SEC18/NSF-independent, protein-sorting pathway from the yeast cortical ER to the plasma membrane

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Classic studies of temperature-sensitive secretory (sec) mutants have demonstrated that secreted and plasma membrane proteins follow a common SEC pathway via the endoplasmic reticulum (ER), Golgi apparatus, and secretory vesicles to the cell periphery. The yeast protein Ist2p, which is synthesized from a localized mRNA, travels from the ER to the plasma membrane via a novel route that operates independently of the formation of coat protein complex II-coated vesicles. In this study, we show that the COOH-terminal domain of Ist2p is necessary and sufficient to mediate SEC18-independent sorting when it is positioned at the COOH terminus of different integral membrane proteins and exposed to the cytoplasm. This domain functions as a dominant plasma membrane localization determinant that overrides other protein sorting signals. Based on these observations, we suggest a local synthesis of Ist2p at cortical ER sites, from where the protein is sorted by a novel mechanism to the plasma membrane.

Introduction

The vesicular transport of integral membrane proteins is mediated by the recognition of cytoplasmic sorting signals or transmembrane (TM) segments by adaptor and coat proteins during vesicle budding (Rayner and Pelham, 1997; Sato et al., 2003; Munro, 2004). Coat protein complex (COP) II vesicles assemble at specialized regions of the ER that are dedicated to sorting proteins for export to the Golgi apparatus (Antonny and Schekman, 2001). These sites are distributed over the entire surface of the cortical and perinuclear ER in Saccharomyces cerevisiae (Rossanese et al., 1999). Generally, it was thought that protein sorting to different cellular locations occurs within the TGN. This view has been challenged by the recent observation in S. cerevisiae that glycosylphosphatidylinositol-anchored proteins are separated from other secretory proteins at the ER by packaging them into specific COPII-coated vesicles (Muniz et al., 2001). This suggests the existence of several distinct mechanisms for the concentration, selection, and exit of cargo proteins from the ER (Watanabe and Riezman, 2004).

In contrast to transport by the so-called classical SEC pathway via the ER, Golgi apparatus, and secretory vesicles, we have shown that the yeast integral membrane protein Ist2p reaches the plasma membrane independently of the formation of COPII-coated vesicles. The transport of Ist2p does not depend on Sec12p and Sec23p, on the transport of vesicles along actin filaments (which is mediated by Myo2p), on the formation of vesicles at the Golgi (which is mediated by Sec7p), or on the Sec1p-dependent fusion of vesicles with the plasma membrane (Jüschke et al., 2004). These observations have led to the hypothesis that a connection between the localization of IST2 mRNA and the unusual trafficking of the protein could exist (Jüschke et al., 2004). IST2 mRNA belongs to a group of transcripts that accumulate at the cortex of daughter cells (Takizawa et al., 2000; Shepard et al., 2003). These mRNAs interact with the RNA-binding protein She2p, which connects mRNA particles with the myosin motor Myo4p via the She3p adaptor and, thereby, mediates the translocation of the RNA along the polarized actin cytoskeleton into the daughter cell (Gonsalvez et al., 2005).

The transport of IST2 mRNA by the She machinery is required for the expression of Ist2p in the plasma membranes of daughter cells (Takizawa et al., 2000; Jüschke et al., 2004). The observed ablation of Ist2p expression in small and medium-sized daughter cells in sheΔ mutants could be explained by a lack of transport and synthesis of Ist2p into daughter cells. This is why, in combination with the diffusion barrier for integral plasma membrane proteins located at the bud-neck region of the plasma membrane, sheΔ mutants that fail to transport IST2 mRNA into daughter cells lack Ist2p in their plasma membranes (Takizawa et al., 2000; Jüschke et al., 2004). These observations suggest that Ist2p is synthesized at the cortical ER and that daughter cells need the transport of RNA for local synthesis.
microscopy. In exponentially growing minal domain of Ist2p is required to target Ist2p to the plasma membranes. Together, these results show that the COOH-ter-

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membrane by light microscopy (Jüschke et al., 2004), we tested the protease accessibility of the fusion protein. Adding pronase to intact yeast cells resulted in the cleavage of the 150-kD band of GFP-Sac1-Ist2C into a 90-kD, protease-protected fragment (Fig. 2 B). This corresponded to a cleavage within the extracel-

Figure 1. Trafficking of Ist2p to the plasma membrane requires its COOH-terminal cytoplasmic domain. Fluorescence of ist2Δ yeast cells, expressing different EGFP-tagged Ist2p variants under the control of their endogenous UTRs. (a) Full-length Ist2p (aa 946). (b) A COOH-terminal truncated variant, lacking the cytoplasmic domain (aa 592–946 are deleted). (c) An NH2-terminal truncated variant lacking the NH2 terminus, and TM segments one to six (aa 1–490 are deleted). (d) An NH2-terminal truncated variant lacking the NH2 terminus, and TM segments one to eight (aa 1–588 are deleted).

