the COOH-terminal tail of Rer1p in fact acts as a Golgi localization signal, it should be capable of relocating other passenger proteins to the Golgi. To test this, we chose Ste2p, the α-factor receptor localized on the plasma membrane of *MAT***a** cells. We constructed an HA-tagged Ste2p and its variant with the COOH-terminal tail replaced by that of Rer1p (28 residues: MRRQI. . . .SHSSN-c) (HA–Ste2-Rer1p) and expressed them in the wild-type *MAT***a** cells at 30°C. As shown in Fig. 4 c, immunofluorescence of HA-Ste2p showed a typical plasma membrane pattern (panel C). In contrast, HA–Ste2-Rer1p was clearly localized to intracellular punctate structures like Rer1p itself (panel D). This staining overlapped well with the immunofluorescence of myc-Emp47p and thus indicates Golgi localization (panels G and H). Weak ER staining was also seen in some cells expressing HA–Ste2-Rer1p (not shown). Thus, the COOH-terminal tail of Rer1p is necessary and sufficient for the localization to the Golgi.

To define the localization signal(s) in the COOH-terminal tail of Rer1p more precisely, we generated a series of mutants from GFP-Rer1p. Deletion of the COOH-terminal 10 residues (Δ10) led to the mislocalization to the vacuole and the failure to complement *rer1*, but the Δ5 construct was normal (Fig. 4 d). We noticed that the amino acid sequence GKKKY (179–183) contains a dilysine-type motif and performed a mutational analysis on this. Single mutations affected the ability to complement *rer1* in different degrees (Fig. 5 a). As shown in Fig. 5 b, K180S and Y183A mutations caused clear mislocalization of GFP-Rer1p to the vacuole, whereas G179A and K182S had a marginal effect on the Golgi localization. K181S mutant was partially missorted to the vacuole. The KKSS double mutant (K180S K181S) completely lost the function as Rer1p (Fig. 5 a) and was severely mislocalized to the vacuole (data not shown). Interestingly, Y183A showed clear mislocalization to the vacuole but retained the Rer1p function to sort Sec12p.

We also realized that a tyrosine-containing YIPL (173–176) motif is present in the tail region of Rer1p. Such a tyrosine-based motif has been known to be involved in recognition by adapter complexes (Ohno et al., 1998), but recent reports (Mallabiabarrena et al., 1995; Cosson et al., 1998) also suggest its role in the ER retention and COPI binding. We constructed the Y173A mutant version of GFP-Rer1p. This mutant not only lost the ability to complement *rer1* but was also markedly mislocalized to the vacuole (Fig. 5 b).

The result of a pulse–chase experiment to follow the processing of GFP-Rer1p derivatives in *PEP4*$^+$ cells (Fig. 5 c) is consistent with the microscopic observations: Rer1Δ10, Rer1Δ15, Rer1Δ20, Rer1Δ25, Y173A, K180S, and Y183A showed rapid processing of GFP in the vacuole.

COPI-dependent Golgi Localization of Rer1p

We further examined the behavior of GFP-Rer1p in coatomer mutants, *ret1-1*, *ret1-3*, and *sec27-1*. The *ret1-1* mutant has a lesion in the α subunit of coatomer and shows a defect in the Golgi-to-ER retrograde transport of dilysine-harboring proteins but not in the anterograde transport of carboxypeptidase Y to the vacuole (Letour-

