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.

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 dilyssine (KKXX) signal (Jackson et al., 1990) of ER proteins, respectively, and retrieve them from the Golgi to the ER by the COP1 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; Fülekrug 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retrieval signals of Sec12p and Sec71p are present in the TMD (Sato et al., 1996; Sato, K., and A. Nakano, unpublished data).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., 1999). Another piece of evidence supporting the link between COPI and Rer1p comes from a recent work on a yeast glycosphingolipidinostitol (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-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.

Key words: retrieval • vesicle recycling • Golgi apparatus • coatomer • Saccharomyces cerevisiae
Table I. Yeast Strains Used in This Study

| Strain    | Genotype                                      | Source               |
|-----------|-----------------------------------------------|----------------------|
| SKY43     | MATa pep4::ADE2 ura3-52 leu2-3,112 trp1-289 his3 his4 suc gal2 | This study           |
| SKY160-13A| MATa ret1-1 sec16-2 ura3 trp1 leu2 his3    | This study           |
| SYM22-10B | MATa dap2::LEU2 mfa1::ADE2 bar1::HIS3 ura3 leu2 trp1 his3 his4 ade2 | M. Bernstein and R. Schekman* |
| MBX3-15A  | MATa sec13-1 ura3-52 leu2-3, 112 trp1-289 his3 his4 | This study           |
| MBY4-1A   | MATa sec16-2 ura3-52 trp1-289 his3 his4      | M. Bernstein and R. Schekman* |
| MBY12-6D  | MATa sec18-1 ura3-52 leu2-3, 112 trp1-289 his3 his4 | M. Bernstein and R. Schekman* |
| MBY6-4D   | MATa sec21-1 ura3-52 leu2-3, 112 trp1-289 his3 his4 | M. Bernstein and R. Schekman* |
| RSY1315   | MATa ret1-1 ura3 leu2 trp1                   | R. Schekman*         |
| RSY1318   | MATa ret1-3 ura3-52 leu2-3, 112 lys2-801     | R. Schekman*         |
| RSY770    | MATa sec27-1 ura3-52 leu2-3, 112 trp1        | R. Schekman*         |
| EGY101    | MATa ret1-1 ura3 leu3 his3 trp1 sucΔ9        | F. Letourneur2       |
| EGY103    | MATa sec21-2 ura3 leu2 his3 lys2 sucΔ9       | F. Letourneur2       |
| SEY6210   | MATa ura3-52 leu2-3,112 trp1-D901 his3-D200 lys2-801 suc2-Δ9 | S. Emr§  |
| SEY6211   | MATa ura3-52 leu2-3,112 trp1-D901 his3-D200 ade2-101 suc2-Δ9 | S. Emr§  |
| SEY2102   | MATa ura3-52 leu2-3,112 his4-519 gal2 suc2-Δ9 | S. Emr§  |
| YPH500    | MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 | Sikorski and Hieter, 1989 |
| SKY64     | MATa ste2::LEU2 ura3-52 leu2-3,112 trp1-D901 his3-D200 ade2-101 suc2-Δ9 | This study |
| SKY65     | MATa sec21-2 ste2::LEU2 ura3 leu2 his3 lys2 sucΔ9 | This study |
| ANY21     | MATa 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.], supplemented appropriately. Polyclonal antibodies against HA (Y11) and the myc 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.

Plasmid Construction

DSD mutants were constructed by PCR-mediated mutagenesis as described previously (Sato et al., 1996). NO-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'-ccgagatccatgtgtagac-aaggggcc3'- and 5'-ggagactctttgactgcctgcc-3'. The obtained fragment was digested with BamHI and BglII and inserted into the TDIH3 promoter and the CMK1 terminator on a single-copy plasmid (pTU1) with the URA3 marker (Sato et al., 1999), resulting in pSK5. The ORF of RER1 or its derivatives was inserted into the BglII site of pSK5. To construct STE2 derivatives, we first replaced the TDIH3 promoter of pTU1 by the PCR-synthesized STE2 promoter. A DNA fragment encoding the hemagglutinin (HA) epitope (MYPPDYVPDYARS) 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 fragment 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 anti-coatomer and anti- sec2p 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 cotranslational 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... SSNSSN188) 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 un-treated glutathione-Sepharose 4B 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 anti-coatomer and anti-sec2p antibodies.
the absence of DSP.

subjected to the same procedures (C, lanes 7 and 8) as controls in 10B cells expressing Rer1-3HAp and Dap2p or DSD were also anti-HA monoclonal (B) and polyclonal (C) antibodies. SMY22-munoblotting with anti-Dap2 polyclonal antibody (B and C), and treated with 50 mM DTT to cleave DSP and then analyzed by 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.

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 between Rer1p 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 diethyldithio reversion microsomal 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 IP Lab software (Scanalytics).

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.

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).

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.

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 NH2 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).
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).
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 degraded 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 MATa 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 MATa 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 GKKY (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-
neur et al., 1994). retl-1 also mislocalizes the Rer1p-dependent ER membrane proteins (Sato et al., 1997). On the other hand, retl-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 retl-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 retl-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 retl-1, retl-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 retl-1 sec16-2 double mutant (Fig. 6 c, panels A–C). MATa cells expressing HA-Ste2p were able to mate with MATα 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/HAA–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 coatamer, we performed an in vitro binding assay using GST fusion proteins. The COOH-terminal 28 residues of

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-Mla1p (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 Tran35S-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.
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 [KKLETFKKKTN] but not to GST-WBP1SS [KKLETF-SSTN] [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
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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 retrieval (Sato et al., 1997). Rer1p fusions of Sec12p, Sec71p, and Sec63p whose correct ER localization depends on Rer1p are all mislocalized to the trans-Golgi in an a-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). The dilysine-like motif (K180K181) and two tyrosine residues (Y173 and Y183) 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 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-e 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 for the 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 go 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 multi-meric 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 (K180K181) and two tyrosine residues (Y173 and Y183) 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-e 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 for the 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.
mechanism (Mallababarena 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 \textit{ret1}-1 and \textit{sec21}-2 but not from \textit{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 \textit{ret1}-1 but not that from \textit{ret2}-1 (β-COP mutant) (Schröder-Köhne et al., 1998), whereas the coatomer from \textit{ret1}-1 or \textit{sec27}-1 is able to bind to a GST fusion containing a new COPI binding motif (WXXXW) which specifically interacts with β-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). 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). Mis-sorting 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 mis-targeted 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 retrometer. 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 Δ\textit{perp} \textit{C} 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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