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Endoplasmic Reticulum Quality Control of Unassembled Iron Transporter Depends on Rer1p-mediated Retrieval from the Golgi

Miyuki Sato,*† Ken Sato,* and Akihiko Nakano*‡§

*Molecular Membrane Biology Laboratory, RIKEN Discovery Research Institute, Saitama 351-0198, Japan; and ‡Department of Biological Sciences, Graduate School of Science, University of Tokyo, Tokyo 113-0033, Japan

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Endoplasmic reticulum (ER) quality control is a conserved process by which misfolded or unassembled proteins are selectively retained in the endoplasmic reticulum (ER). Failure in oligomerization of multisubunit membrane proteins is one of the events that triggers ER quality control. The transmembrane domains (TMDs) of unassembled subunits are determinants of ER retention in many cases, although the mechanism of the TMD-mediated sorting of unassembled subunits remains elusive. We studied a yeast iron transporter complex on the cell surface as a new model system for ER quality control. When Fet3p, a transmembrane subunit, is not assembled with the other membrane subunit, Ftr1p, unassembled Fet3p is exclusively localized to the ER at steady state. The TMD of Fet3p contains a determinant for this process. However, pulse-chase analysis and in vitro budding assays indicate that unassembled Fet3p rapidly escapes from the ER. Furthermore, Rer1p, a retrieval receptor for ER-resident membrane proteins in the Golgi, is responsible for the TMD-dependent ER retrieval of unassembled Fet3p. These findings provide clear evidence that the ER quality control of unassembled membrane proteins can be achieved by retrieval from the Golgi and that Rer1p serves as a specific sorting receptor in this process.

INTRODUCTION

The eukaryotic secretory pathway initiates from the endoplasmic reticulum (ER), where newly synthesized proteins are assisted in their folding and oligomerization by molecular chaperones. The ER has a surveillance system termed ER quality control (ERQC), which prevents transport of immature proteins beyond the ER (Ellgaard *et al*., 1999; Ellgaard and Helenius, 2003). ERQC is generally observed in yeast and higher eukaryotes and is now known to relate to a number of diseases, including cystic fibrosis and α1-antitrypsin deficiency (Ward *et al*., 1995; Teckman and Perlmutter, 1996). Molecules recognized by ERQC are often subject to rapid degradation by the ER-associated degradation (ERAD) mechanism, in which proteins are dislocated from the ER and targeted for ubiquitin-proteasome–dependent degradation (Brodsky and McCracken, 1999; Tsai *et al*., 2002). On the other hand, some misfolded proteins such as a variant of vesicular stomatitis virus G protein (VSV-G) tsO45 are rather stably localized in the ER for a prolonged period (Doms *et al*., 1987; Loayza *et al*., 1998). ER-resident chaperones and glycosylation events are also involved in recognition of ERQC substrates (Ellgaard *et al*., 1999; Ellgaard and Helenius, 2003).

Compared with the ERAD and chaperone-dependent mechanisms, how the substrates of ERQC are retained in the ER is poorly understood in terms of protein transport. ER localization of ERQC substrates could occur either by static retention in the ER or retrieval from post-ER compartments. Retrograde transport from the Golgi to the ER, which is mediated by COPI-coated vesicles, plays a critical role in correct localization of authentic ER-resident proteins. Protein sorting in the early-Golgi is accomplished by multiple mechanisms. Erd2p recognizes the C-terminal HDEL (KDEL in mammals) sequence of lumenal proteins and recruits them into COPI-vesicles (Semenza *et al*., 1990). The C-terminal di-lysine motif of type I transmembrane proteins also acts as a retrieval signal through its direct binding to the COPI complex (Jackson *et al*., 1993; Letourneur *et al*., 1994). In addition to these mechanisms, we and others have elucidated the Rer1p-dependent retrieval pathway (Nishikawa and Nakano, 1993; Boehm *et al*., 1994; Sato *et al*., 1995). Rer1p is a Golgi-resident protein with four transmembrane domains (TMDs) that is required for the ER localization of a variety of ER membrane proteins (Sato *et al*., 1997; Massaad *et al*., 1999). Rer1p recognizes polar amino acid residues specifically arranged in the TMD of its ligands and returns them to the ER via COPI vesicles (Sato *et al*., 1996, 1997, 2001). Recently, there were reports in yeast showing that retrieval from the Golgi is involved in ERQC of mutant forms of soluble proteins but not in that of misfolded membrane proteins (Vashist *et al*., 2001; Haynes *et al*., 2002). In

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† Present address: Department of Molecular Biology and Biochemistry, Rutgers University, Piscataway, NJ 08854.
§ Corresponding author. E-mail address: nakano@postman.riken.go.jp.

Abbreviations used: ERAD, endoplasmic reticulum-associated degradation; ERQC, endoplasmic reticulum quality control; GFP, green fluorescent protein; PM, plasma membrane; TMD, transmembrane domain; VSV-G, vesicular stomatitis virus G protein.