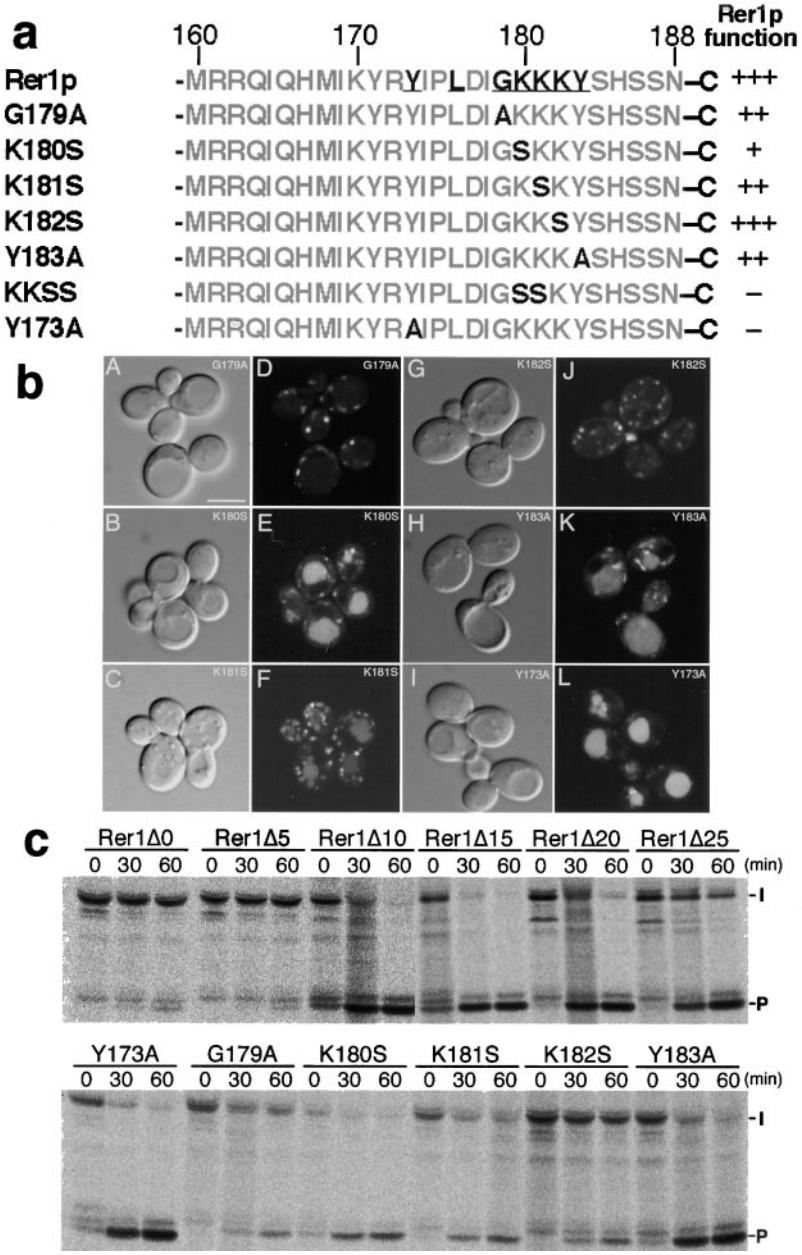

Figure 5. Dilysine-like motif and two tyrosines are required for the correct localization of Rer1p. (a) Mutational analysis of the COOH-terminal tail of GFP-Rer1p. The ability of each fusion to complement *rer1-2* in terms of missorting of Sec12-Mfα1p (Sato et al., 1995) is shown on the right. (b) Localization of a series of point mutants of GFP-Rer1p. Wild-type cells (ANY21) expressing each of GFP-Rer1p point mutants were harvested and subjected to fluorescence microscopy. Nomarski images (panels A–C and G–I) and GFP images (panels D–F and J–L) are shown. (c) Pulse–chase analyses of GFP-Rer1p mutants. Wild-type cells (ANY21) expressing each GFP-Rer1p derivative were labeled with Tran^{35}S-label (ICN Biochemicals) at 30°C for 10 min and chased for the indicated times. GFP fusions were immunoprecipitated with the anti-GFP antibody and analyzed by SDS-PAGE and radioimaging. I and P indicate intact and processed forms, respectively. Bar, 5 μm.

