Endoplasmic Reticulum Localization of Sec12p Is Achieved by Two Mechanisms: Rer1p-dependent Retrieval That Requires the Transmembrane Domain and Rer1p-independent Retention That Involves the Cytoplasmic Domain

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Abstract. Yeast Sec12p is a type II transmembrane protein in the ER, which is essential for the formation of transport vesicles. From biochemical and morphological lines of evidence, we have proposed that Sec12p is localized to the ER by two mechanisms: static retention in the ER and dynamic retrieval from the early Golgi compartment. We have also shown that Rer1p, a membrane protein in the Golgi, is required for correct localization of Sec12p. In the present study, we have performed a systematic analysis to determine the ER localization signals in Sec12p corresponding to these two mechanisms. Both the transmembrane domain (TMD) and the NH_2-terminal cytoplasmic domain of Sec12p show the ability to localize the protein to the ER. The effect of the TMD is potent and sufficient by itself for the ER localization and is strongly dependent on Rer1p. On the other hand, the cytoplasmic domain shows a moderate ER-localization capability which is independent of Rer1p. The rate of mannosyl modification has been measured to distinguish between retention and retrieval. The cytoplasmic domain significantly delays the transport from the ER to the *cis*-Golgi. In contrast, the TMD shows only a subtle retardation in the transport from the ER to the *cis*-Golgi but strictly prevents the transport beyond there. From these observations, we conclude that the TMD mainly acts as the retrieval signal and the cytoplasmic domain contains the retention signal. This study not only supports the two-mechanisms hypothesis but also provides powerful tools to dissect the two.

THE secretory pathway of eukaryotic cells consists of a series of membrane-bound organelles. The correct sorting of proteins is essential to maintain complex structure and function of these organelles and requires a specific localization signal in each protein. To date, a few examples of such signals have been identified for ER proteins. One is the COOH-terminal Lys-Asp-Glu-Leu (KDEL, mammals) or His-Asp-Glu-Leu (HDEL, yeast) sequence (Munro and Pelham, 1987; Pelham et al., 1988). The *cis*-Golgi receptor for this K(H)DEL sequence, Erd2p, has also been identified (Semenza et al., 1990; Lewis and Pelham, 1990). Other signals are the COOH-terminal dilysine motif of type I transmembrane proteins (Jackson et al., 1990) and the NH_2-terminal diarginine motif of type II transmembrane proteins (Schutze et al., 1994). It is believed that the ER proteins harboring the K(H)DEL or dilysine motifs are retrieved from the early Golgi to the ER (Pelham, 1988; Dean and Pelham, 1990; Hsu et al., 1991; Lewis and Pelham, 1992; Jackson et al., 1993).

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Yeast Sec12p is a type II transmembrane glycoprotein and is essential for formation of transport vesicles from the ER (Nakano et al., 1988; d'Enfert et al., 1991; Rexach and Schekman, 1991; Oka and Nakano, 1994). Most of Sec12p is localized to the ER in the steady state (Nakano et al., 1988; Nishikawa and Nakano, 1993) and not detected on the purified transport vesicles (T. Oka and A. Nakano, unpublished; Barlowe et al., 1994). However, a significant portion of Sec12p receives $\alpha 1 \rightarrow 6$ mannose modification on its N-linked oligosaccharide, which takes place in the early Golgi (Nakano et al., 1988; d'Enfert et al., 1991; Nishikawa and Nakano, 1993). Based on these observations, we have proposed that the ER localization of Sec12p involves two different mechanisms: static retention in the ER and dynamic retrieval from the early Golgi. Sec12p molecules are largely excluded from the transport vesicles during budding from the ER, and those escaping from this retention are sent back from the Golgi to the ER by the retrograde pathway (Nishikawa and Nakano, 1993; Sato et al., 1995).

In order to understand molecular mechanisms underlying these sorting events, we designed a screening to isolate mutants that mislocalize Sec12p beyond the early Golgi. A fusion protein of Sec12p and the precursor of α-mating

factor (Sec12-Mfα1p) was used as a marker protein. If this fusion protein is mislocalized to the late Golgi, the Mfα1 moiety is processed to mature α-factor, which is then secreted to the medium. Using this trick, we have isolated two *rer* mutants (Nishikawa and Nakano, 1993). The *RER1* gene encodes a protein of 188 amino acid residues (Rer1p) containing four putative transmembrane domains. The null mutant of *RER1* is viable, and even in this mutant, a significant portion of Sec12p is still located in the ER. Immunofluorescence microscopy and subcellular fractionation experiments demonstrated that Rer1p is mainly localized to the Golgi complex. This suggests that Rer1p functions at the retrieval step (Sato et al., 1995).

On the other hand, little is known about structural requirements for Sec12p to be localized to the ER. According to our hypothesis, Sec12p should possess two localization signals corresponding to the two mechanisms, retention and retrieval. To identify these signals, we performed a systematic analysis on Sec12p localization using chimeric proteins between Sec12p and Dap2p. Dap2p is a type II transmembrane protein in the vacuole and has been claimed to have no particular localization information in itself (Roberts et al., 1992). In this paper, we report the results of our analysis on the chimeric proteins and present evidence that two different regions of Sec12p are important for the ER localization.

Materials and Methods

Yeast Strains and Culture Conditions

Saccharomyces cerevisiae strains used in this study are listed in Table I. Yeast cells were grown in YPD [1% (wt/vol) Bacto yeast extract (Difco Laboratories Inc., Detroit, MI), 2% (wt/vol) polypeptone (Nihon Seiyaku, Tokyo, Japan), and 2% (wt/vol) glucose] or in MVD [0.67% yeast nitrogen base without amino acids (Difco Laboratories) and 2% glucose] medium supplemented appropriately. MCD medium is MVD containing 0.5% casamino acids (Difco Laboratories Inc.).

Plasmid Construction

A yeast multicopy plasmid, pSQ326, has been described elsewhere (Qadota et al., 1992). pSHF9-1 and pSHF9-4 were constructed by subcloning the *SEC12-MFα1* fusion into pBluescript II SK+ (Stratagene Cloning Systems, La Jolla, CA) and pSQ326, respectively (Nishikawa and Nakano, 1993).

The PstI to SalI fragment of *MFα1* was subcloned into pBluescript II SK+ (pBα1). The *DAP2-MFα1* fusion was constructed by inserting the blunted BamHI to BbiII fragment of pGP3 (Roberts et al., 1989) (obtained from T. Stevens of the University of Oregon) into the SmaI site of pBα1 (pMD1-1). An NcoI site was introduced at the start codon of *DAP2-MFα1* by PCR-mediated, site-directed mutagenesis using the oligonucleotide, 5′-CCCCGGGCCATGGAAGGTGGCGAAGAAG-3′ (D-1). The *DAP2* promoter region (BamHI to NcoI) was replaced by the *SEC12* promoter fragment (XbaI to BspHI) (Nishikawa et al., 1994) to give pDDD-1.

Site-direct mutagenesis was performed to introduce XbaI and AflII sites at the 354th and the 373rd codons of *SEC12*. A BspHI site was created at the start codon of *SEC12* (Nishikawa et al., 1994). *SEC12-MFα1* bearing these three restriction sites was subcloned into pBluescript II SK+ whose BspHI site was destroyed by blunting and religation. The resulting plasmid was named pMS2-3 (also called pSSS-1). The start to the 29th codon fragment of *DAP2* was made by PCR using primers D-1 and 5′-CCTCTAGACCTTATGAGCTTATCTAACAAATG-3′ (D-5), digested by NcoI and XbaI and used to replace the BspHI to XbaI region of pSSS-1, and thus produced pDSS-1. For pDDS-1, PCR was performed using primers D-1 and 5′-GCCTTAAGTATACTTTTTAGCAACAAAAC-3′ (D-3). The resulting fragment (start to the 48th codon of *DAP2*) was digested by NcoI and AflII and inserted into the BspHI and AflII sites of pSSS-1. For pSDS-1, a fragment encoding the transmembrane domain (TMD)[1] of Dap2p was synthesized by PCR using primers D-3 and 5′-GCTCTAGAGTCGGAATAATCCTTGTAC-3′ (D-4). This fragment was digested by XbaI and AflII and introduced into the XbaI and AflII sites of pSSS-1. An AflII site was created just before the 48th codon of *DAP2-MFα1* by PCR-mediated mutagenesis and subcloned into pBluescript II SK+ (named pMD3-3). The AflII to SacI (in vector) fragment of pMD3-3 was used to replace the corresponding fragments of pSSS-1, pDSS-1, and pSDS-1 to produce pSSD-1, pDSD-1, and pSDD-1, respectively. These chimeric genes were also subcloned into pSQ326 to yield pXXX-2, where XXX stands for SSS, DDD, SDS, DDS, DSS, DSD, SDD, or SSD.

The plasmids for the mutational analysis of the Sec12p TMD were constructed by PCR-mediated mutagenesis using oligonucleotides containing mutations corresponding to each mutant. The XbaI to AflII region of pDSD-1 was replaced by the XbaI/AflII mutated fragments made by PCR. All mutant genes were subcloned into pSQ326.

For the purpose of examining ER localization signals of other ER membrane proteins, we chose *SED4* and *SEC20*. *SED4* was cloned from a λ phage containing the corresponding region of the yeast chromosome III (Yoshikawa and Isono, 1990) (provided by S. Tanaka and K. Isono of Kobe University). *SEC20* was a kind gift from J. Nikawa of Kyushu Institute of Technology. PCR fragments encoding the TMD of Sed4p and Sec20p were made using pairs of primers, 5′-CCTCTAGATTTTTCTTGAATTTTGTTTTGT and 5′-TGCTTAAGGCCTAGTTGAATAGCGCCAG (*SED4*), and 5′-TGTCTAGAGATGTCTATTTATCACTT and 5′-TGCTTAAGCCATAGAACCCACGAGAC (*SEC20*). These fragments were digested by XbaI and AflII and substituted for the XbaI-AflII region of DSDm-1 to produce pD4Dm-1 and pD20Dm-1, respectively. *SED4* was subcloned in pBluescript II SK+, and an SpeI site was made just behind the 345th codon by PCR-mediated mutagenesis. The XbaI-XbaI (in vector) fragment from pSDD1-1 was inserted at the SpeI site of *SED4* to yield p4DDm-1. (This chimera is under the *SED4* promoter.) These chimeras were subcloned into pSQ326 (pD4Dm-2, pD20Dm-2, and p4DDm-2).