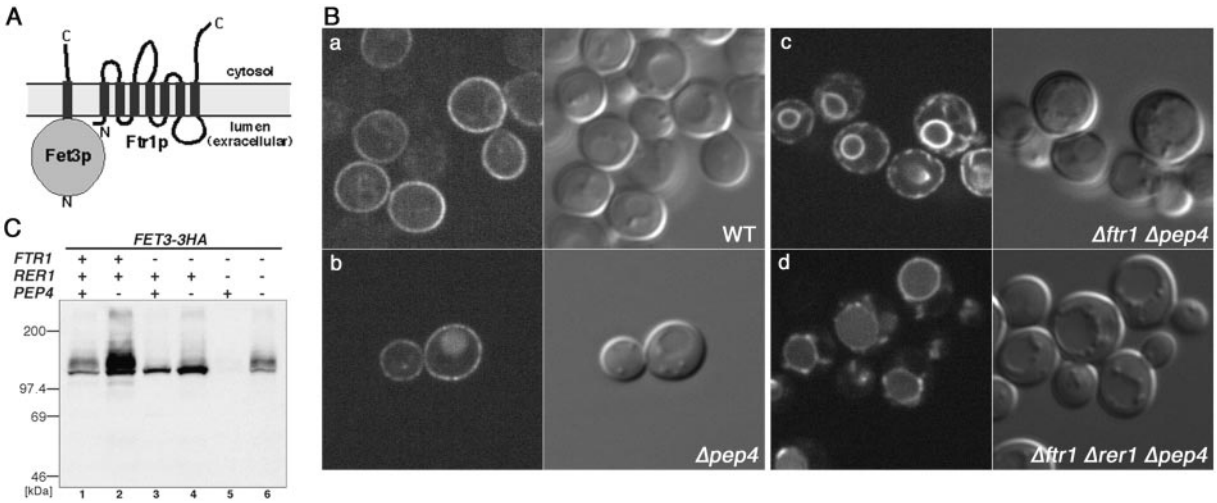

Figure 1. Unassembled Fet3p is localized to the ER. (A) Schematic representation of a yeast high-affinity iron transporter complex on the PM. (B) Rer1p-dependent ER localization of unassembled Fet3p-GFP. *FET3-GFP* was integrated into the *FET3* locus of the wild-type (SMY502; a), Δ*pep4* (SMY501; b), Δ*ftr1* Δ*pep4* (SMY531; c), or Δ*ftr1* Δ*rer1* Δ*pep4* (SMY541; d). The GFP signal was observed in living cells by confocal microscopy. The right panels in the pair show Nomarski images in the same field. (C) Immunoblotting of Fet3-3HAp. *FET3-3HA* was integrated into the *FET3* locus of the wild-type (YPH500; lane 1), Δ*pep4* (SMY501; lane 2), Δ*ftr1* (SMY53; lane 3), Δ*ftr1* Δ*pep4* (SMY531; lane 4), Δ*ftr1* Δ*rer1* (SMY54; lane 5), or Δ*ftr1* Δ*rer1* Δ*pep4* (SMY541; lane 6). Total cell lysates were prepared and analyzed by immunoblotting by using the anti-HA antibody.

mammalian cells, the static retention mechanism was suggested for several membrane proteins arrested by ERQC, including VSV-G tsO45 and the unassembled major histocompatibility complex (MHC) class I molecule (Nehls *et al*., 2000; Spiliotis *et al*., 2002). Thus, the current idea is that soluble ERQC substrates travel to the Golgi but transmembrane substrates are statically retained in the ER. Molecular mechanisms of these sorting events are not understood.

Here, we focus our attention to the sorting of a membrane protein subunit missing its intrinsic partner. Failure in oligomerization is known to be a general cause of ERQC for multisubunit membrane proteins. An interesting feature of this type of ERQC in mammalian cells is that the TMDs of unassembled subunits are the determinants of ER retention in many cases (Bonifacino *et al*., 1990, 1991; Cosson *et al*., 1991; Reth *et al*., 1991; Letourneur *et al*., 1995). Although such a specific region has been identified as an ER localization signal, it remains unknown how this TMD-mediated molecular sorting of unassembled proteins is accomplished. To address this question, we chose an iron transporter complex of *Saccharomyces cerevisiae* as a new model system for ERQC of unassembled membrane proteins. Two subunits, Fet3p and Ftr1p, constitute a high-affinity iron transporter on the plasma membrane (PM) (Figure 1A) (Askwith *et al*., 1994; Dancis *et al*., 1994). Ftr1p is an iron permease with multiple TMDs, whereas Fet3p is a type I transmembrane protein containing a large lumenal (extracellular) domain. The lumenal domain of Fet3p exhibits similarity to human ceruloplasmin and possesses a multicopper oxidase activity essential for iron transport across membranes. This complex and related factors have an important role in iron homeostasis, which is associated with many disease processes (Dancis *et al*., 1994; Yuan *et al*., 1995; Stearman *et al*., 1996). A previous study suggested that Ftr1p and Fet3p form a complex in the ER and that this is prerequisite for exit from the ER (Stearman *et al*., 1996). In the absence of Fet3p, Ftr1p is not transported to the PM but is retained in the ER. On the other hand, Ftr1p expression is required for Fet3p to be loaded with copper, which takes place in the Golgi or a post-Golgi compartment, suggesting that in the absence of Ftr1p Fet3p is retained in an earlier compartment. Thus, Fet3p and Ftr1p depend on each other, and the lack of the partner seems to result in a typical ERQC case of unassembled subunits.

To reveal mechanisms underlying the sorting events of unassembled membrane protein, we analyzed the fate of Fet3p in Δ*ftr1* cells by using morphological and biochemical approaches. We confirmed morphologically that, in the cells lacking Ftr1p, Fet3p is exclusively localized to the ER at steady state. However, our in vivo and in vitro analysis demonstrated that unassembled Fet3p is rapidly exported from the ER and reaches the early-Golgi compartment. We further showed that the ER localization of orphan Fet3p strongly depends on its TMD and that Rer1p, the sorting receptor in the early-Golgi, is required for the retrieval of Fet3p to the ER by recognizing a specific residue in the TMD. These results indicate that Rer1p-dependent retrieval is a molecular mechanism responsible for TMD-mediated sorting of an unassembled membrane protein. We suggest that the ER localization of ERQC substrates is fulfilled by multiple mechanisms depending upon the substrate.

MATERIALS AND METHODS

Plasmids Construction

The *FET3* gene was amplified by polymerase chain reaction (PCR) by using primers that correspond to 1 kb upstream and 0.7 kb downstream of the *FET3* open reading frame and cloned into the *Sac*I and *Xho*I sites of pRS306 (Sikorski and Hieter, 1989). A *Spe*I site was created just before the stop codon and the fragments encoding 3HA or green fluorescent protein (GFP) were inserted into this *Spe*I site to produce pFET3-42 and pFET3-43, respectively. For integration of *FET3-3HA* and *FET3-GFP* into the *FET3* locus, pFET3-42 and pFET3-43 were digested with *Sac*I and *Bsi*WI, blunted and self-ligated to yield pFET3-52 and pFET3-53 that contain only the 3′ halves of the tagged *FET3* genes.