neur et al., 1994). *ret1-1* also mislocalizes the Rer1p-dependent ER membrane proteins (Sato et al., 1997). On the other hand, *ret1-3*, another mutant allele of α subunit, and *sec27-1*, a mutant of β′ subunit, show accumulation of the ER form of carboxypeptidase Y at the restrictive temperature (Duden et al., 1994, 1998) perhaps due to a secondary defect in the ER-to-Golgi anterograde traffic. As shown in Fig. 6 a (panels A–D), a large portion of GFP-Rer1p was mislocalized to the vacuole in the *ret1-1* mutant even at a permissive temperature (20°C). Accumulation of GFP-Rer1p in the ER was not detected at all with this mutant during the incubation at 37°C for 30 min. Similar results were obtained in *ret1-3* (panel E), *sec27-1* (panel F), *sec21-1* (Hosobuchi et al., 1992), and *sec21-2* (Letourneur et al., 1994) (data not shown). We also examined the transport kinetics of GFP-Rer1p in these mutants by a pulse–chase experiment at a semirestrictive temperature of 32°C (Fig. 6 b). After 60-min chase, ~75% of GFP-Rer1p was processed in *ret1-1*, *ret1-3*, and *sec27-1*, and 50% was processed in *sec21-1* or *sec21-2*. The relocalization of GFP-Rer1p to the ER seen in *sec16-2* (Fig. 6 c, panels D–F) was no longer observed in the *ret1-1 sec16-2* double mutant (Fig. 6 c, panels A–C). *MAT***a** cells expressing HA-Ste2p were able to mate with *MAT*α cells but those expressing HA–Ste2-Rer1p were not (Fig. 6 d, panel A) as expected from the localization experiment (Fig. 4 c). Strikingly, a mutation of γ-COP, *sec21-2*, remedied the inability of *Δste2*/HA–Ste2-Rer1p cells to mate (Fig. 6 d, panel B). These results strongly support the role of COPI in the correct Golgi localization of Rer1p.

As a direct test for the physical interaction between the COOH-terminal tail of Rer1p and the components of coatomer, we performed an in vitro binding assay using GST fusion proteins. The COOH-terminal 28 residues of