Escherichia coli strains used for recombinant DNA experiments were DH5α (Sambrook et al., 1989), SCS1 (Stratagene Cloning Systems), and XL1-Blue (Bullock et al., 1987). DNA manipulations, including restriction enzyme digestions, ligations, plasmid isolation, and *E. coli* transformation, were carried out by standard methods (Sambrook et al., 1989). In all constructions, DNA sequences were determined by the dideoxy method using a sequencer (model 373A; Applied Biosystems, Tokyo, Japan). Yeast transformation was performed by the lithium thiocyanate method (Keszenman-Pereyra and Hieda, 1988).

Immunofluorescence Microscopy

Indirect immunofluorescence microscopy was performed as described previously (Nishikawa and Nakano, 1991; Sato et al., 1995). Affinity-puri-

1. *Abbreviations used in this paper*: endo H, endoglycosidase H; TMD, transmembrane domain.

Table I. Yeast Strains Used in This Study

Strain	Genotype	Source
SNY9	*MATα mfα1::ADE2 mfα2::TRP1 bar1::HIS3 ura3 trp1 ade2 his3 leu2 lys2*	A
SKY7	*MATα rer1::LEU2 mfα1::ADE2 mfα2::TRP1 bar1::HIS3 ura3 trp1 ade2 his3 leu2 lys2*	B
SMY6-9C	*MATα dap2::LEU2 mfα1::ADE2 mfα2::TRP1 bar1::HIS3 ura3 trp1 ade2 his3 leu2 lys2*	C
SMY8-1B	*MATa dap2::LEU2 pep4::LEU2 mfα1::ADE2 mfα2::TRP1 bar1::HIS3 ura3 trp1 ade2 his3 leu2 lys2*	C
SMY8-4C	*MATα dap2::LEU2 pep4::LEU2 mfα1::ADE2 mfα2::TRP1 bar1::HIS3 ura3 trp1 ade2 his3 leu2 lys2*	C

A, Nishikawa and Nakano, 1993; B, Sato et al., 1995; C, this study.

fied anti–Dap2p antibody was kindly provided by Y. Wada of the University of Tokyo and Y. Amaya of Yokohama City University. To amplify the fluorescent signals, the system using biotinylated goat anti–rabbit antibody and streptavidin-fluorescein (Sato et al., 1995) was employed.

Pulse-chase Experiments

Metabolic labeling of yeast cells, preparation of cell extracts, and immunoprecipitation were performed as described previously (Nishikawa and Nakano, 1991). In each immunoprecipitation, affinity-purified anti–Dap2p antibody or anti–Sec12p antiserum was added to the cell extract equivalent to 4×10^7 cells. The first immunoprecipitates were dissolved in 210 μl of 1% SDS, divided into three aliquots, diluted with 10X volume of 2% Triton X-100, and subjected to the second immunoprecipitation with either the same antibody, anti–α1→6 mannose antiserum or anti–α1→3 mannose antiserum. Endoglycosidase H (Endo H) treatment and analysis by SDS-PAGE and fluorography were performed as described (Nishikawa et al., 1990). Radioimage was also observed and quantified with an image analyzer (model BAS-1000; Fuji Photo Film Co., Tokyo, Japan).

Other Methods

Halo assays were performed on MCD plates with a tester *MAT***a** *sst2* strain as described previously (Nishikawa and Nakano, 1993). Four to ten independent spots were examined to quantify the amount of α-factor secreted. Expression levels of various chimeras were always analyzed by SDS-PAGE and immunoblotting using antibodies against Sec12p, Dap2p, or α-factor. In the case of α-factor fusions, the density of each band was evaluated by scanning and used to normalize the secretion of α-factor.

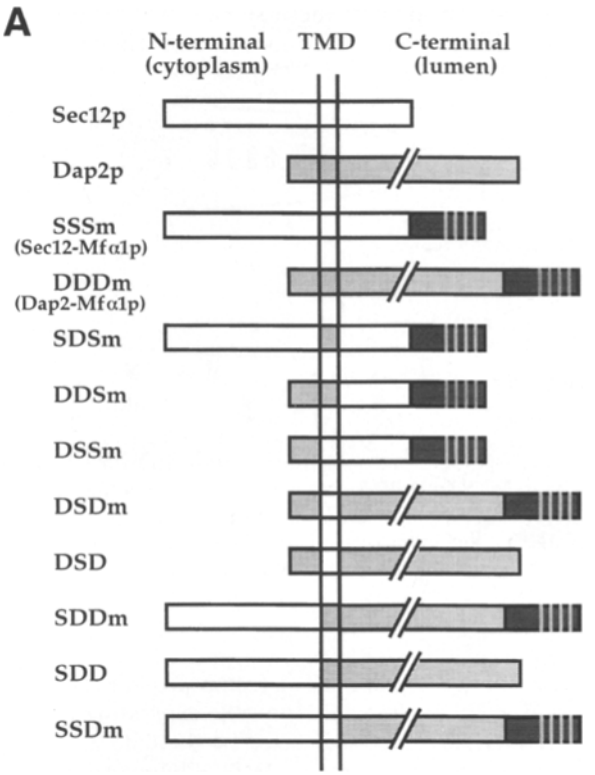
Results

Construction of Chimeric Proteins between Sec12p and Dap2p

To determine which region of Sec12p is important for the ER localization, we planned to construct chimeric proteins between Sec12p and an appropriate passenger protein that is innocent for its destination. As such a passenger protein, we chose Dap2p (dipeptidyl aminopeptidase B), a vacuolar membrane protein. Dap2p is a transmembrane protein with the same topology as Sec12p (type II) but is transported to the vacuole quickly after biosynthesis (Roberts et al., 1989). It has been shown that Dap2p has no particular localization signal in itself and thus is transported to the vacuole by default (Roberts et al., 1992). This gives us a nice opportunity to identify the ER localization signal(s) of Sec12p by examining which chimeric constructs are localized to the ER. We dissected Sec12p and Dap2p into three parts: the NH_2-terminal cytoplasmic domain, the TMD, and the COOH-terminal lumenal domain by introducing appropriate restriction sites in their corresponding genes. For convenience, we refer to the constructs with three letters composed of S and D. S stands for Sec12p and D is for Dap2p. The first letter indicates the NH_2-terminal domain, the second is for the TMD, and the third is for the COOH-terminal domain. For example, SDD refers to the N (Sec12p)-TMD (Dap2p)-C (Dap2p) chimera. All the combinations of the three parts of the two proteins were made and named by this nomenclature (Fig. 1). They were all placed under the *SEC12* promoter.

Analysis Using the α-factor Fusion

The subcellular localization of these chimeras was examined in three different ways: halo assay, indirect immunofluorescence, and pulse–chase experiments. First, we undertook the halo assay method that was used to isolate *rer* mutants (Nishikawa and Nakano, 1993). As illustrated in Fig. 2, this method utilizes fusions containing Mfα1p in the lumenal domain. If a particular fusion protein is transported to the late Golgi, Mfα1p moiety would be processed by the Kex2 protease leading to the secretion of mature α-factor. Cells secreting α-factor form a halo when placed on an agar plate with a lawn of tester **a** strain as a result of the growth arrest of the **a** cells ($Halo^+$). Accordingly, the $Halo^-$ phenotype should indicate that the fusion is retained in the ER or the early Golgi. Sec12-Mfα1p (SSSm) behaves very much like the authentic Sec12p. It is mostly localized in the ER and does not yield a halo in the wild-type cells (Nishikawa and Nakano, 1993; Fig. 2, *black arrows*; see also Fig. 3, *A* and *B*, and Fig. 4, *upper spot 1*).



B

Sec12p	**SMKQKISR**	**FFTNFILIVLLSYILQFSL KHNLHSMLF**
Dap2p	HLLDKLIR	VGIILVLLIWGTVLLLKSI PHSNTPDY
SDSm	**SMKQKISR**	VGIILVLLIWGTVLLLKSIL **KKHNLHSMLF**
DDSm	HLLDKLIR	VGIILVLLIWGTVLLLKSIL **KKHNLHSMLF**
DSSm	HLLDKLIRSR	**FFTNFILIVLLSYILQFSL KHNLHSMLF**
DSDm	HLLDKLIRSR	**FFTNFILIVLLSYILQFSL** KPHSNTPDY
SDDm	**SMKQKISR**	VGIILVLLIWGTVLLLKSIL KPHSNTPDY
SSDm	**SMKQKISR**	**FFTNFILIVLLSYILQFSL** KPHSNTPDY

Figure 1. (*A*) Schematic representation of chimeras between Sec12p and Dap2p. DSD and SDD have the complete luminal domain of Dap2p, while other chimeras with the suffix "m" are fused to Mfα1p at their COOH terminus. All constructs were expressed by the *SEC12*-promoter on a multicopy plasmid. (*B*) Sequences of the junction regions of chimeras. The black letters are derived from Sec12p and the gray letters are from Dap2p. Underlines indicate the TMD regions. For the details of construction, see *Materials and Methods.*

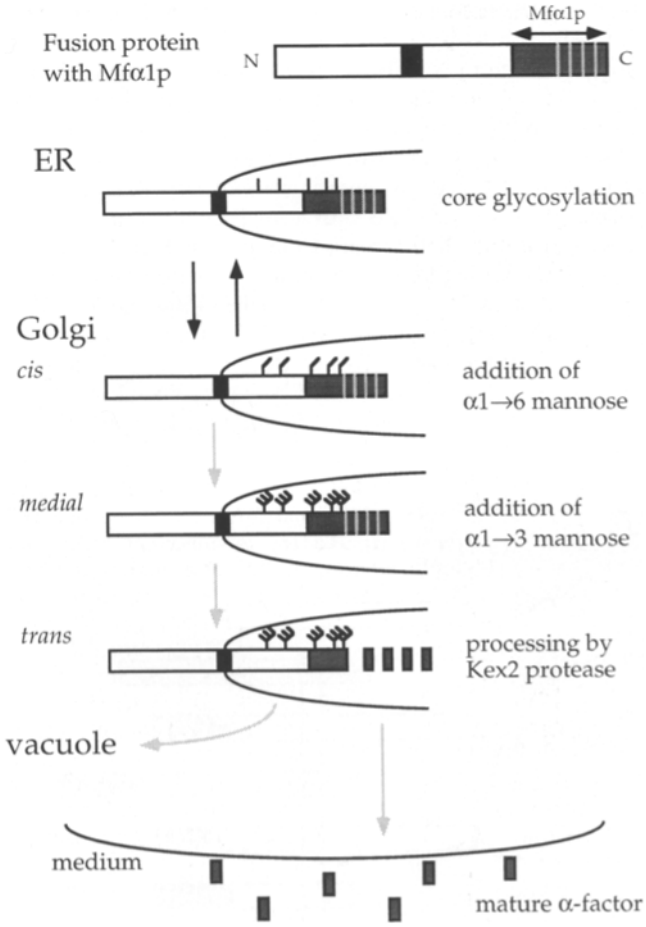


Figure 2. Biosynthetic pathway of fusion proteins with Mfα1p. In wild-type cells, Sec12-Mfα1p does not go beyond the *medial*-Golgi (*black arrows*). On the other hand, Dap2-Mfα1p travels through the whole Golgi apparatus on the way to the vacuole and thus gives rise to the secretion of mature α-factor (*gray arrows*).