The TMD mutants were made as follows. In pFET3-42, *Nhe*I and *Afl*II sites were inserted after the 560th and 584th codons of *FET3*, respectively (pFET3-802NA). The *Nhe*I to *Afl*II region corresponding to the fragment encoding the TMD was replaced with the DNA fragment encoding 24 leucines (pFET3-802L24). Silent mutations were introduced at the 596th and 597th codons to create a *Bgl*II site (GAT CTG) in pFET3-42 (pFET3-42B). The PCR-based site-directed mutagenesis was performed to change each polar residue in the

TMD to leucine, and these fragments were inserted into the *Bam*HI (intrinsic)-*Bgl*II site of pFET3-42B. *FET3-GFP* versions of these mutants were constructed by the same strategy by using pFET3-43 instead of pFET3-42. To integrate these constructs into the *ura3* locus, plasmids were linearized by the use of *Nsp*V in the *URA3* gene of pRS306 and used for transformation.

The open reading frame and the downstream sequence of *FET3-3HA* and its mutant forms (L24 and S567L) were cloned into the *Eco*RI and *Xho*I sites of pTU1, which contains the *TDH3* promoter on a single-copy plasmid pRS316. *GFP-RER1* under the *TDH3* promoter (pSKY5-RER1) was described previously (Sato *et al*., 2001).

Strains and Culture Conditions

The Δ*fet3*::kanMX and Δ*ftr1*::kanMX4 loci were amplified by PCR by using the genomic DNA of the Δymr058w and Δyer145c strains from EUROSCARF as templates. SNY9 (the wild-type) (Nishikawa and Nakano, 1993) and SKY7 (Δ*rer1*) (Sato *et al*., 1995) were transformed with these DNA fragments, and transformants were screened on YPD plates containing 0.2 mg/ml G418. The correct disruption was checked both by PCR and by the sensitivity to a Fe(II) chelator, bathophenanthroline sulfonate (Sigma-Aldrich, St. Louis, MO). They are crossed with the wild-type YPH499 (Sikorski and Hieter, 1989), and *MAT*α Δ*fet3* (SMY51), *MAT*α Δ*ftr1* (SMY53), *MAT*α Δ*ftr1* Δ*rer1* (SMY54), and *MAT*α Δ*rer1* (SMY55) were selected after tetrad dissection. The Δ*fet3* and Δ*ftr1* Δ*rer1* cells were crossed, and *MAT*α Δ*fet3* Δ*ftr1* (SMY60) and *MAT*α Δ*fet3* Δ*ftr1* Δ*rer1* (SMY61) were selected from nonparental ditype asci of tetrad dissection. In YPH500 (Sikorski and Hieter, 1989), SMY51, SMY53, SMY54, SMY55, SMY60, and SMY61, *PEP4* was disrupted by the *ADE2* marker (resulting in SMY501, SMY511, SMY531, SMY541, SMY631, SMY601, and SMY611, respectively), or the *ADE2* gene was reintroduced into the *ade2* locus (SMY502, SMY512, SMY532, SMY542, SMY632, SMY602, and SMY612, respectively).

To replace the *FET3* locus with *FET3-3HA*, yeast cells were transformed with pFET3-52 digested with *Bam*HI. The correct transformants were streaked onto plates containing 5′-fluoroorotic acid to loop out *URA3* (Guthrie and Fink, 1991), and the cells expressing *FET3-3HA* were screened by immunoblotting using the anti-hemagglutinin (HA) antibody. *FET3-GFP* was also integrated into the *FET3* locus by using pFET3-53 after *Bam*HI digestion and correct integration was checked by PCR.

Yeast cells were grown in SC (0.67% yeast nitrogen base without amino acids [Difco, Detroit, MI], 2% glucose, and 0.2% Drop-out mix) (Kaiser *et al*., 1994) supplemented with 10 μM $FeSO_4$ to keep the expression level of *FET3* constant.

Immunoblotting and Cross-linking Experiment

Cells were cultured at 30°C to the middle logarithmic phase. Total cell lysates (60 μg) were prepared by agitation with glass beads in 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% (wt/vol) glycerol, and 1 mM phenylmethylsulfonyl fluoride and boiling at 70°C for 10 min. They were subjected to immunoblotting by using the anti-HA monoclonal antibody (mAb) (16B12; Covance Research Products, Berkeley, CA) and visualized by the ECL-plus chemiluminescent detection system (Amersham Biosciences, Piscataway, NJ).

Chemical cross-linking between Rer1p and Fet3-3HAp was performed using the thiol-cleavable linker dithiobis-(succinimidylpropionate) (DSP) as described previously (Sato *et al*., 2001). SMY611 expressing GFP-Rer1p and Fet3-3HAp (or its mutant form) under the *TDH3* promoter on a single-copy plasmid (8×10^7 cells) was lysed and divided. An aliquot was treated 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 subjected to the immunoprecipitation with the anti-GFP polyclonal antibody (Sato *et al*., 2001). The immunoprecipitates were treated with 5% β-mercaptoethanol to cleave DSP and analyzed by immunoblotting with the anti-GFP mAb (BD Biosciences Clontech, Palo Alto, CA) and the anti-HA mAb (16B12). By using the other aliquots, total Fet3-HAp was immunoprecipitated with the anti-HA mAb (16B12) and detected by the anti-HA polyclonal antibody (Y11; Santa Cruz Biotechnology, Santa Cruz, CA).

Confocal Laser Microscopy

GFP fluorescence was visualized under an Olympus BX-60 fluorescence microscope equipped with a confocal laser scanner unit CSU10 (Yokogawa Electronic, Tokyo, Japan). Images were acquired by a high-resolution digital charge-coupled device camera (ORCA-ER; Hamamatsu Photonics, Hamamatsu, Japan), and processed by the IPLab software (Scanalytics, Fairfax, VA).