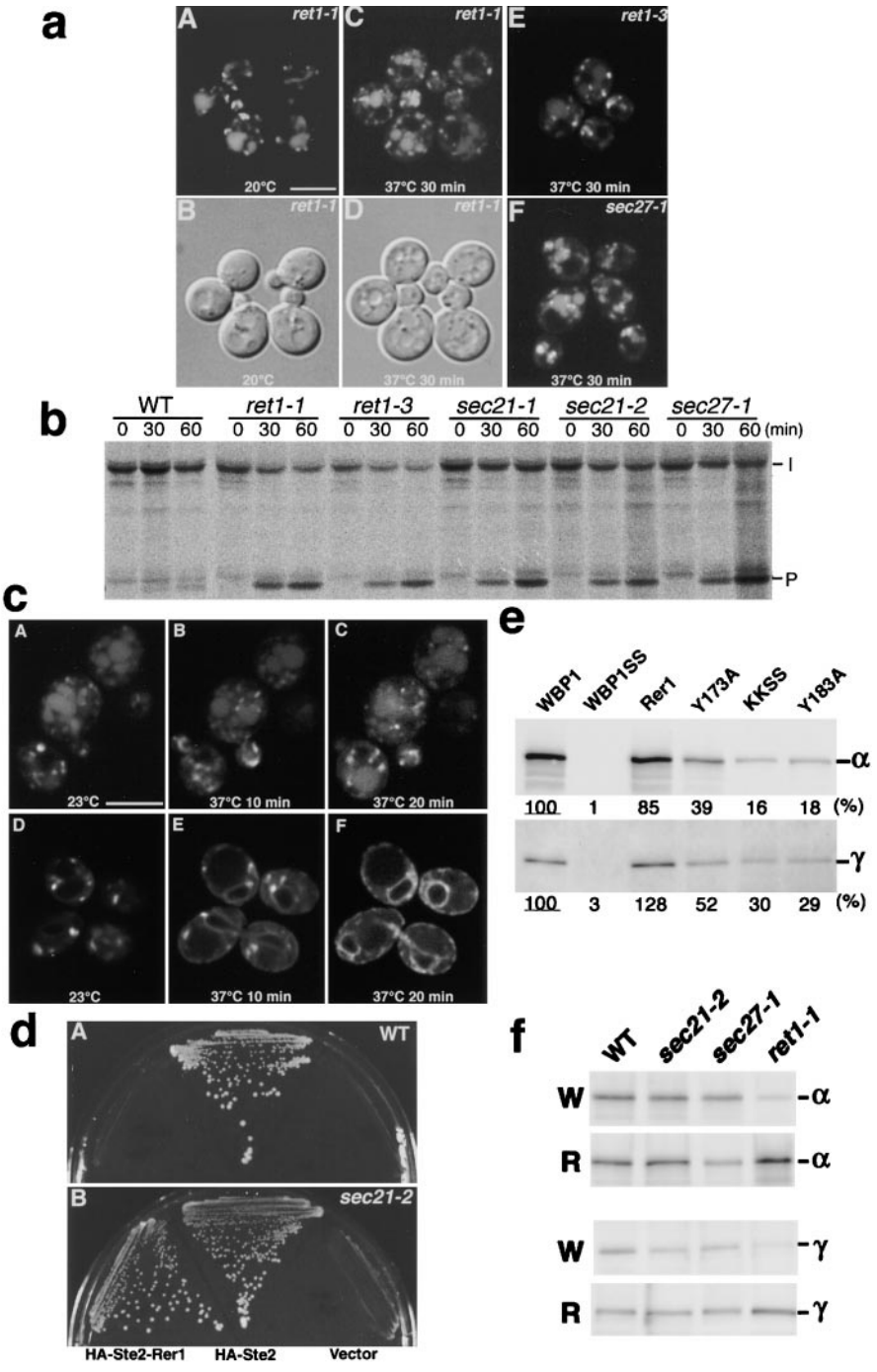

Figure 6. Coatomer-dependent Golgi localization of GFP-Rer1p. (a) Localization of GFP-Rer1p in coatomer mutants. *ret1-1* (RSY1315; panels A–D), *ret1-3* (RSY1318; panel E), and *sec27-1* (RSY770; panel F) cells expressing GFP-Rer1p were observed at 20°C or after the shift to 37°C for the indicated times. Panels B and D are Nomarski images of panels A and C. (b) Pulse–chase analysis of GFP-Rer1p in coatomer mutants. The mutant cells (*ret1-1*, *ret1-3*, *sec21-1*, *sec21-2*, and *sec27-1*) expressing GFP-Rer1p were preincubated at 32°C for 45 min, labeled with Tran^{35}S-label at 32°C for 10 min, and then chased for the indicated times. GFP fusions were immunoprecipitated with the anti-GFP antibody and analyzed by SDS-PAGE and radioimaging. I and P indicate intact (49-kD) and processed (27-kD) forms, respectively. (c) GFP-Rer1p does not move to the ER in *ret1-1 sec16-2* cells. *ret1-1 sec16-2* (SKY60-13A; panels A–C) and *sec16-2* (MBY4-1A; panels D–F) cells expressing GFP-Rer1p were first observed at 23°C (panels A and D) by confocal fluorescence microscopy. The temperature of the microscope stage was then raised to and kept at 37°C for the indicated times. The same cells (panels A–C and D–F) are shown. (d) A mating assay of *Δste2* expressing Ste2p derivatives. *Δste2* (SKY64) and *sec21-2 Δste2* (SKY65) cells of the *MAT***a** mating type, which express either HA-Ste2p or HA–Ste2-Rer1p, were mixed with *MAT*α cells (SEY2102) on YPD at 30°C for 2 d, then streaked on appropriate MVD plates selective for only mated diploid cells, and incubated at 30°C for 3 d. (e) In vitro COPI-binding assay. Purified GST-WBP1p, GST-WBP1SSp, GST-Rer1C28p, GST-Rer1C28pY173A, GST-Rer1C28pKKSS, and GST-Rer1C28p Y183A were immobilized on the glutathione-Sepharose beads and incubated with the wild-type yeast (YPH500) cytosol (see Materials and Methods). After washing, bound proteins were eluted and subjected to immunoblotting analysis with anticoatomer and anti-Sec21p antibodies. The coatomer subunits bound to each fusion were quantified and expressed as proportions relative to that bound to GST-WBP1 (100%). (f) Purified GST-WBP1p (W) and GST-Rer1p (R), which were immobilized on the glutathione-Sepharose beads, were incubated at 4°C with an 20S fraction from the lysates of wild-type (WT, SEY6210), *sec21-2* (EGY103), *sec27-1* (RSY770), and *ret1-1* (EGY101) in Hepes-Triton buffer. Bound proteins were eluted and analyzed as in panel e. α, α-COP (Ret1p); γ, γ-COP (Sec21p).