We constructed a Dap2-Mfα1 fusion protein (DDDm) and tested whether the wild-type cells expressing this fusion (WT/DDDm) secrete α-factor by the halo assay. As shown in Fig. 4, upper spot *2*, WT/DDDm produced a large halo, indicating that this fusion protein was transported to the late Golgi quite efficiently. Furthermore, DDDm was found to be subject to degradation in the vacuole. Immunoblotting using the anti-Dap2p antibody showed that the amount of DDDm was higher than in the wild-type *PEP4* cells when the major vacuolar proteolytic activities were disrupted (*Δpep4*) (data not shown). Finally, the immunofluorescence observation of DDDm showed clear vacuolar staining in the *Δpep4* cells (Fig. 3, *C–E*). These all indicate that DDDm is processed in the late Golgi, transported to the vacuole, and eventually degraded there (Fig. 2, *gray arrows*). The presence or absence of the vacuolar proteolytic activities (*PEP4* or *Δpep4*) did not affect the size of halo produced from DDDm (data not shown). The radius of the halo is proportional to the logarithm of the amount of secreted α-factor and thus is a good measure of the passage of the fusion protein through the late Golgi compartment.

Sec12p TMD Contains an ER Localization Signal That Depends on Rer1p

Since SSSm is localized to the ER, whereas DDDm is transported through the late Golgi to the vacuole, we went on to ask, in Sec12/Dap2-Mfα1p chimeric proteins, which part of Sec12p could retain the whole molecule in the ER or the early Golgi? This was tested by performing halo assays on Mfα1p fusions with all six chimeras we constructed. The fusion proteins were named with three letters composed of S and/or D followed by m (Fig. 1). As Rer1p plays an important role in the ER localization of Sec12p, probably in the retrieval from the Golgi, all the experiments were done for both wild-type (SNY9) and *Δrer1* (SKY7) cells. Expression of each chimera was examined by immunoblotting with the anti-Mfα1p antibody. Although the cellular levels tended to be higher for the chimeras containing the NH_2-terminal cytoplasmic domain of Sec12p (SSSm, SDSm, SDDm, and SSDm), no appreciable difference was observed between *Rer1*$^+$ and *Δrer1* cells (data not shown). The band densities were quantified by image scanning and used for further analysis (see below).

The result of the halo assay is shown in Fig. 4, spots *3–8*. Using the synthetic pure α-factor as the standard, the amounts of secreted α-factor were calculated by measuring the radii of halos and their relative proportions to WT/DDDm (*upper spot 2*). These results are summarized in Table II. Considering the different levels of chimeras as measured by immunoblotting, the relative values were corrected by normalization. The corrected values are shown in parentheses in Table II. This normalization does not affect our argument henceforth.

Among the six chimeras that are composed of both Sec12p and Dap2p fragments, DSSm, DSDm, and SSDm did not produce any detectable halo in the wild-type cells (see Fig. 4, *spots 5, 6,* and *8*). This indicates that these proteins were retained in the cells before they reach the Kex2 compartment of the late Golgi. All these constructs contain the TMD of Sec12p (SRFFTNFILVLLSYILQFSL). It is striking that the DSDm chimera, whose only part from Sec12p is the TMD, is completely retained before the late Golgi. Complementary to this result, SDSm secreted α-factor in the wild-type cells (see Fig. 4, *spot 3*), again indicating that the TMD region of Sec12p is very important for its localization. The same interpretation can be made for SDDm (compare with SSDm). By replacing the Sec12p TMD by the Dap2p TMD, the retention of the molecule was seriously impaired. It should be noted, however, that the amount of the α-factor secreted by SDSm or SDDm is only 27–38% (12–13% if corrected by normalization of expression levels) as compared to DDDm, suggesting that another mechanism of retention operates for these proteins (see below). It seems that the lumenal domain of Sec12p has no effect on the localization as long as we could test by this halo assay. DDSm and SSDm were almost indistinguishable from DDDm and SSSm, respectively, in α-factor secretion.

To further examine the effect of the Sec12p TMD on localization, we performed indirect immunofluorescence microscopy on DSDm in *Δdap2* cells. As shown in Fig. 5 *A*, staining with the anti–Dap2p antibody exhibited the typical ER pattern (nuclear envelope and peripheral ER) as

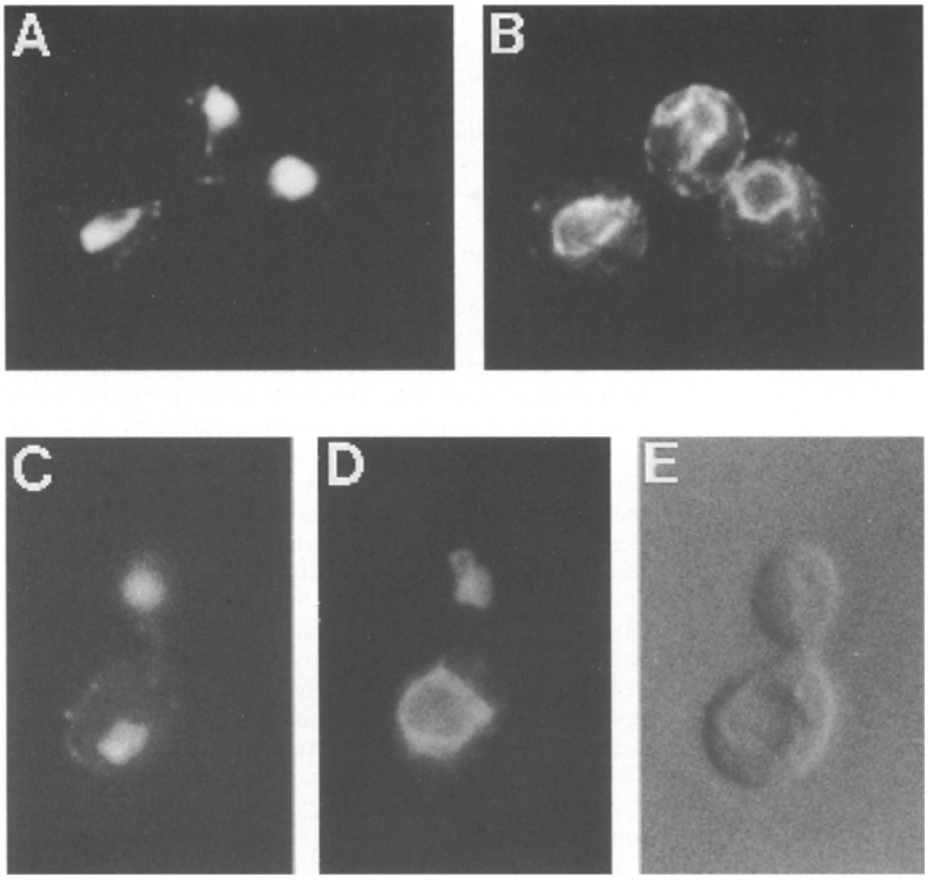


Figure 3. Subcellular localization of SSSm (Sec12-Mfα1p) and DDDm (Dap2-Mfα1p). Wild-type cells (SNY9) expressing SSSm (*A* and *B*) and *Δdap2 Δpep4* cells (SMY8-1B) expressing DDDm (*C–E*) were fixed and prepared for immunofluorescence microscopy with anti–Sec12p and anti–Dap2p antibodies, respectively. (*A* and *C*) DNA staining with 4′, 6-diamidino-2-phenylindole (DAPI) to locate nuclei. (*B* and *D*) Fluorescence images with the antibodies. (*E*) Nomarski image to visualize vacuole. The SNY9 cells contain one copy of the authentic *SEC12* in the chromosome but its product's fluorescence signal is not detectable under this condition.

seen with the authentic Sec12p or SSSm (see Fig. 3 *B*). No indication of vacuolar or Golgi staining was observed. Thus, we conclude that the TMD of Sec12p has sufficient information to localize the molecule to the ER.

The disruption of the *RER1* gene showed various effects on the chimeric proteins (see Fig. 4, Table II). The three constructs, DSSm, DSDm, and SSDm, which contain the Sec12p TMD, secreted significant amounts of α-factor in the *Δrer1* cells like SSSm. This indicates that Rer1p is very important for the retention of these proteins in the early compartments. In contrast, the secretion of α-factor by SDSm or SDDm was not markedly affected by the *RER1* disruption. In the case of SDDm, the amount of the secreted α-factor was about 35–38% (12–13% if corrected) of that of DDDm, regardless of *RER1*$^+$ or *Δrer1*. In other words, a significant amount of the molecule is still retained in the early compartments independent of the Rer1p function.

Sec12p Cytoplasmic Domain Contains an Rer1p-independent ER Localization Signal

It is evident that the TMD region of Sec12p is sufficient to localize the chimeric proteins to the ER in the *RER1*$^+$ cells. However, is it solely necessary for the ER localization of Sec12p? The answer is obviously no. Even with the Dap2p TMD, SDSm and SDDm constructs secrete only 30–40% (10–15% if corrected) of α-factor as compared to

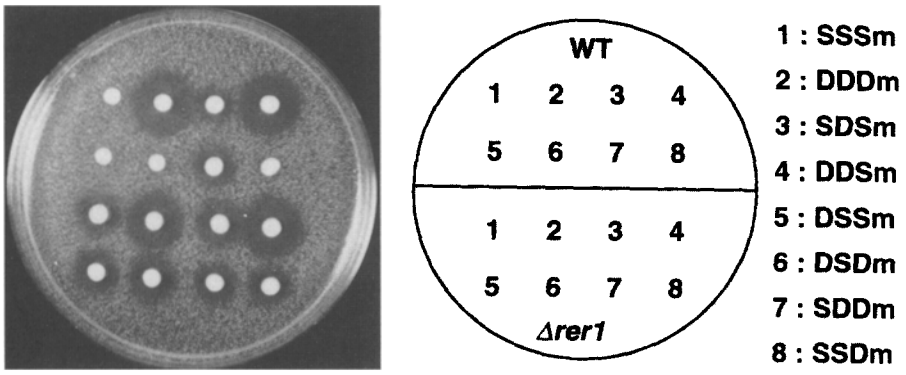


Figure 4. Secretion of mature α-factor produced from Sec12/Dap2-Mfα1p chimeras. Wild-type (SNY9) and *Δrer1* (SKY7) cells harboring various chimeras were tested by the halo assay.