Pulse-Chase Analysis

Cells grown in SC to the early logarithmic phase were washed and preincubated in SC without methionine and cysteine for 1 h at 30°C. Cells were pulse-labeled with Redivue Promix [^{35}S] (Amersham Biosciences) for 15 min, centrifuged, and resuspended in SC supplemented methionine and cysteine. All medium contained 10 μM $FeSO_4$. Cells were harvested at appropriate time points, and cell lysates were prepared as described previously (Sato *et al*., 1999) and subjected to immunoprecipitation with the anti-HA mAb (16B12). The first immunoprecipitates were divided into three aliquots and subjected to the second immunoprecipitation with either the anti-HA antibody, the anti-α1,6 mannose antiserum or the anti-α1,3 mannose antiserum (Sato *et al*., 1996). Radioimages were observed and quantified with a Fuji Film image analyzer BAS-1000.

In Vitro Vesicle Budding Assay

Microsomes and cytosol were prepared as described previously (Wuestehube and Schekman, 1992). Microsomes (60 μg of protein) were incubated in B88 with 150 μg of wild-type cytosol, 0.2 mM GTP, 1 mM ATP, and an ATP degeneration system at 25°C for 45 min. The reactions were placed on ice for 5 min, and the vesicle fractions were separated by centrifugation at 15,000 × *g* for 5 min and analyzed by immunoblotting. Polyclonal antibodies against Sec22p and Sec61p were described previously (Stirling *et al*., 1992; Bednarek *et al*., 1995).

RESULTS

Fet3p Is Localized to the ER in Δftr1 Cells

It has been suggested that the oligomerization of Fet3p and Ftr1p is required for the correct surface targeting of the iron transporter complex and that failure of oligomerization results in the ER localization of the both subunits (Stearman *et al*., 1996). Because Fet3p acquires N-linked oligosaccharide modification, which serves as an indicator of intracellular transport, but Ftr1p does not (Yuan *et al*., 1995), we decided to select Fet3p as a marker to study the ERQC of this complex. To follow the intracellular behavior of Fet3p, a GFP- or 3HA-tag was fused to the C terminus of Fet3p. Expression of either fusion from a single-copy plasmid fully remedied the enhanced sensitivity of Δ*fet3* to an Fe(II) chelator, bathophenanthroline sulfonate (Askwith *et al*., 1994), indicating that these tagged versions of Fet3p are functional (our unpublished data). We found that imbalanced expression of *FET3* and *FTR1* resulted in the ER localization of the excess protein (our unpublished data). For the subsequent experiments, the tagged *FET3* gene was integrated into the genomic *FET3* locus by replacing the authentic gene to maintain the appropriate expression level.

Using the Fet3p-GFP fusion, the subcellular localization of Fet3p was directly examined in wild-type and Δ*ftr1* strains (Figure 1B). To detect a possible Fet3p signal in the vacuole, we used a Δ*pep4* background in which most vacuolar proteolytic activity was inactivated (Zubenko *et al*., 1983). In wild-type cells, the majority of Fet3p-GFP was localized to the PM, although faint intracellular GFP fluorescence was observed. When *FTR1* was deleted, Fet3p-GFP clearly showed the typical ER pattern consisting of the nuclear membrane and the peripheral ER. A similar *FTR1*-dependent change of subcellular localization was observed by immunofluorescence microscopy of Fet3-3HAp (our unpublished data). These results showed that unassembled Fet3p was retained in the ER. Next, the protein level of Fet3-3HAp was examined by immunoblotting with an anti-HA antibody (Figure 1C). In wild-type cells, Fet3-3HAp was detected as a smear band around 120–145 kDa due to elongation of sugar chains in the Golgi as commonly observed for glycoproteins passing through the secretory pathway. The amount of high-molecular-weight Fet3p was increased in the Δ*pep4* background, suggesting that a significant fraction of Fet3p is normally turned over in the vacuole. In the Δ*ftr1* strain, a substantial amount of Fet3-3HAp was detected, but it occurred as a sharp 120-kDa band, implying that Fet3-3HAp was not converted to the Golgi form in the absence of Ftr1p.

The fate of Fet3-3HAp was further analyzed in a pulse-chase experiment. In the *FTR1* Δ*pep4* strain, Fet3-3HAp was first detected as a 120-kDa band corresponding to the core-glycosylated ER form. Then, the molecular mass increased to 127–145 kDa in a time-dependent manner (Figure 2A). In

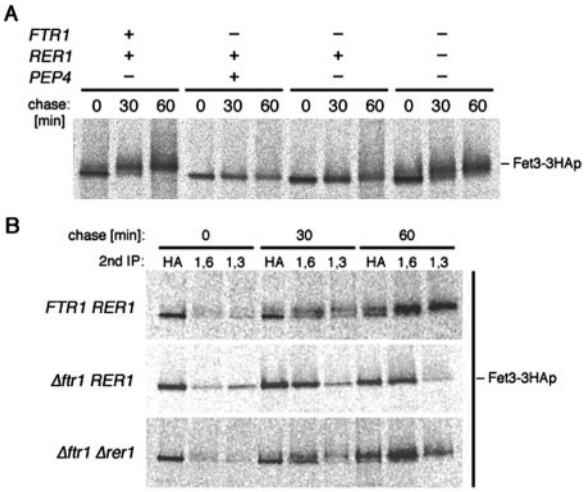

Figure 2. Intracellular transport of Fet3–3HAp. (A) Pulse-chase analysis of Fet3-3HAp in Δ*pep4* (SMY501), Δ*ftr1* (SMY53), Δ*ftr1* Δ*pep4* (SMY531), and Δ*ftr1* Δ*rer1* Δ*pep4* (SMY541) strains. Cells were pulse labeled for 15 min and chased for 60 min. Immunoprecipitates with the anti-HA antibody were separated by SDS-PAGE and analyzed by autoradiography. (B) Unassembled Fet3-3HAp receives mannosyl modification in the early-Golgi. The immunoprecipitates were prepared as described in A, divided into three aliquots, and subjected to second immunoprecipitation with antibodies against HA, α1,6 mannosyl linkages (α1,6) and α1,3 mannosyl linkages (α1,3).