Rer1p (Rer1C28p) or its mutant versions (Y173A, KKSS [K180S, K181S], and Y183A) fused to GST were immobilized on glutathione-Sepharose beads and incubated with the wild-type yeast cytosol. Proteins bound to the beads were eluted and examined by immunoblotting with anticoatomer (anti-Ret1p) and anti-Sec21p antibodies to detect the coatomer complex(es) (Fig. 6 e). As reported by Cosson and Letourneur (1994), Ret1p and Sec21p were observed in proteins bound to GST-WBP1 (Wbp1p [KKLETFKKTN] but not to GST-WBP1SS [KKLETFSSTN] [Fig. 6 e, lanes 1 and 2]). As shown in Fig. 6 e, lane 3, both Ret1p and Sec21p were also detected in proteins bound to GST-Rer1C28p. KKSS and Y183A mutants showed lower COPI-binding ability (Fig. 6 e, lanes 5 and 6). Y173A was also low in the ability to bind COPI (Fig. 6 e, lane 4). The binding ability of GST-Rer1C28p to the coatomer was also assessed for the cytosol from COPI mutants, *ret1-1*, *sec21-2*, and *sec27-1* (Fig. 6 f). GST-WBP1

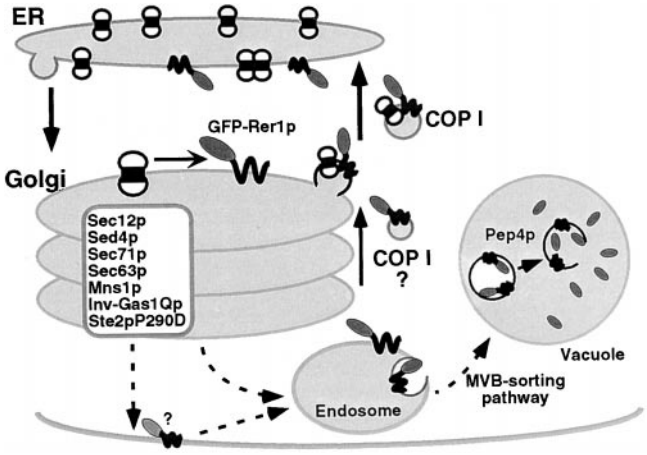

Figure 7. Model of the dynamic function of Rer1p. Rer1p recognizes the signal in the TMD of cargo proteins and actively recycles between the early Golgi and the ER. When it is mislocalized to the late Golgi, it is retrieved to the early Golgi by the COPI vesicles (solid arrows). In COPI mutants, Rer1p is no longer able to localize to the Golgi and is transported to the vacuole via the MVB sorting pathway (broken arrows).

bound Ret1p (α) and Sec21p (γ) from the cytosol of wild-type (SEY6210) and *sec21-2* and *sec27-1* mutants but not efficiently in the *ret1-1* cytosol as described previously (Letourneur et al., 1994). Interestingly, GST-Rer1C28p binds both Ret1p (α) and Sec21p (γ) even better in the *ret1-1* cytosol, whereas its binding to Ret1p (α) was lower in the *sec27-1* cytosol. This result suggests that the COOH-terminal tail of Rer1p interacts with the coatomer in a different fashion from the typical dilysine motif.