trafficking of Ist2p through the ER

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Results

The COOH-terminal domain of Ist2p is required for its trafficking to the plasma membrane

To investigate the cis-acting elements that are responsible for directing Ist2p to the plasma membrane, we constructed yeast strains that expressed different NH2- and COOH-terminally truncated versions of Ist2p. All constructs were tagged with GFP at the NH2 terminus and were analyzed by fluorescence microscopy. In exponentially growing ist2Δ yeast cells, full-

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When we induced the expression of CFP-tagged Prm1p, no signal was detected at the ER, but some Prm1-CFP accumulated at sites of cell fusion after 90 min (Fig. 3 A, a). Small amounts of Prm1-CFP were also seen at other sites of the plasma membrane, but the majority was located inside the vacuole. We suggest that the slower folding of CFP, compared with the folding of GFP, accounts for the observed absence of visible Prm1-CFP at the perinuclear ER. An isogenic strain expressing Prm1-GFP showed the previously described perinuclear ER accumulation of Prm1p (unpublished data).

It is important to note that the mating partner cells, which expressed Prm1-GFP-Ist2C, showed a different staining; the majority of the protein was located at the cell periphery in a patchlike pattern that resembled the typical Ist2p localization (Fig. 3 A, b). These observations are consistent with the dominant function of Ist2C as a specific plasma membrane sorting signal. The presence of the Ist2C domain redirects Prm1p from sites of cell–cell contact to a patchlike distribution at the plasma membrane and prevents its accumulation in the vacuole.

To determine whether Prm1-GFP-Ist2C passes through the ER, we compared the apparent molecular mass of Prm1-CFP and Prm1-GFP-Ist2C with that of the calculated molecular mass. The modification of 14 predicted consensus sites for N-linked glycosylation should lead to a decreased mobility of the protein in SDS-PAGE and would indicate a passage through the ER. Prm1-CFP showed a major band of 115 kD with a faint, diffuse smear above it, whereas Prm1-GFP-Ist2C migrated as a band of 180 kD (Fig. 3 B). The treatment of membranes from these cells with peptide N-glycosidase F, an enzyme that removes N-linked sugar moieties, shifted both Prm1-CFP and Prm1-GFP-Ist2C bands into faster migrating species of 95 and 150 kD, respectively, indicating that both proteins had received N-linked core glycosylation at the ER. These results demonstrate that adding Ist2C to the COOH terminus of a membrane protein does not prevent its trafficking through the ER nor prevents its accessibility to the core glycosylation machinery.

**Trafficking of Ist2p through the Golgi apparatus**

To determine whether Ist2p is directly transferred from the ER to the plasma membrane or if the trafficking of the protein involves passage through the Golgi apparatus, we investigated whether the N-linked glycosylation sites receive Golgi-specific mannose modifications. Modifications of N-linked oligosaccharides in the yeast Golgi complex is initiated by the transfer of a mannose residue to the core oligosaccharide in an α-1,6-linkage (Nakayama et al., 1992). This modification is followed by further heterogeneous elongation and branching, resulting in a final addition of α-1,3-linked mannose residues to the branched chain (Raschke et al., 1973). These reactions are initiated in distinct compartments of the Golgi complex: α-1,6-

**Figure 3. The trafficking of Ist2p is initiated by insertion into ER membranes.** Wild-type cells of opposite mating types were mixed and incubated at 25°C for 90 min. (A) Schematic topology and the predicted position of N-linked glycosylation sites of Prm1p (Heiman and Walter, 2000) as well as fluorescence of mating cells expressing either CFP-tagged Prm1p (Prm1-CFP in red) or GFP-tagged Prm1-Ist2C (Prm1-GFP-Ist2C in green). (B) Membranes corresponding to 1 OD600 cells, expressing Prm1-CFP or Prm1-GFP-Ist2C, were treated with peptide N-glycosidase F according to the manufacturer’s instructions and were separated on SDS-PAGE. Proteins were decorated with GFP-specific antibodies. Prm1-CFP* and Prm1-GFP-Ist2C* mark the deglycosylated products of Prm1-CFP and Prm1-GFP-Ist2C, respectively.
linkage occurs at the cis-Golgi, and α-1,3-linkage occurs at the medial- and trans-Golgi (Brigance et al., 2000).

We introduced constructs encoding Prm1-GFP-Ist2C and Prm1-CFP in MATa and MATα strains and induced the expression by mixing the cell cultures of opposite mating types. 75 min after induction, Prm1-CFP and Prm1-GFP-Ist2C were immunoprecipitated with GFP-specific antibodies, and the isolated proteins were probed with an antibody recognizing GFP to determine the recovery of the proteins. Prm1-CFP was seen as a major 115-kD band with some additional faint, diffuse bands that had reduced mobility (Fig. 4, lane 1). These diffuse bands were also recognized by antibodies specific for α-1,6- or α-1,3-mannose modifications (Fig. 4, lanes 2 and 3), indicating that only a minor part of Prm1-CFP reached the cis- and trans-Golgi compartments at the time of induction.