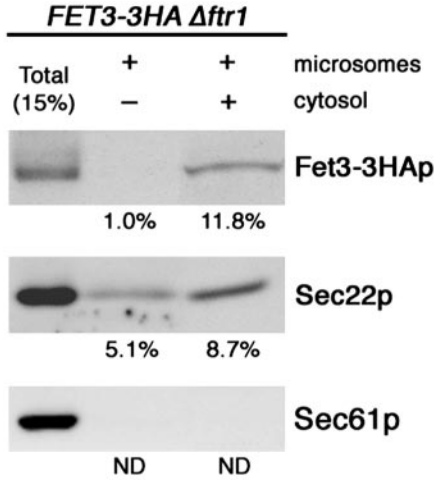

Figure 3. Unassembled Fet3-3HAp is incorporated into the ER-derived vesicles. In vitro budding assay was performed with microsomes prepared from Δ*ftr1* Δ*pep4* (SMY531) expressing *FET3-3HA*. Fifteen percent of total reaction and budded vesicle fractions were analyzed by immunoblotting with anti-HA, anti-Sec22p, and anti-Sec61p antibodies. Sec22p and Sec61p represent a positive and negative control, respectively.

yeast, carbohydrate modifications with α1,6 and α1,3 mannosyl linkages occur in the early- and late-Golgi compartments, respectively (Orlean, 1997). We confirmed that Fet3p with high molecular weight contained both α1,6 and α1,3 mannose modifications (Figure 2B). This indicates that the mobility shift in Fet3p reflects transport of Fet3-3HAp through the Golgi where Fet3-3HAp receives further mannose modifications. When *FTR1* was intact, most of Fet3-3HAp became fully glycosylated within 30 min of the chase. In contrast, in Δ*ftr1* cells, Fet3-3HAp was not converted to the high-molecular-weight species even after a 60-min chase (Figure 2A). This result is consistent with our morphological findings showing that Fet3p-GFP or Fet3-3HAp missing Ftr1p is not found at the PM but is localized to the ER.

Unassembled Fet3p Is Exported from the ER

To examine whether unassembled Fet3p is statically retained in the ER or recycles between the ER and post-ER compartments, we analyzed carbohydrate modifications in more detail by a second immunoprecipitation by using anti-α1,6 or anti-α1,3 mannose antibodies. As shown in Figure 2B, in Δ*ftr1* cells, a significant part of Fet3-3HAp received α1,6 mannose, suggesting that Fet3-3HAp travels to the early-Golgi. Strikingly, the rate of α1,6 modification to Fet3-3HAp in Δ*ftr1* was comparable with that in wild type. In spite of this, transport of Fet3p beyond the early-Golgi seemed to be impaired because α1,3 modification was significantly lower than in wild-type cells.

As an alternative strategy to prove ER exit of Fet3-3HAp, we performed an in vitro budding assay from the ER (Wuestehube and Schekman, 1992). Microsomes were prepared from Δ*ftr1* cells expressing *FET3-3HA* and let react with wild-type cytosol. The vesicles released from the microsomes were separated by centrifugation and analyzed by immunoblotting. Figure 3 demonstrates that Fet3-3HAp was incorporated into the vesicle fraction as efficiently as the established cargo protein Sec22p. The negative control, Sec61p, was not detected in the vesicle fraction in this condition, showing that this reaction is selective. Together with the exclusive ER localization of Fet3p-GFP at the steady state, both in vivo and in vitro results suggest that unassembled Fet3-3HAp is exported from the ER but selectively retrieved from the early-Golgi back to the ER.

TMD Confers the ER Localization of Unassembled Fet3p

We examined whether any particular sequence within the TMD is required for the ER localization of unassembled Fet3p as is the case for ERQC of unassembled membrane proteins in mammalian cells (Figure 4). First, the whole TMD of Fet3p-GFP corresponding to the 561–584 residues of Fet3p was replaced by a stretch of 24 leucine residues (L24; Figure 4A) and the localization of this mutant Fet3p was observed in Δ*ftr1* cells (Figure 4B). L24 was no longer localized in the ER, and most of the GFP signal was observed in the vacuole, demonstrating that the specific sequence of the Fet3p TMD is required for the ER localization in the absence of Ftr1p. Next, we looked at the four polar residues in the Fet3p TMD. Each residue was mutated to leucine (Figure 4A). First, the effect on the subcellular localization of unassembled Fet3p-GFP was examined (Figure 4B). Among four mutants, S567L lost its ER localization almost completely and resulted in redistribution of Fet3p to the vacuole. The other mutations (T565L, T577L, and Y581L) showed only minor defects in the ER localization of Fet3p-GFP in Δ*ftr1* cells. These results were confirmed for all mutant forms of Fet3p expressed in the context of Fet3-3HAp in Δ*ftr1* and Δ*ftr1* Δ*pep4* cells (Figure 4C). Wild-type Fet3-3HAp was detected as a sharp band of 120 kDa either in Δ*ftr1* and Δ*ftr1* Δ*pep4* cells. In contrast, slow migrating bands corresponding to the Golgi-modified form of Fet3-3HAp were obvious in L24 and S567L mutants. These bands became faint in the *PEP4*$^+$ background, suggesting that these mutants are transported through the Golgi to the vacuole and degraded there. As for T565L, T577L, and Y581L mutants, most of the protein