Table II. Secretion of α-Factor by Sec12/Dap2-Mfα1p Fusion Proteins

Chimera	Relative amount of α-factor secreted			
	Wild-type		%	*Δrer1*	
SSSm (Sec12-Mfα1p)	<5	(<2)	33	(13)
DDDm (Dap2-Mfα1p)	**100**	(**100**)	95	(101)
SDSm	27	(12)	39	(16)
DDSm	100	(71)	105	(81)
DSSm	<5	(<3)	44	(23)
DSDm	<5	(<3)	56	(31)
SDDm	38	(13)	35	(12)
SSDm	<5	(<2)	35	(15)

The amounts of secreted α-factor in Fig. 4 were quantified and expressed as proportions relative to that secreted by DDDm in the wild-type cells. A value below 5 means that it was below the detection limit. The figures in parentheses are after correction, considering the amount of each chimera estimated by immunoblotting.

DDDm, leaving a considerable amount of the protein retained in the early compartment(s). In fact, these chimeric genes were able to complement the lethality of the *SEC12* gene disruption on a multicopy plasmid. Since the NH_2-terminal cytoplasmic domain is the only region originating from Sec12p in the case of SDDm, these observations strongly suggest that this domain also has a signal to localize the protein.

To examine whether SDDm is in fact retained in the ER, we performed immunofluorescence microscopy. As shown in Fig. 5 *C*, the anti–Dap2p antibody again stained the ER in the *Δdap2* cells expressing SDDm. This led us to conclude that the cytoplasmic domain of Sec12p also has an ability to localize the protein to the ER. The efficiency of the ER localization attained by the cytoplasmic domain appears to be lower than that of the TMD because the secretion of α-factor from the Mfα1p fusion is not completely prevented by this domain alone. The effect of the cytoplasmic domain does not require the presence of Rer1p. This raises an intriguing possibility that the retention by the cytoplasmic domain may be due to the static mechanism at the exit from the ER. It should also be noted that, in the absence of Rer1p, the cytoplasmic domain is not the only region that causes the retention. As seen in Table II, the Sec12p TMD also has an effect in the *Δrer1* cells (see DSSm and DSDm). This Rer1p-independent mechanism may involve both the cytoplasmic and TMD regions.

It is known that the ER retention of the proteins could also result from the quality control of the ER. The Dap2-Mfα1p (DDDm) construct showed no enzymatic activity of dipeptidyl aminopeptidase. The Mfα1p moeity might hinder correct folding of the lumenal domain of Dap2p and thus activate the potent quality control system in the ER for some of the chimeric proteins. Although the fact that DSDm forms a large halo in the *Δrer1* cells strongly argues against this possibility, we decided to test the ER localization effects of the TMD and the cytoplasmic domain in more native forms. We constructed DSD and SDD, which contain the complete COOH-terminal lumenal domain of Dap2p devoid of the Mfα1p moiety. This version of the chimeric proteins expressed in the *Δdap2* cells showed full dipeptidyl aminopeptidase activities as the authentic Dap2p (data not shown). SDD is also completely functional as Sec12p since it could complement the *Δsec12* mutant on a single-copy plasmid. Immunofluorescence microscopy (Fig. 6, *A* and *B*) showed that, like DSDm, DSD was strictly localized to the ER. SDD was mainly localized to the ER, but weak vacuolar staining was also observed (Fig. 6, *C–E*). This is consistent with the fact that SDDm had less ability of retention than DSDm as measured by the halo assay. Because DSD and SDD retain the full activity as Dap2p and SDD fucntions like the

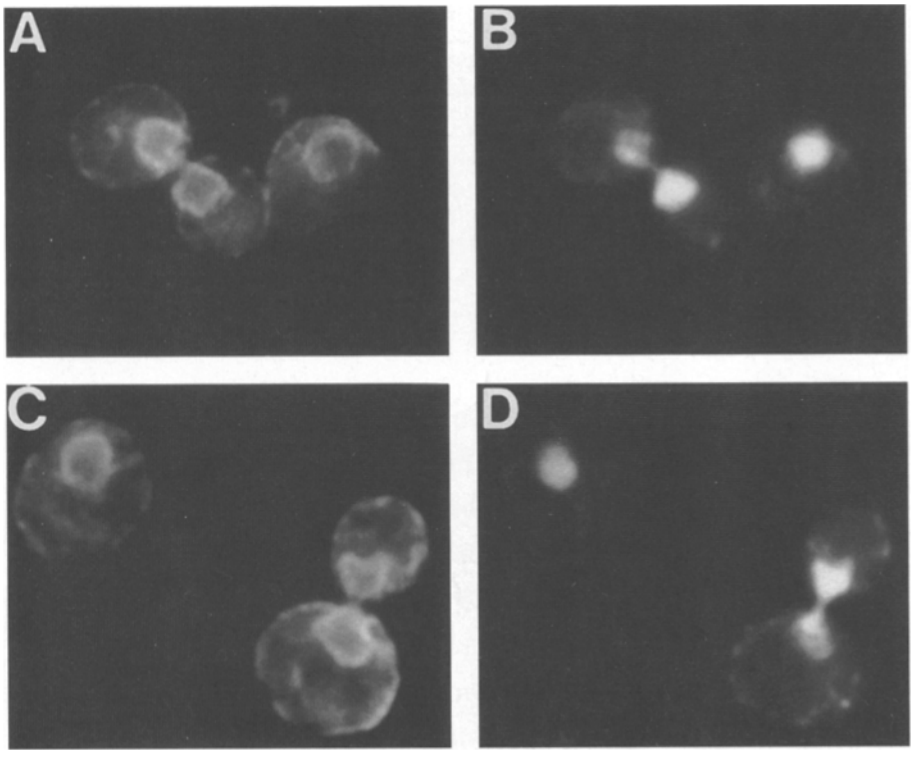


Figure 5. Subcellular localization of DSDm and SDDm. *Δdap2 Δpep4* (SMY8-1B) cells harboring DSDm (*A* and *B*) or SDDm (*C* and *D*) were analyzed by immunofluorescence microscopy using the anti–Dap2p antibody. (*A* and *C*) Fluorescence images with the antibody. (*B* and *D*) DNA staining (with DAPI) of the same fields.

authentic Sec12p, it is unlikely that these proteins invoke the quality control of the ER as unfolded proteins.

We also observed immunofluorescence of DSD in *Δrer1* cells. As shown in Fig. 7, *D–F*, the ER staining was still obvious in most cells, although the vacuolar staining was also seen (compare with DDD, Fig. 7, *A–C*). This indicates that the TMD of Sec12p has an ability of ER localization even in the absence of Rer1p, supporting the aforementioned possibility that it is involved in both Rer1p-dependent and independent mechanisms.

Rate of Mannosyl Modification Distinguishes between Retention and Retrieval

To determine which mechanism of ER localization, retention or retrieval, utilizes these two domains in Sec12p, we carried out metabolic pulse-labeling and chase experiments on the chimeric proteins. Since Och1p, yeast α1→6 mannosyl transferase, is located in the early Golgi compartment, the rate of α1→6 mannose modification should reflect the rate of transport from the ER to the early Golgi.

First, as a control, the cells harboring *SEC12* or *DAP2* on a multicopy plasmid were pulse-labeled for 10 min and chased for 0–60 min. Sec12p and Dap2p were immunoprecipitated with the anti–Sec12p or anti–Dap2p antibody and then subjected to the second immunoprecipitation with either the same antibody, anti-α1→6 mannose antibody, or anti-α1→3 mannose antibody (Fig. 8). To avoid ambiguity due to heterogeneous glycosylation, a half of each sample was treated with endo H. After SDS-PAGE, the radioactivity of each band was quantified by radioimaging analysis. The relative amounts of α1→6 and α1→3 mannosyl modifications are shown in Table III as percentage over the second Sec12p or Dap2p immunoprecipitate. As described previously (d'Enfert et al., 1991; Nishikawa and Nakano, 1993), Sec12p acquires a significant amount of α1→6 mannose modification after synthesis, 18% at 60 min chase in this particular experiment (Fig. 8 *A*). This modification is obvious, but its rate is much slower than those of typical secretory proteins. This indicates that the exit from the ER is rate limiting for Sec12p. Furthermore, modification by α1→3 linkage was hardly detected even at 60 min chase, indicating that the retrieval process operates quite efficiently as well.

In contrast, mannosyl modification of Dap2p is very fast (Fig. 8 *B*). Dap2p appeared as the 110-kD ER form containing 5–8 N-linked oligosaccharide chains and underwent further modification to the 120-kD mature (vacuolar) form during chase (Roberts et al., 1989). Endo H treatment unveiled emergence of a smaller species, which is perhaps due to the degradation in the vacuole. Quantification of mannosyl modification indicates that 29% of Dap2p already acquired the α1→6 mannosyl linkage during 10-min pulse, and the proportion increased to 70% at 60 min chase (see Table III). The modification with α1→3 linkage was also evident even at 0 min and extended during chase.