Discussion

The purpose of this study is to demonstrate that Rer1p is a sorting receptor in the cis-Golgi which recognizes signals present in TMDs of a set of ER membrane proteins and retrieves them to the ER (Fig. 7). We have shown the first biochemical evidence for the direct interaction between Rer1p and the TMD of Sec12p (Fig. 1). The following observations support the role of Rer1p as a receptor. First, the TMDs of Sec12p, Sed4p, and Gas1p contain information for the Rer1p-dependent retrieval to the ER, and the spatial locations of polar residues in these TMDs are important for the recognition by Rer1p (Sato et al., 1996; Letourneur and Cosson, 1998; Sato, K., and A. Nakano, unpublished data). Second, there is an apparent competition for Rer1p between Sec12p and Sec71p, suggesting a saturable mechanism for the retrieval (Sato et al., 1997). Rer1p is a limiting component in this competition. We have also demonstrated that coatomer plays a critical role for the function and localization of Rer1p. Mfα1p fusions of Sec12p, Sec71p, and Sec63p whose correct ER localization depends on Rer1p are all mislocalized to the trans-Golgi in an α-COP mutant (Sato et al., 1997). Rer1p itself is vigorously recycling between the Golgi and the ER in a COPI-dependent fashion (Fig. 3 and Fig. 6 c) and is mistargeted to the vacuole in α-, β′-, and γ-COP mutants (Fig. 6, a and b). Most importantly, coatomer subunits bind to the COOH-terminal tail of Rer1p in vitro (Fig. 6 e). All of these observations led us to conclude that Rer1p is a novel type of receptor that recognizes a retrieval signal in the lipid bilayer. Such a ligand–receptor interaction in the lipid milieu has been long proposed to explain membrane protein sorting. The binding of Rer1p with the Sec12p TMD would provide an ideal example to study the mode of interaction from a structural viewpoint as well.

Two possibilities can be considered for the coatomer-dependent function of Rer1p. First, Rer1p that has bound a ligand (ER membrane protein) could recruit coatomer and goes to the ER as a complex via the COPI vesicle. In this case, the role of Rer1p may be similar to that of Erd2p, the receptor of the KDEL/HDEL signal (Lewis and Pelham, 1990; Lewis et al., 1990; Semenza et al., 1990). Alternatively, the function of Rer1p may be to package the ligand into the COPI vesicle without entering by itself. This could be regarded as a packaging chaperone as in the case of Shr3p which loads amino acid permeases into the COPII vesicle but is left behind in the ER (Gilstring et al., 1999). In this model, the recycling of Rer1p between the Golgi and the ER is not necessary for the retrieval function itself but is rather important for the steady-state cis-Golgi localization of Rer1p. The difference between these models lies in the timing at which Rer1p releases the ligand. We are in favor of the former model because the strong physical interaction between the Rer1p tail and coatomer subunits implies the presence of a stable complex, but further studies will be required. The in vitro COPI vesicle formation assay from the Golgi (Spang and Schekman, 1998) may be useful to address this question. It is also conceivable that Rer1p plays a role as a folding chaperone in the Golgi. Sec71p and Sec63p form a multimeric complex in the ER membrane required for the post-translational translocation of newly synthesized secretory proteins (Deshaies et al., 1991). If one of them is mislocalized to the Golgi, the protein might expose some polar residues of the TMD to the hydrophobic environment in the lipid bilayer and become unstable. Rer1p could recognize such a circumstance and conceal these residues by binding to the TMD. When the Rer1p–ligand complex arrives at the ER, Rer1p can pass the ligand to its original partner which has a higher affinity than Rer1p. This idea might explain why the mutants of invertase Gas1p and Ste2p show the Rer1p-dependent ER localization (Letourneur and Cosson, 1998).