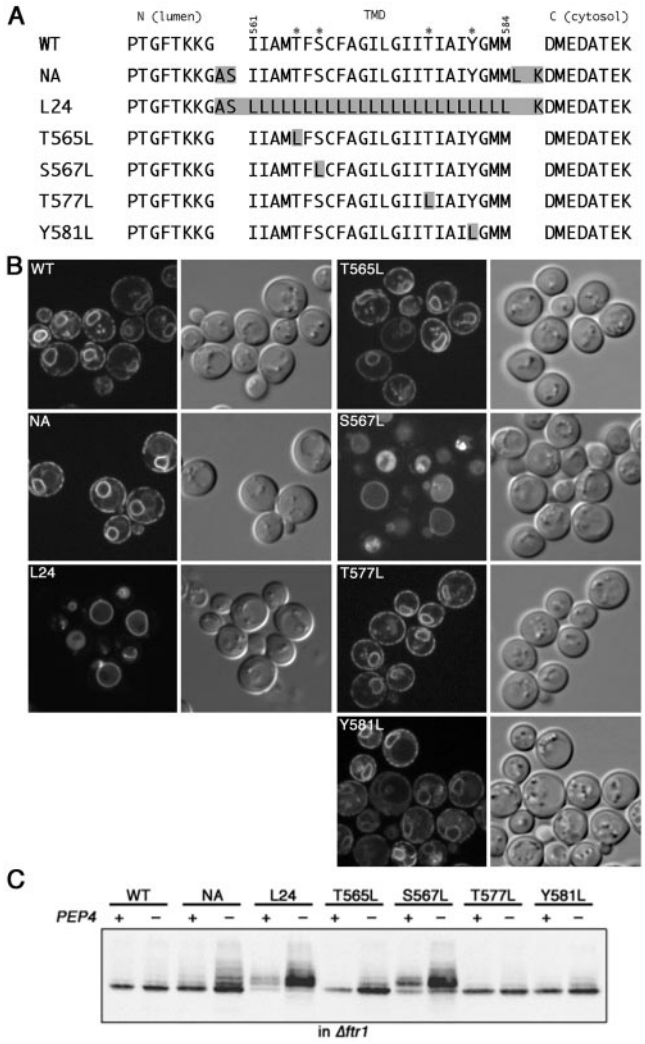

Figure 4. Fet3p TMD is required for ER localization of Fet3p in Δ*ftr1*. (A) Sequences of the wild-type Fet3p TMD and its mutant forms. (B) Subcellular localization of Fet3p-GFP and its mutant forms. Wild-type *FET3-GFP* or indicated mutants were integrated into the *ura3* locus of Δ*fet3* Δ*ftr1* Δ*pep4* (SMY611) and observed by confocal microscopy. Fluorescence (left panel in the pair) and Nomarski (right panel in the pair) images are shown. (C) *PEP4*-dependent degradation of mutant Fet3p. Wild-type *FET3-3HA* and indicated mutants were integrated into the *ura3* locus of either Δ*fet3* Δ*ftr1* (SMY60) or Δ*fet3* Δ*ftr1* Δ*pep4* (SMY611) and detected by the anti-HA antibody.

remained in the 120-kDa band, which is consistent with the morphological observation by using GFP fusions. Thus, the TMD of Fet3p, especially the S567 residue within the TMD, is important for the ER localization of unassembled Fet3p.

Rer1p Is Responsible for the ER Localization of Fet3p in Δftr1

Our data suggest that the ER localization of unassembled Fet3p is achieved by retrieval from the Golgi. Among the known retrieval pathways, the Rer1p-dependent pathway has a unique feature in that it retrieves a variety of ER-resident membrane proteins from the early-Golgi in a TMD-dependent manner (Sato *et al*., 2001). Although these ligands show no apparent similarity in amino acid sequence, Rer1p recognizes their TMDs, which contain polar residues in particular positions (Sato *et al*., 1996; Sato *et al*., 2003). Because the ER localization of unassembled Fet3p depends on the TMD containing a polar residue essential for ERQC, we tested whether Rer1p is involved in the retrieval process of unassembled Fet3p. First, subcellular localization of Fet3p-GFP was observed in a Δ*ftr1* Δ*rer1* Δ*pep4* strain (Figure 1B, d). In these cells, unassembled Fet3p-GFP was clearly localized to the vacuole, indicating that the ER retention of unassembled Fet3p-GFP depends strongly upon Rer1p. It should be noted that, in Δ*rer1*, most unassembled Fet3p-GFP is transported to the vacuole instead of the PM, the normal destination of the Fet3p–Frt1p complex. This implies the existence of a second quality control system that targets unassembled Fet3p-GFP escaping ER retention to the vacuole. In the vacuole, GFP signal was observed both on the limiting membrane and in the lumen. The luminal GFP signal may be due to internalization of Fet3p-GFP via the multivesicular body sorting pathway.

We further analyzed the effect of the deletion of *RER1* by immunoblotting (Figure 1C) and pulse-chase analysis (Figure 2). As shown in Figure 1C, in the absence of Rer1p, unassembled Fet3-3HAp was degraded in a *PEP4*-dependent manner, supporting vacuolar targeting and *PEP4*-dependent degradation of Fet3-3HAp. Pulse-chase analysis also demonstrated that, when *RER1* was disrupted, unassembled Fet3-3HAp was converted to the high-molecular-weight Golgi form containing α1,3 mannose linkage like the assembled Fet3-3HAp. Importantly, the kinetics of the α1,6 mannosyl modification was not affected by the deletion of *RER1*, suggesting that Rer1p is involved in the sorting event in the early-Golgi compartment.

A chemical cross-linking experiment was performed to prove physical interaction between Rer1p and unassembled Fet3p. Because physical interaction between Rer1p and its ligands is expected to be transient, Fet3-3HAp and GFP-Rer1p were overexpressed in Δ*ftr1* to increase the chances of detecting their physical interaction. Cell lysates were prepared from these cells and reacted with a thiol-cleavable linker DSP. Cross-linked products were immunoprecipitated with an anti-GFP antibody, treated with β-mercaptoethanol to cleave the linker, and subjected to immunoblotting with the anti-HA antibody. Figure 5 demonstrates that Fet3-3HAp was cross-linked with GFP-Rer1p in Δ*ftr1* cell lysates. This physical interaction strongly suggests that Rer1p directly recognizes unassembled Fet3p. Moreover, we corroborated that the TMD of Fet3p is required for the interaction between Rer1p and Fet3p. Fet3-3HA with a L24 or S567L mutation was expressed in Δ*ftr1* cells and cross-linking was carried out by the same procedure. As shown in Figure 5, L24 and S567L were no longer cross-linked with Rer1p. These results suggest that S567 is critical for recognition by Rer1p, which confers ER localization to unassembled Fet3p.