A similar pulse–chase experiment was performed on DSD and SDD using anti–Dap2p and anti–Sec12p anti-

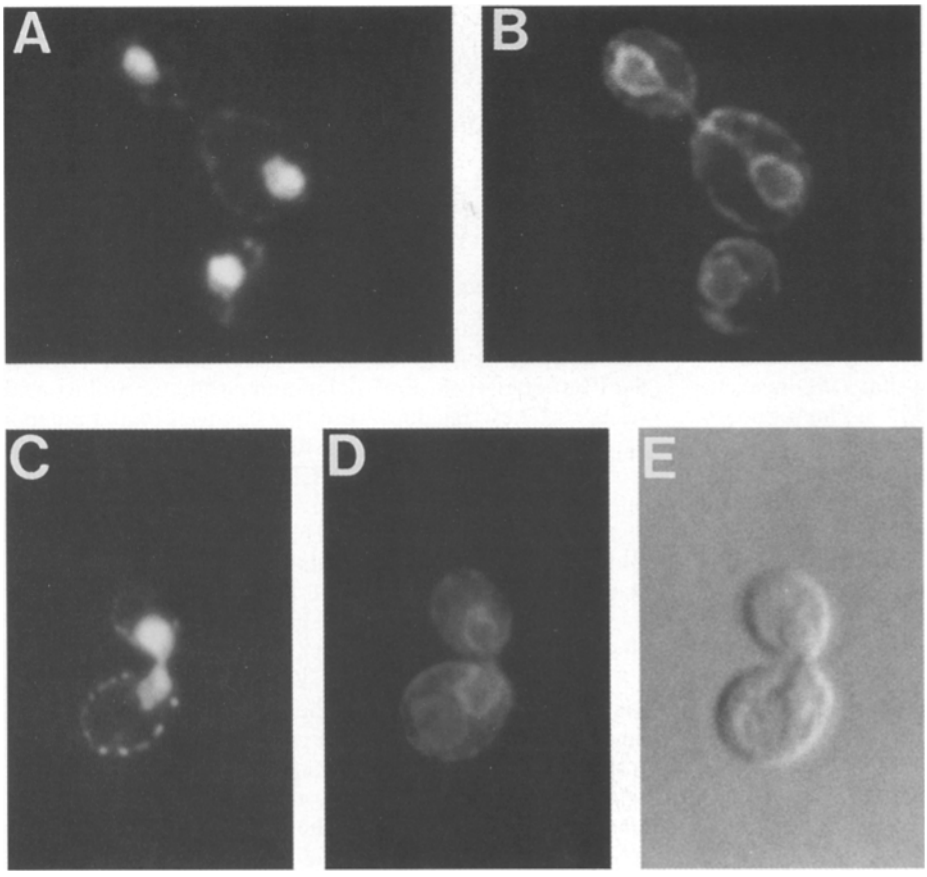


Figure 6. Subcellular localization of DSD and SDD. *Δdap2 Δpep4* (SMY8-1B) cells harboring DSD (*A* and *B*) or SDD (*C–E*) were analyzed by immunofluorescence microscopy using the anti–Dap2p antibody. (*A* and *C*) DAPI staining. (*B* and *D*) Fluorescence images with the antibody. (*E*) Nomarski image.

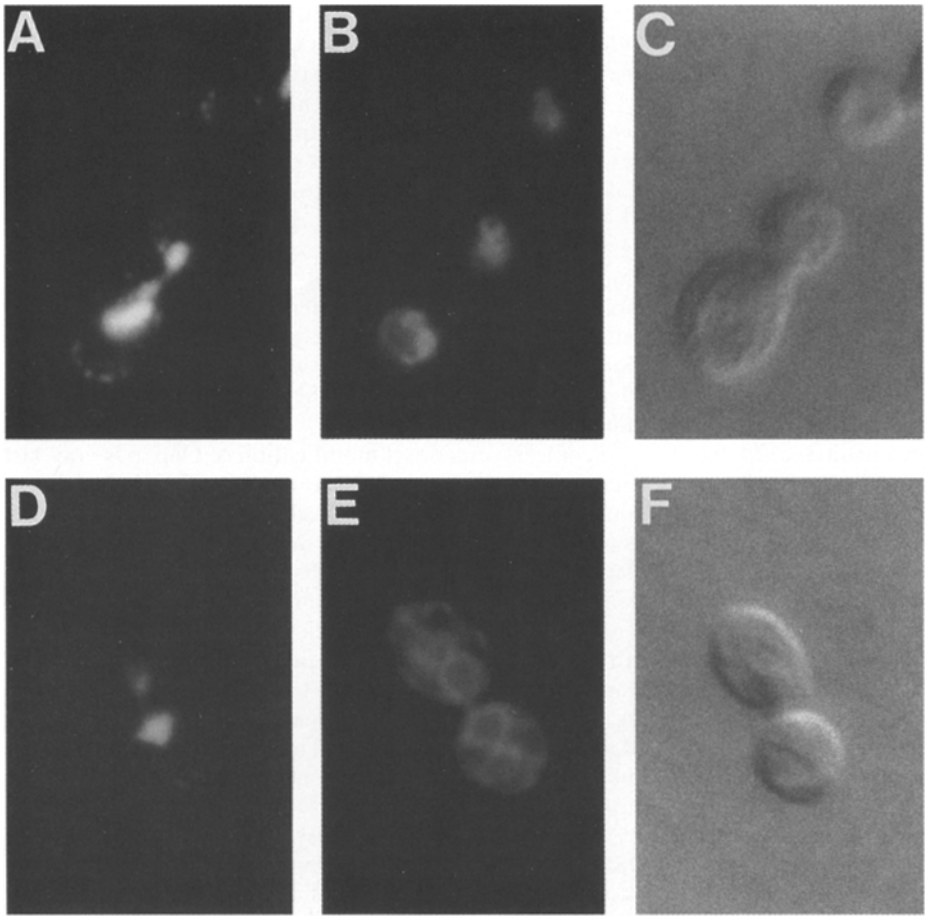


Figure 7. Subcellular localization of DDD and DSD in *Δrer1* cells. SKY7 cells expressing DDD (*A–C*) or DSD (*D–F*) were fixed and prepared for immunofluorescence microscopy with the anti-Dap2p antibody. (*A* and *D*) DAPI staining. (*B* and *E*) Fluorescence images with the antibody. (*C* and *F*) Nomarski images.

bodies, respectively (Fig. 9). DSD was detected as triplet bands at around 110 kD (Fig. 9 *A*). This heterogeneity is due to the N-linked oligosaccharides because they coalesced to a single band by endo H treatment. Apparently, the rate of α1→6 modification of DSD was much faster than that of Sec12p. 40% of DSD became precipitable by the α1→6 mannose antibody at 60 min. Even at 0 min, a population (~6%) that was modified with α1→6 linkage was detectable after endo H treatment. DSD was little modified with α1→3 mannosyl linkage, indicating that this chimera efficiently arrives at the early Golgi but does not go beyond there. Considering the fact that DSD is exclusively localized to the ER as observed by immunofluorescence, this result strongly suggests that it is the retrieval from the Golgi to the ER that operates predominantly to localize DSD to the ER.

SDD was detected as a 152-kD band (Fig. 9 *B*). In contrast to DSD, the rate of α1→6 mannosyl modification on SDD was very slow. Only 19% was modified with the α1→6 linkage after 60 min, which is very similar to the case of Sec12p (see Table III). It appears that the acquisition of the α1→3 modification of SDD (8% at 60 min) is significantly faster than either Sec12p or DSD. Since the ER localization of SDD does not require Rer1p, which is involved in the retrieval, it may be reasonable that the SDD molecules are not sent back to the ER efficienctly once they have left. In fact, the immunofluorescence of SDD (see Fig. 6 *D*) indicates that some population of SDD is transported to the vacuole. The results of the halo assay on SDDm also supports this. All these observations are consistent with the idea that SDD is localized to the ER by moderate static retention. A small portion escapes from this mechanism, and the molecules that depart from the ER do not return but reach the vacuole.

Mutational Analysis of the TMD

We tried to determine which residues in the TMD of Sec12p are important for the ER localization. The TMD of Sec12p contains several polar and aromatic amino acid residues. These residues were first changed to alanine in a variety of combinations (Fig. 10). The mutations were introduced into DSDm, whose ER localization was fulfilled only by virtue of the Sec12p TMD, and the effect was tested by the halo assay. F-A (F355A, F356A, and F359A) and SY-A (S366A, Y367A, and S372A) mutants did not produce any halo in the wild-type cells, indicating that they were completely retained in the ER/*cis*-Golgi. FSY-A (F-A plus SY-A), N-A (N358A), and Q-A (Q370A) formed very small halos. The halo of NQ-A (N-A plus Q-A) was slightly larger than that of either N-A or Q-A. These mutants appear to be leaking out of the ER, but the majority is still localized to the ER. Even the A10 mutant, in which all of the polar and aromatic residues were replaced by alanine, is mostly located in the ER in the wild-type cells. It appears that none of these alanine mutants disrupts the

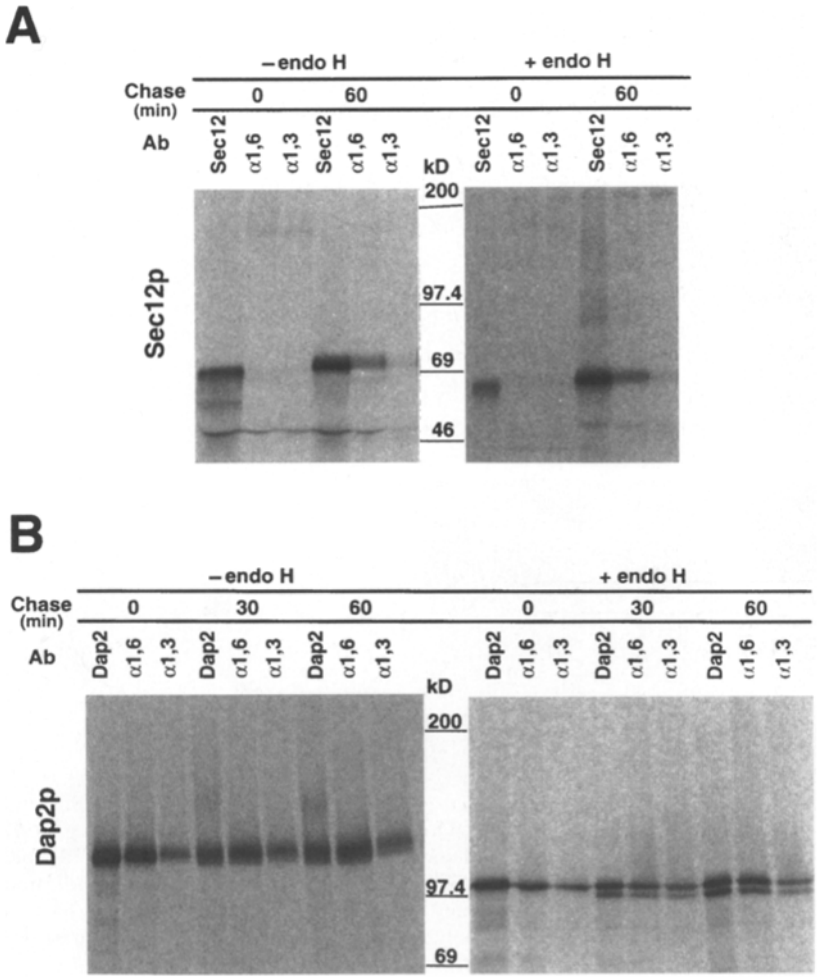


Figure 8. Mannosyl modification of Sec12p and Dap2p. Wild-type (SNY9) cells expressing Sec12p (*A*) and *Δdap2* (SMY6-9C) cells expressing Dap2p (*B*) were labeled with tran^{35}S-label at 30°C for 10 min and chased for the indicated times. Sec12p and Dap2p were first immunoprecipitated with the respective antibodies. The immunoprecipitates were dissolved in 1% SDS, divided into three aliquots, and subjected to the second immunoprecipitation with the antibodies against Sec12p or Dap2p, α1→6 mannosyl linkages (α1,6), and α1→3 mannosyl linkages (α1,3). Each sample was further divided into two, treated with or without endo H, and analyzed by SDS-PAGE and radioimaging.