The dilysine-like motif ($K^{180}K^{181}$) and two tyrosine residues (Y^{173} and Y^{183}) are important for the steady-state localization and function of Rer1p. The former tyrosine is in the sequence YIPL, which is similar to tyrosine-based motifs involved in adaptor and COPI recognition. Replacement of the COOH-terminal tail of Ste2p with that of Rer1p led to the Golgi localization of the chimeric protein (Fig. 4 c). Since a mutation in any of these motifs causes the mislocalization of GFP-Rer1p to the vacuole and the inefficient binding of GST-Rer1C28p to the coatomer subunits, they may form a single site recognized by the coatomer. This is reminiscent of the case of the unassembled CD3-ε chain of the T cell receptor which is not transported to the plasma membrane and retained in the ER. Its COOH-terminal five amino acid residues (NQRRI) in addition to the tyrosine-based motif (YSGL) are required for the efficient ER localization possibly by a retrieval

mechanism (Mallabiabarrena et al., 1995). Cosson et al. (1998) have recently reported that the tyrosine motif is sufficient for the binding of COPI in vitro. Interestingly, we find that the cytoplasmic tail of Rer1p efficiently binds the coatomer from *ret1-1* and *sec21-2* but not from *sec27-1* in vitro (Fig. 6 f), although GFP-Rer1p is mistargeted to the vacuole in these mutants (Fig. 6, a and b). This result implies that the coatomer complex or its subcomplexes may recognize the COOH-terminal tail of Rer1p in a different way from the case of the typical dilysine motif. A GST fusion with Emp47 tail is reported to bind the coatomer from *ret1-1* but not that from *ret2-1* (δ-COP mutant) (Schröder-Köhne et al., 1998), whereas the coatomer from *ret1-1* or *sec27-1* is able to bind to a GST fusion containing a new COPI binding motif (WXXXW) which specifically interacts with δ-COP in vitro (Cosson et al., 1998). Such a diversity in the mode of coatomer binding may reflect the presence of multiple pockets in the coatomer complex for the recognition of substrates (Fiedler et al., 1996). However, it should be noted that such in vitro binding experiments may not reflect quantitative differences in the affinity of the mutant coatomer and the binding motifs. Further careful analysis needs to be performed under more quantifiable conditions to discuss these observations more rigorously.

We should also consider the possibility that any of these motifs may function as a retrieval signal to the cis-Golgi from the later Golgi. Previous studies by other groups failed to observe the mislocalization of Rer1p in coatomer mutants (Boehm et al., 1997; Schröder-Köhne et al., 1998). However, our sensitive assay adopting GFP-Rer1p clearly shows that the correct Golgi localization of Rer1p depends on the coatomer function (Fig. 6, a and b). Missorting of Rer1p in COPI mutants suggests the role of the coatomer in intra-Golgi recycling. The Y183A mutant of Rer1p is quite interesting in this regard because it is largely mistransported to the vacuole but does not show a significant deficiency of the activity to retrieve Sec12p. This may imply that Tyr183 is important for the intra-Golgi recycling of Rer1p via the COPI vesicles but not for the Golgi-to-ER retrieval. Another candidate that may regulate the Rer1p function through the tyrosine-based motif is a complex called retromer. Retromer is shown to localize on the endosome and function for the retrieval of Vps10p, a sorting receptor containing a tyrosine-based motif, from the endosome to the trans-Golgi (Seaman et al., 1998). If Rer1p is transported to the endosome, it may well be recycled back to the Golgi by the retromer. Multiple mechanisms of recycling may be required for the correct localization of Rer1p in the cis-Golgi (Fig. 7).

In the COPI mutants we have examined, GFP-Rer1p does not relocate to the ER even at the restrictive temperature. This is consistent with the idea that COPI functions in the retrograde protein transport from the Golgi to the ER. The use of GFP-Rer1p as a monitor protein will be helpful to examine whether a mutant has a defect in the anterograde or retrograde transport. Furthermore, the use of a real-time visualization system has enabled us to observe very rapid movement of membranes. The quite mobile structures found in the vacuole of *Δpep4* cells expressing GFP-Rer1Δ25p are indicative of the MVB sorting pathway. Analysis of GFP-Rer1p along with the development of a high performance visualization system would provide further insights into the mechanisms of membrane protein localization and sorting.

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