Finally, we asked whether Rer1p-dependent recycling is also involved in oligomerization of Fet3p and Ftr1p in the wild-type cells. When *RER1* was deleted in the presence of *FTR1*, the steady-state amount of Fet3-3HAp was markedly decreased compared with that in *RER1 FTR1* cells (Figure 6). The amount of Fet3-3HAp was partially restored by disruption of *PEP4*, suggesting that degradation of Fet3-3HAp in the vacuole is enhanced in *FTR1* Δ*rer1* cells. One possible explanation is that in the absence of Rer1p, the Fet3p–Frt1p complex is not formed efficiently, and unassembled Fet3p is susceptible of degradation in the vacuole and other compartments.

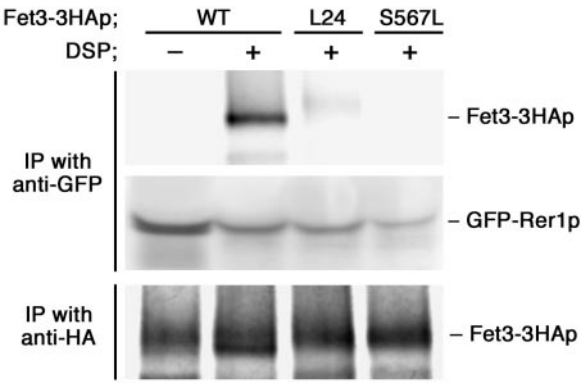

Figure 5. Physical interaction of Rer1p and unassembled Fet3p. GFP-Rer1p and Fet3-3HAp or its mutant forms (L24 and S567L) were co-overproduced under the *TDH3* promoter in Δ*fet3* Δ*ftr1* Δ*rer1* (SMY611). Total cell lysates were subjected to chemical cross-linking with DSP. The immunoprecipitates with the anti-GFP antibody were examined by immunoblotting with the anti-GFP and anti-HA antibodies. Total Fet3-3HAp was also immunoprecipitated from an aliquot of cell lysates by using the anti-HA antibody and detected by the anti-HA antibody.

DISCUSSION

ERQC is a process of conformation-dependent molecular sorting in the ER, leading to the selective ER localization of misfolded or unassembled proteins and eventual degradation (Ellgaard *et al*., 1999; Ellgaard and Helenius, 2003). In contrast to the degradation step, molecular mechanisms are still elusive that should explain the selective ER localization of ERQC substrates in terms of protein transport.

In this article, we studied the Fet3p–Ftr1p complex, the iron transporter on the PM, as a new model of ERQC of incompletely assembled transmembrane subunits. Failure in complex formation has been known as a general trigger of ERQC in mammalian systems. In many cases, including T-cell receptor, CD8, and B-cell receptor, TMDs contain determinants of ER localization of incompletely assembled subunits, and a common feature of such determinants is the presence of one or several hydrophilic or polar residues within the TMD (Bonifacino *et al*., 1990, 1991; Cosson *et al*., 1991; Reth *et al*., 1991; Hennecke and Cosson, 1993; Letourneur *et al*., 1995). In yeast, we demonstrated by using tagged Fet3p that the absence of Ftr1p causes relocalization of Fet3p to the ER. Similar to previous studies of mammalian systems, the TMD of Fet3p, particularly a single polar residue in the TMD, is critical for this ER localization. Interestingly, in vivo pulse-chase analysis monitoring the processing of sugar chains indicates that unassembled Fet3p is exported from the ER and modified in the early-Golgi in spite of its extensive ER localization at steady state. ER exit of Fet3p was further supported by the in vitro budding assay. Furthermore, Rer1p, a retrieval receptor for ER-resident membrane proteins (Sato *et al*., 2001), turns out to be responsible for the ER localization of unassembled Fet3p. This finding not only demonstrates that ER retention of unassembled Fet3p is achieved by continuous retrieval from the Golgi but also provides a new insight into the molecular mechanism underlying ER localization of ERQC substrates.

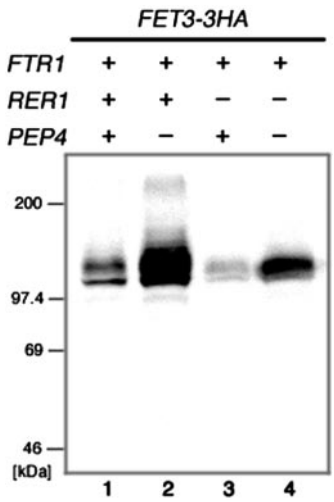

Figure 6. Deletion of *RER1* causes enhanced degradation of Fet3-3HAp in the presence of Ftr1p. *FET3-3HA* was integrated into the *FET3* locus of the wild-type (YPH500; lane 1), Δ*pep4* (SMY501; lane 2), Δ*rer1* (SMY632; lane 3), and Δ*rer1* Δ*pep4* (SMY631; lane 4). Total cell lysates were prepared and analyzed by immunoblotting by using the anti-HA antibody.