ER localization information in the TMD completely, although some mutations resulted in the decrease of efficiency. Interestingly, all these alanine mutants kept the Rer1p dependency. In the *Δrer1* cells, the mutants produced halos as large as DSDm did. Upon this surprising result, we decided to construct another series of replacement using leucine instead of alanine. The result is also shown in Fig. 10. N-L (N358L), Q-L (Q370L), and NQ-L (N-L plus Q-L) mutations formed larger halos than the corresponding alanine mutants, indicating that hydrophobicity of these residues is an important parameter. The Rer1p dependency was still seen with these mutants but became less apparent, especially with NQ-L. With the L7 mutant, in which serine, threonine, asparagine, and glutamine residues were all replaced by leucine, the Rer1p dependency finally disappeared. The difference between the alanine and leucine mutants suggests that the distribution of hydrophobicity in the TMD somehow affects its ability to localize the protein to the ER in the Rer1p-dependent manner. On the other hand, even with this L7 mutant, the secretion of α-factor was not as efficient as DDDm. We further proceeded to construct the LeuX19 mutant, which contains only 19 leucine residues in the TMD. This artificial TMD showed Rer1p-independent halo formation, but again the efficiency was not as good as DDDm. Since the expression levels of these mutant proteins were almost the same as DSDm and DDDm as determined by immunoblotting (data not shown), the decrease of the size of halos was not due to their reduced synthesis. In fact, immunofluorescence observation of the cells expressing L7 or LeuX19 showed staining of both the ER and the vacuoles (data not shown). The ER localization was not as strict as with the Sec12p TMD, but it was still obvious. The length of the stretch of leucine (LeuX13, LeuX15, LeuX17, LeuX21, LeuX23, and LeuX26) did not affect its localization property as long as we could test by the halo assay (M. Sato, unpublished data). We also introduced some of these mutations in the TMD of SSSm and performed the halo assay. Similar tendencies were observed, though the halos were smaller because the defect was partly masked by the reten-

Table III. Rate of Mannosyl Modification of Sec12/Dap2 Chimeras

	Mannosyl modification					
	%					
	α1→6			α1→3		
Chimera	0	30	60	0	30	60 min
Sec12p	<2	–	18	<2	–	<2
Dap2p	29	54	70	11	40	22
DSD	6	19	40	<2	<2	<2
SDD	<2	7	19	<2	3	8

Radioactivity of each band was quantified from the +endo H panels of Figs. 8 and 9.

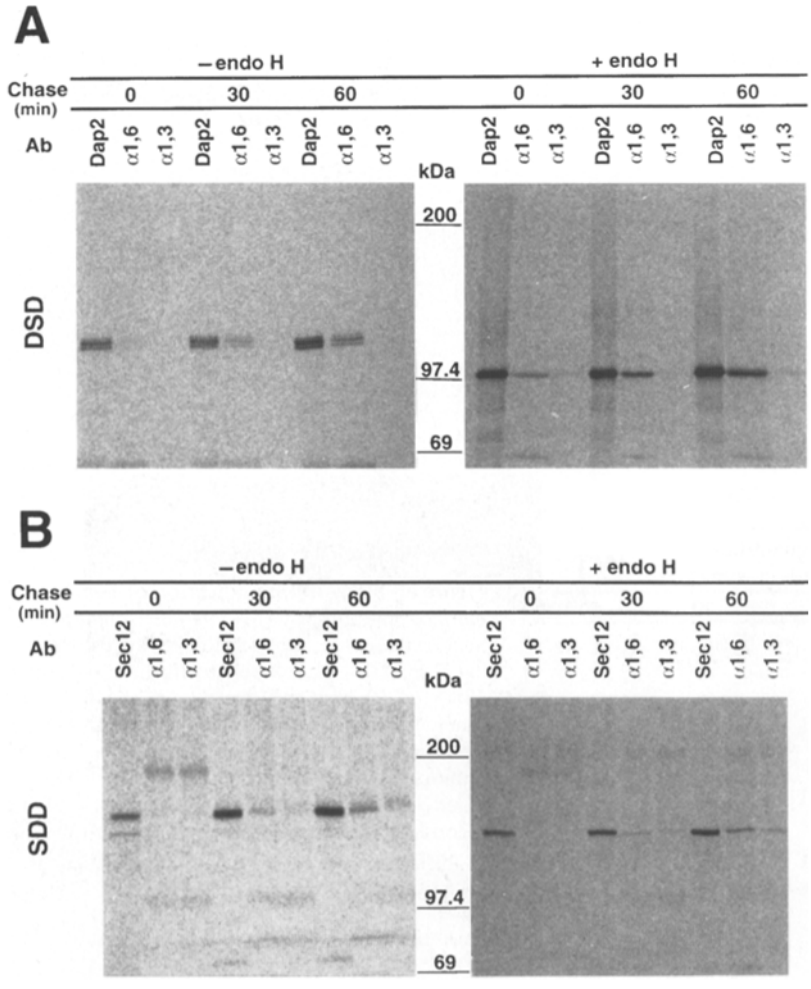


Figure 9. Mannosyl modification of DSD and SDD. *Δdap2* (SMY6-9C) cells expressing DSD (*A*) or SDD (*B*) were labeled with tran ^{35}S-label at 30°C for 10 min and chased for the indicated times. Modification with α1→6 and α1→3 was analyzed by double immunoprecipitation as described in the legend of Fig. 8.

tion effect of the Sec12p cytoplasmic domain (data not shown).

ER Localization Signals of Other ER Membrane Proteins

We further examined whether any parts of other ER membrane proteins could act as a signal to localize Dap2p to the ER. Sed4p and Sec20p are also type II transmembrane proteins in the ER but have the HDEL sequence at their COOH terminus, unlike Sec12p (Hardwick et al., 1992; Gimeno et al., 1995; Sweet and Pelham, 1992). Sed4p shows a striking structural similarity to Sec12p in the NH_2-terminal cytoplasmic domain and the TMD (45% identical) but not in the COOH-terminal lumenal domain. Sec20p has no homology to Sec12p. It has been shown that the HDEL sequence of Sec20p is important for its ER localization (Sweet and Pelham, 1992). We constructed D4Dm and D20Dm (Fig. 11 *A*) by replacing the TMD of Dap2p by that of Sed4p or Sec20p. The behavior of these chimeras in wild-type and *Δrer1* cells was tested by the halo assay (Fig. 11 *B*). D4Dm did not produce any halo in the wild-type cells, indicating that the TMD of Sed4p is sufficient for the ER localization. Subcellular localization of D4Dm was also examined by immunofluorescence, which showed strict ER staining similar to that of DSDm (Fig. 12 *B*). This effect was dependent on Rer1p because D4Dm produced a halo in *Δrer1* (Fig. 11 *B*). Thus, the Rer1p-dependent mechanism of ER localization commonly operates on the TMD of Sec12p and Sed4p. In contrast, D20Dm formed a large halo in either wild-type or *Δrer1* cells (Fig. 11 *B*). This clearly indicates that the Rer1p-dependent system is not effective to all transmembrane ER proteins. It apparently discriminates Sec20p from Sec12p and Sed4p. In the case of Sec20p, other motifs including the HDEL sequence might decide the localization.

Since the cytoplasmic domain of Sed4p is homologous to that of Sec12p, we tested its effect on the ER localization as well. 4DDm (Fig. 12 *A*) was expressed in wild-type cells, and indirect immunofluorescence microscopy was performed (Fig. 12 *B*). 4DDm was mainly located in the ER, but weak vacuolar staining was also observed in some cells. This is very similar to SDDm and SDD, suggesting that the cytoplasmic domain of Sed4p also has an ability to localize the molecule in the ER in a similar manner to that of Sec12p.

Discussion

Two ER Localization Signals of Sec12p

Signals, or special structural motifs, are essential for correct sorting of proteins in a cell. During membrane traffic

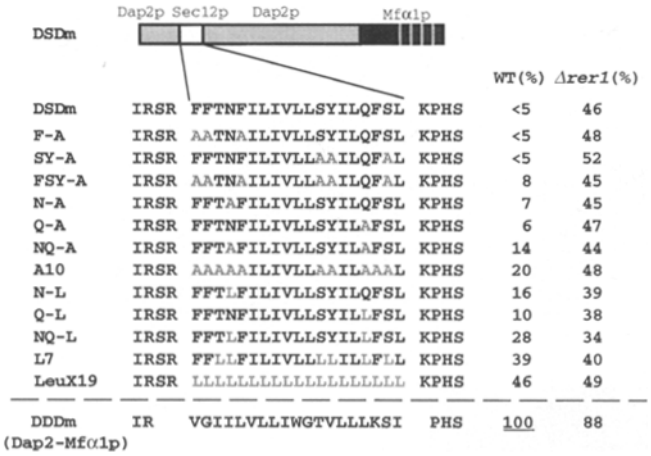


Figure 10. Mutational analysis on the TMD of Sec12p in DSDm. Amino acid sequences of the TMD regions of the various mutants are shown in the left. The values in the right are quantification of the secreted α-factor in the halo assay performed on wild-type (SNY9) or *Δrer1* (SKY7) cells. The average values of two independent experiments are shown.

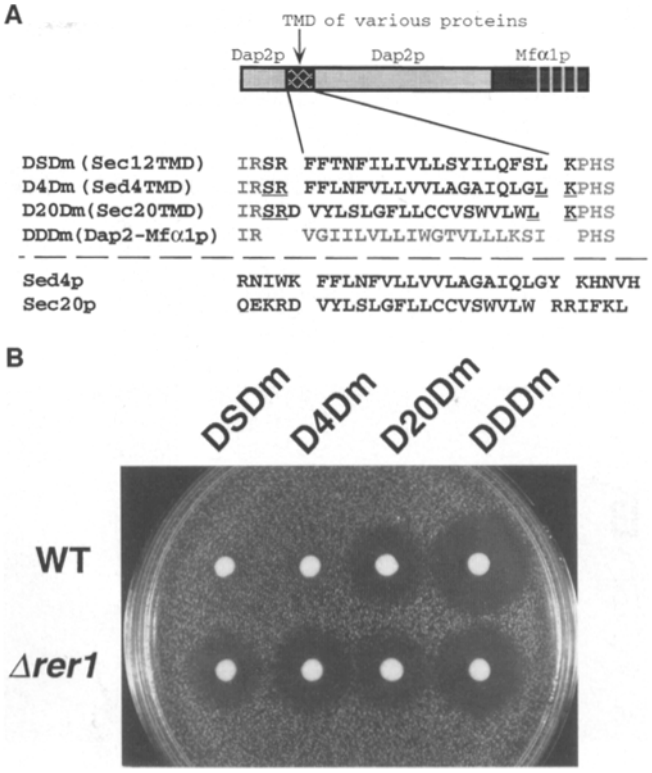


Figure 11. The effect of the TMDs of Sed4p and Sec20p on ER localization. (*A*) The TMD of DDDm was replaced by that of Sed4p and Sec20p to construct D4Dm and D20Dm, respectively. Amino acid sequences of the junction regions are shown. (*B*) The secretion of mature α-factor was examined by the halo assay using wild-type (SNY9) and *Δrer1* (SKY7) cells.

in the secretory pathway, vesicles dynamically cycle between organelles. The sorting processes in such vesicular traffic should also be understood from the viewpoint of recognition of signals.

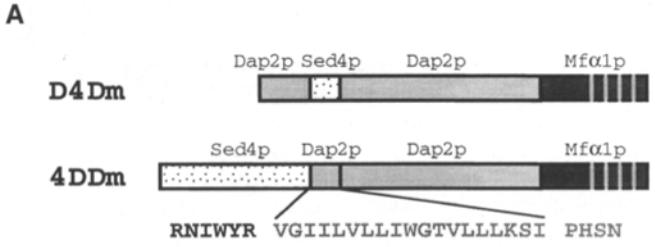
We have been investigating how yeast Sec12p is localized to the ER. Sec12p is a pivotal membrane component required for vesicle budding from the ER and needs to be strictly localized to the ER membrane, but there is also evidence that it recycles between the ER and the Golgi apparatus (Nakano et al., 1998; Nishikawa and Nakano, 1993; Oka and Nakano, 1994). We have shown in this paper that such localization of Sec12p is achieved by two mechanisms: static retention in the ER that involves the NH_2-terminal cytoplasmic domain of Sec12p and dynamic retrieval from the Golgi to the ER that requires the TMD. We shall call the structural motifs contained in these domains the retention and retrieval signals, respectively. A Golgi membrane protein, Rer1p, plays an important role in the retrieval process (Sato et al., 1995). The retrieval by the TMD signal depends on the presence of Rer1p in the Golgi, whereas the retention by the cytoplasmic signal is Rer1p-independent (Fig. 13).

Retention and Retrieval

The above conclusion has been obtained from the experiments using chimeric fusions between Sec12p and a reporter protein Dap2p. Since Dap2p, a vacuolar membrane protein, is believed to be innocent for its destination and is transported to the vacuole by default under normal conditions (Roberts et al., 1992), the effects of the Sec12p domains on localization can be directly tested on chimeric proteins. The results of biochemical and morphological experiments demonstrate that either the TMD or the cytoplasmic domain of Sec12p causes ER localization when included in the chimera. The actions of these two domains are independent of each other. The TMD alone can localize the protein to the ER quite efficiently, while the cytoplasmic domain can retain the majority of the molecules in the ER as well.

The fact that the effect of the TMD is Rer1p-dependent suggests that this is due to the retrieval mechanism. In a previous study, we showed that Rer1p is an integral membrane protein in the Golgi apparatus, most probably residing in the *cis* region of the Golgi (Sato et al., 1995). Rer1p could serve for ER localization of Sec12p either by directly sending back the molecule to the ER or by preventing its progression to the later compartments of the Golgi. The role of Rer1p in such retrieval processes is in good agreement with the biochemical observations in this study. On DSD, a chimeric construct whose ER localization is achieved by the Rer1p-dependent effect of the TMD, the rate of α1→6 mannosyl modification is quite fast, unlike Sec12p, whereas that of α1→3 mannosyl modification is strictly inhibited. Since the α1→6 and α1→3 modifications take place in the *cis* and *medial* compartments of the Golgi apparatus, respectively (Nakayama et al., 1992; Graham et al., 1994), this observation indicates that DSD is rapidly reaching the *cis*-Golgi but does not move on to the *medial*-Golgi because it is returned to the ER (see Fig. 13). This has led us to conclude that the signal contained in the TMD is for retrieval.

In contrast, the effect of the NH_2-terminal cytoplasmic domain is quite different. It does not require the presence of Rer1p and appears to be less efficient than the TMD for ER localization. The analysis on mannosyl modification



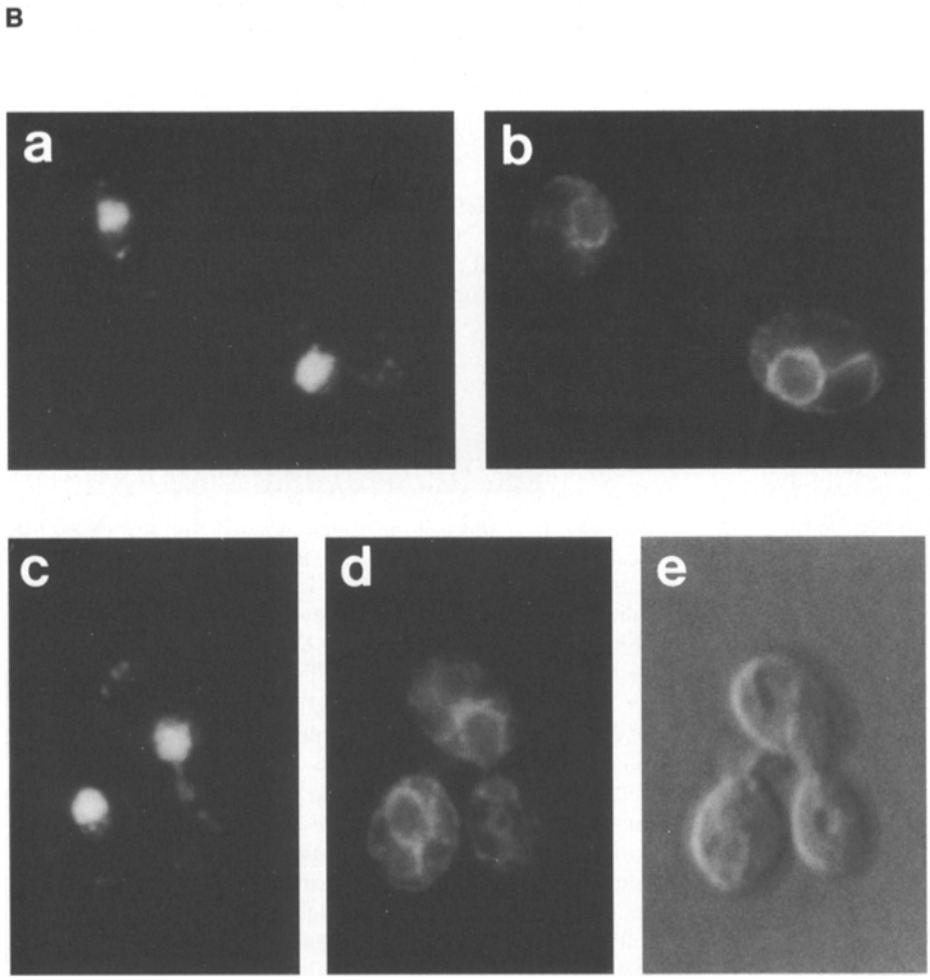


Figure 12. (*A*) Chimeras between Sed4p and Dap2p. (*B*) Subcellular localization of D4Dm and 4DDm. *Δdap2 Δpep4* (SMY8-4C) cells expressing D4Dm (*a* and *b*) or 4DDm (*c–e*) were fixed and prepared for immunofluorescence microscopy with the anti-Dap2p antibody. (*a* and *c*) DAPI staining. (*b* and *d*) Fluorescence images with the antibody. (*e*) Nomarski image.

on SDD, a construct localized to the ER by the cytoplasmic domain, indicates that this molecule is largely detained in the ER. The rate of α1→6 modification is as slow as that of the authentic Sec12p. This strongly suggests that the ability of the cytoplasmic domain to localize the protein in the ER is due to the static retention mechanism. Interestingly, the SDD molecules that have escaped the retention and acquired the α1→6 linkage appear to be further modified by the α1→3 mannose in time. This may be because the retrieval system does not work effectively on SDD. This is also consistent with the fact that the ER localization of SDD is not affected by the deletion of *RER1*.

Dissection of Signals

Increasing numbers of reports suggest that some membrane proteins have multiple signals for localization. For example, Graham and Krasnov (1995) showed that the COOH-terminal lumenal domain and the TMD of yeast Mnn1p are important for the Golgi localization; Itin et al. (1995) reported that both the lumenal and cytoplasmic domains of human ERGIC-53 are involved in ER-ERGIC-*cis*-Golgi recycling; and Szczesna-Skorupa et al. (1995) claimed that the TMD and the COOH-terminal cytoplasmic domain of rabbit cytochrome P450 2C1 and 2C2 contain signals for the ER localization. However, the functional differentiation of these signals remains unclear. In this paper, we have shown for the first time that yeast Sec12p contains two distinct signals that correpond to two different mechanisms, retention and retrieval. Previously, Boehm et al. (1994) implied the importance of the Sec12p TMD by comparing the secretion of invertase from Sec22-α-factor-invertase and Sec22/12-α-factor-invertase fusions. They showed that secretion of the invertase activity was suppressed by introduction of the Secp TMD and that this

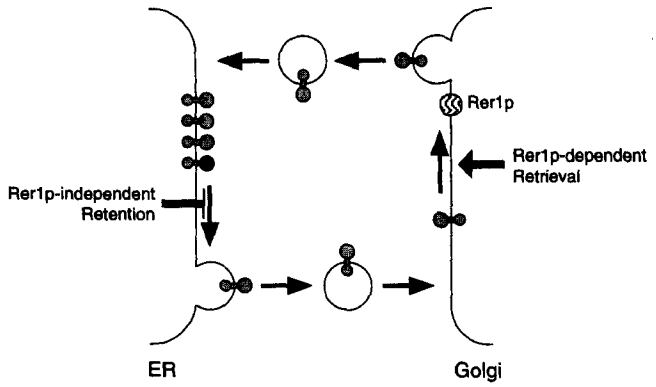


Figure 13. A schematic model of the two mechanisms that localize Sec12p to the ER. The NH_2-terminal cytoplasmic domain of Sec12p is important to be excluded from the budding vesicles on the ER, whereas the TMD of Sec12p is required for the efficient Rer1p-dependent retrieval from the Golgi to the ER. The parts of Sec12p that act as signals of retention and retrieval are filled in black.

effect was diminished by the deletion of *RER1*. In this context, their findings are consistent with our study, although the effect of the TMD was only partial in their work. In normal cells, the retention mechanism would mask the retrieval effect and vice versa. Successful dissection of two such signals has become possible by the systematic analysis of chimeric proteins and the use of the *Δrer1* mutant in the present study.