Contribution of the retrieval pathway to ERQC is still controversial. Recent studies suggest the static retention mechanism for transmembrane substrates, including VSV-G tsO45 and unassembled MHC class I molecule in mammalian cells (Nehls *et al*., 2000; Spiliotis *et al*., 2002) and Ste6-166p in yeast (Vashist *et al*., 2001). On the other hand, several lines of evidence showed involvement of the retrieval pathway from the post-ER compartments. It was reported that, under a certain experimental condition, VSV-G tsO45 and unassembled MHC class I are recycled between the ER and the intermediate compartment (Hsu *et al*., 1991; Hammond and Helenius, 1994). As for soluble misfolded proteins, a more positive role of the retrieval pathway was reported especially for the ERAD step in yeast. CPY* (mutant version of carboxypeptidase Y) and KHN (simian virus 5 hemagglutinin neuraminidase with the Kar2p signal sequence), well-characterized soluble substrates of ERQC, are transported to the Golgi before degradation. This transport is an obligatory step for efficient degradation by ERAD, although the exit rate from the ER is lower than that of normal cargo proteins (Vashist *et al*., 2001; Haynes *et al*., 2002). There are also morphological studies in which post-ER localization was demonstrated for important ERQC components such as UDP-glucose:glycoprotein glucosyltransferase and endo-α-mannosidase (Zuber *et al*., 2000, 2001). Thus, at least a part of ERQC substrates should be retrieved from the post-ER compartment. Our study provides clear evidence that the retrieval pathway is involved in ERQC of an unassembled transmembrane protein. Fet3p in Δ*ftr1* cells is exported from the ER as efficiently as in wild-type cells. Furthermore, analysis of mannosyl modification indicates that protein sorting of unassembled Fet3p takes place mainly in the early-Golgi. Unassembled Fet3p could represent a novel type of ERQC substrate whose ER localization strongly depends on retrieval from the Golgi. It should be noted that Fet3p itself is intact in this system and is potentially competent to form a complex with Ftr1p. One possibility is that even unassembled Fet3p is exported from the ER due to a presumed ER export signal in its cytosolic region. If it is the case, retrieval from the Golgi would restrict transport of the immature protein to further destinations and increase the chance of complex formation. A more positive role of retrieval might be that recycling between the ER and Golgi prevents rapid degradation of Fet3p by ERAD. Unlike the

case of the mutant Ste6-166 protein that is rapidly degraded by ERAD, unassembled Fet3p is relatively stable in *RER1* Δ*ftr1* cells. Recycling might ensure enough time to oligomerize with Ftr1p in wild-type cells (also discussed below). Multiple mechanisms of ERQC must operate depending on the status of substrates, and misfolded and unassembled proteins could follow different pathways.

An important finding is that Rer1p plays a central role in the sorting of the unassembled Fet3p in the Golgi. Rer1p is a conserved protein originally identified as a Golgi membrane protein required for correct ER localization of Sec12p, a type II transmembrane protein essential for budding of COPII vesicles from the ER (Sato *et al*., 1995, 1999; Fullekrug *et al*., 1997). Subsequent studies uncovered that Rer1p recognizes a variety of ER membrane proteins including type III and polytopic membrane proteins and sends them back to the ER via COPI vesicles (Sato *et al*., 1997; Massaad *et al*., 1999). When *RER1* was deleted, most unassembled Fet3p-GFP was relocalized to the vacuole. This effect is direct because the physical interaction between Rer1p and unassembled Fet3p was shown by chemical cross-linking experiments. This finding not only reinforces the idea that ER retention of unassembled Fet3p is dependent on retrograde transport but also gives a mechanical insight into sorting and localization of unassembled proteins. A previous report suggested the involvement of Rer1p in a particular type of ERQC (Letourneur and Cosson, 1998). Glycosylphosphatidylinositol (GPI)-anchored proteins are synthesized as a precursor with a membrane-anchoring domain. In the ER, this precursor is cleaved at the luminal site adjacent to the membrane anchor and attached to a GPI-anchor. When the cleavage and subsequent attachment of GPI is inhibited by a mutation of the cleavage site, this mutant protein is retained in the ER in Rer1p- and COPI-dependent manner. Rer1p-dependent retrieval seems to contribute commonly to ERQC of a subset of membrane proteins.

Rer1p recognizes the TMDs of its ligands that contain polar residues (Sato *et al*., 1996, 2003). This holds true for unassembled Fet3p because L24 and S567L mutations in the TMD diminish binding to Rer1p and cause vacuolar localization. It is likely that the complex formation with Ftr1p masks S567 in the TMD, which releases the assembled complex from Rer1p-dependent recycling between the ER and the Golgi. Even in wild-type cells, Rer1p could prevent transport of premature Fet3p beyond the Golgi and return it to the ER to provide a next chance of complex formation, which improves the efficiency of correct complex formation. Supporting this possibility, the deletion of *RER1* remarkably reduced the stability of Fet3-3HAp even in the presence of Ftr1p, implying that inefficient complex formation in Δ*rer1* results in degradation of unassembled Fet3p in the vacuole and other compartment. In this context, the function of Rer1p could be similar to that of molecular chaperons. Rer1p may conceal polar residues exposed in the lipid bilayer and stabilizes premature proteins in a hydrophobic environment. The deletion of *RER1* causes the sensitivity to hygromycin B, an inhibitor of protein synthesis (Sato, unpublished observation). This might be explained if the efficiency of complex formation is generally reduced in Δ*rer1*, which causes shortage of functional proteins when protein synthesis is also inhibited. As mentioned above, TMD-mediated ERQC in mammalian cells requires hydrophilic or polar residues within the TMD, which is similar to the Rer1p-dependent retrieval signal in yeast. We would speculate that Rer1p is also involved in ERQC of unassembled membrane proteins in higher eukaryotes.

A previous study suggested another quality control mechanism in the late-Golgi (Hong *et al*., 1996). It showed that a soluble misfolded protein is captured by Vps10p at the Golgi and selectively targeted to the vacuole. In the present study, we found that unassembled Fet3p, which escapes ERQC either by the deletion of *RER1* or by mutations in the TMD, is transported not to the PM but to the vacuole, implying that unassembled Fet3p is subjected to second quality control in the late secretory pathway. Multiple quality control systems through the secretory pathway may monitor the folding status of proteins to prevent potentially toxic effects caused by accumulation of unfolded proteins.

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