Further determination of the signals of Sec12p has been attempted at the level of amino acid residues. We have performed an extensive mutational analysis on the residues in the TMD and obtained a surprising result: Even the mutant TMD that has had all the polar and aromatic residues replaced by alanine (*A10* of Fig. 12) can still localize the DSD molecule to the ER in an Rer1p-dependent manner. Since this TMD contains only alanine, valine, leucine, and isoleucine residues, one might argue that the recognition of the TMD signal by the retrieval system is not quite strict in terms of structure. However, we have also realized that the dependency on Rer1p is completely lost when most of these polar residues are replaced by leucine (*L7* of Fig. 12). Apparently, there is a difference between leucine and alanine residues in the Rer1p-dependent localization effect of the mutant TMD. One obvious difference between these two amino acids is the bulkiness of the side chain. In other words, they differ in the degree of hydrophobicity. By scrutinizing the Sec12p TMD and its mutant versions that retain the Rer1p dependency, we find that there is a gradient of hydrophobicity which peaks in the middle of the TMD. The hydrophobic core in the center that is flanked by less hydrophobic residues appears to be a feature of the Rer1p-dependent signals. From such a point of view, the Rer1p-dependent TMD of Sed4p has a similar profile, whereas the Rer1p-independent TMDs of Dap2p and Sec20p do not. In support of this idea, the artificial TMD consisting of 19 leucine residues does not show the Rer1p-dependent localization effect. Further experimental tests are possible and will be necessary to prove or disprove this hypothesis.

Apart from the Rer1p dependency, it is also puzzling that even the LeuX19 TMD can localize the protein to the ER to some extent. Although weak, this artificial TMD has an ability to retain the chimeric protein in the ER, which is not present in the Dap2p TMD. How could this observation be explained? Let us presume here that this is due to the retention in the ER because it is Rer1p-independent. Possibly, the introduction of this artificial stretch of leucines interferes with the assembly of the DSDm protein into the transport vesicles and thus retards the ER-to-Golgi traffic. The quality control system of the ER might be somehow involved in this process. Alternatively, the partial ER retention may take place without any particular signal, and the fast exit of Dap2p from the ER may depend on a selective mechanism that positively transports the protein to the Golgi apparatus. This is a revival of the selectivity model of Lodish (1988) and may be consistent with the arguments that cargo molecules are selectively concentrated in transport vesicles (Mizuno and Singer, 1993; Balch et al., 1994). Of course, we cannot exclude the possibility that the effect of the LeuX19 TMD in ER localization is due to another retrieval mechanism that does not require Rer1p.

It is known that many Golgi membrane proteins are localized by virtue of the TMD signals. However, the structural requirements of such signals are still unclear (Swift and Machamer, 1991; Bretscher and Munro, 1993; Nilsson et al., 1993; Weisz et al., 1993). If the TMD alone can distinguish ER and Golgi proteins, what is the difference between them? Munro (1995) has recently demonstrated that the length of the TMD is critical for the Golgi retention in the case of sialyltransferase and suggested the importance of the lipid-based sorting. In his experiment, the LeuX17 TMD functions as a Golgi localization signal. We have performed a similar experiment to test whether such a length effect of the TMD could be involved in the case of Sec12p as well. The result indicates that all of the leucine-only TMDs we have examined show a partial retention ability that is independent of Rer1p. There was no special preference to the length of leucine stretch (M. Sato, unpublished data). The different requirement of similar hydrophobic TMD between the ER and the Golgi localization remains to be clarified experimentally.

Mechanism of Retention

We have also tried to determine which part of the cytoplasmic domain of Sec12p is important for the ER retention by a systematic deletion analysis. However, most of the constructed mutant proteins are unstable in the yeast cells, and we have been unable to narrow down the region that is ascribed to for the retention mechanism. The cytoplasmic domain of Sec12p contains the catalytic site of the protein that functions as the guanine-nucleotide exchange factor toward the Sar1 GTPase (Barlowe and Schekman, 1993). Mutations in this region may hamper the interaction with Sar1p and other molecules that are essential for vesicle budding and thus accelerate the degradation of the mutant protein. It is also conceivable that the interaction of the cytoplasmic domain of Sec12p with other component(s) of the budding machinery may be the mechanism of the static retention. If so, the signal in this domain may require its overall conformation rather than particular residues.

A hint on this possibility comes from the result on Sed4p. Sed4p shares high homology with Sec12p in the cytoplasmic domain and the TMD (Hardwick et al., 1992). In fact, both of these domains are capable of localizing the Dap2p chimeras in the ER as those of Sec12p do. If the ER retention by the cytoplasmic domain of Sec12p is fulfilled by interaction with other molecules, is it also the case with the cytoplasmic domain of Sed4p? If so, are there common partners with which Sec12p and Sed4p can interact? Sed4p has been shown not to possess the guanine-nucleotide exchanger activity toward Sar1p despite the high similarity to Sec12p (Barlowe and Schekman, 1993). However, Gimeno et al. (1995) reported that synthetic lethal interaction exists between one mutant allele of *SAR1* and the disruptant of *SED4* and suggested the possibility that their products may function in a multisubunit complex in the wild-type cells. Sed4p has also been shown to interact with Sec16p (Gimeno et al., 1995). Since *SAR1* has a strong suppressor activity on *sec12* and *sec16* ts mutants (Nakano and Muramatsu, 1989), there may be a link between Sec12p and Sec16p. Sar1p and Sec16p could be somehow involved in the ER retention of Sec12p and Sed4p. How Sar1p and Sec16p are associated with the ER membrane is totally unknown. To solve this kind of chicken-and-egg problem, further efforts to dissect the structural requirements of the cytoplasmic domains of Sec12p (and Sed4p) will be necessary.

Role of Rer1p

The analyses of signals are always complementary to the analyses of receptors. In the case of the KDEL/HDEL signal, Erd2p has been identified as its receptor (Semenza et al., 1990). In the retrieval of membrane proteins with the dilysine motifs, the coatomer (COP I) binds to this signal and executes the Golgi-to-ER retrograde transport (Cosson and Letourneur, 1994; Letourneur et al., 1994). What about the Sec12p signals? If the recognition of these signals is a receptor-mediated process, massive overproduction of the signals would give rise to saturation and overflow of the sorting mechanism. In the case of the authentic Sec12p, enormous overproduction does not lead to its missorting but rather complex phenomena including proliferation of the ER membrane, inhibition of ER-to-Golgi anterograde transport, and formation of the BiP bodies in the lumen of the ER (Nishikawa et al., 1994). Such consequences are probably due to the imbalance of the Sar1p GTPase cycle caused by the increase of the catalytic sites of Sec12p as the guanine nucleotide exchange factor. Since the TMD of Sec12p does not have such an effect on Sar1p, we could test the effect of its overproduction. In fact, when the DSDm construct was expressed by the strong promoter of the glyceraldehyde-3-phosphate dehydrogenase gene on a multicopy plasmid, secretion of α-factor was clearly observed, indicating that this chimeric protein was mislocalized to the late Golgi (M. Sato, unpublished data). This suggests that the retrieval of Sec12p by the TMD signal is a saturable process, again implying the existence of a receptor.

On the other hand, the structural requirement of the TMD remains to be clarified as discussed above. While the Sec12p TMD accepts rather extensive alterations, a distribution of the hydrophobicity with the central core may be the feature of the signal. Rer1p is an important player in this retrieval process. Is it possible that this molecule recognizes a variety of TMD signals as a receptor? Since Rer1p is an integral membrane protein with four membrane spans, there is a chance that such transmembrane regions physically interact with the Sec12p TMD. Such a possibility is testable by biochemical means and is now being investigated. Rer1p has highly charged regions in both the termini and the middle of the molecule. Such structural properties of Rer1p might have some relation to the hydrophobicity gradient in the TMD signal.

It is also possible that Rer1p is a component of the general machinery that functions in the sorting of proteins in the *cis*-Golgi. It could help concentrate the ER-resident proteins to be retrieved or might block their forwarding to the later compartments. If the role of Rer1p is in fact general for multiple signals, its mutant should show pleiotropic effects on a variety of cargos. The small amount of missecreted BiP seen in the *Δrer1* cells (Sato et al., 1995) could reflect such a general defect. On the other hand, missorting of the dilysine signal was not observed in the same cells (Gaynor et al., 1994). Apparently, understanding of the structure and function of such general machinery requires further identification of components by genetic and biochemical studies. The *RER2* gene we identified previously (Nishikawa and Nakano, 1993) appears to encode a hydrophilic protein which is peripherally associated with the ER membrane (M. Sato, S. Nishikawa, K. Sato, and A. Nakano, unpublished). Its role could be implicated in the retention rather than in retrieval.

It should also be mentioned here that the effect of the Sec12p TMD is not completely Rer1p-dependent. Even in the absence of Rer1p, a significant amount of DSD is localized to the ER. This might indicate that multiple pathways exist in the retrieval system. The fact that the disruption of the *RER1* gene is not lethal could also suggest the redundancy of the retrieval mechanism. Perhaps the deletion of Rer1p only partially impairs the function of the retrieval machinery. Alternatively, the TMD of Sec12p may also harbor a weak signal for static retention. The observation that the rate of α1→6 mannosyl modification on DSD is not as fast as Dap2p supports this possibility. More extensive analysis on the amino acid residues in the TMD might separate such different effects in terms of Rer1p dependency.

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