Prm1-GFP-Ist2C was seen as a 180-kD band (Fig. 4, lane 4) that was reactive with GFP- and α-1,6-mannose–specific antibodies, indicating that Prm1-GFP-Ist2C enters the cis-Golgi compartment. However, probing the precipitated protein with α-1,3-mannose–specific antibody resulted in a very weak signal (Fig. 4, compare bands lane 5 with 6), suggesting that most of the Prm1-GFP-Ist2C was not transported to the trans-Golgi. This retention of Prm1-GFP-Ist2C in the early Golgi could be explained by the retrograde transport of Ist2p. The extreme COOH terminus of Ist2p, KKKL, contains a strong KXXK ER-retrieval signal (Coxson and Letourneur, 1994). This signal could mediate the relocation of Ist2p from the cis-Golgi to the ER, abolishing further trafficking along the SEC pathway through the Golgi apparatus.

To further investigate the trafficking of Ist2p through the Golgi, we chose the chloride channel protein Gef1p as another reporter protein. Gef1p is processed during its transport in the TGN by the furin protease Kex2p, which recognizes amino acid KR at positions 136 and 137 as cleavage sites in Gef1p (Fig. 5 A; Wachter and Schwappach, 2005). KR indicates a Kex2p protease recognition site that allows us to monitor passage through the TGN. To determine whether Gef1-GFP-Ist2C was transported through late Golgi cisternae, we investigated its processing by Kex2p protease. Gef1-GFP, with the Kex2p cleavage site deleted (KR to AA mutation, Gef1(KR→AA)-GFP), migrated as a 110-kD band, whereas the majority of wild-type Gef1-GFP was cleaved into a 90-kD band (Fig. 5 B, first and second lanes). This processing by Kex2p was not observed in cells expressing Ist2C-tagged Gef1-GFP. Gef1-GFP-Ist2C migrated as bands of identical size (Fig. 5 B, third and fourth lanes). Proteins were separated on SDS-PAGE and were decorated with PC epitope–specific antibodies. Gef1-4PC* indicates the protease cleavage product of Gef1-4PC. (E) A schematic representation of possible dimers that can be detected by PC-specific antibodies is shown.
cleavage of a protein C (PC) epitope–tagged version of Gef1p dimer. We took advantage of this fact and analyzed the Kex2p trans as a dominant plasma membrane sorting signal for the Ist2C-tagged Gef1p. Diploids that coexpressed Gef1-RFP and Gef1-GFP-Ist2C showed a diminished dotlike, intracellular localization (Fig. 5 A, b), indicating that the addition of Ist2C to Gef1p targets the resulting chimera to the plasma membrane.

The dimerization allowed us to study the sorting function of Ist2C in trans by coexpressing a wild-type as well as an Ist2C-tagged Gef1p. Diploids that coexpressed Gef1-RFP and Gef1-GFP-Ist2C showed a diminished dotlike, intracellular localization of Gef1-RFP, which partially overlapped with Gef1-GFP-Ist2C at the cell periphery (Fig. 5 C). These observations indicate that both subunits assemble and that Ist2C functions in trans as a dominant plasma membrane sorting signal for the dimer. We took advantage of this fact and analyzed the Kex2p cleavage of a protein C (PC) epitope–tagged version of Gef1p (Gef1-4PC) in cells that coexpress either Gef1-GFP or Gef1-GFP-Ist2C. In a situation that led to the homodimerization of Gef1-4PC or to the heterodimerization of Gef1-4PC with Gef1-GFP, the majority of Gef1-4PC migrated as the processed form (Fig. 5 D, first lane). In the case that Gef1-4PC formed a heterodimer with Gef1-GFP-Ist2C, a significant portion of the Gef1-4PC was shifted into a slower migrating species of the same size as Gef1(Thr > Ala)–4PC (Fig. 5 D, second and fourth lanes). This means that the presence of one copy of Ist2C targets the dimer from the ER to the plasma membrane and prevents the wild-type subunit from being cleaved, which suggests that this transport occurs without passing through the Kex2p-positive TGN compartment. This is consistent with the previously observed bypassing of the medial- and trans-Golgi compartment.

The COOH-terminal domain of Ist2p mediates COPII-independent trafficking to the plasma membrane.

Because Ist2p trafficking occurs independently of SEC12, SEC23, SEC7, and SEC1-mediated transport (Jüschke et al., 2004), we asked if adding Ist2C to membrane proteins is sufficient to bypass the classical SEC pathway to the plasma membrane. To investigate this question, we chose Ste6p, the α-factor pheromone transporter and member of the ATP-binding cassette superfamily, because its membrane topology has been well established by gene fusion experiments (Geller et al., 1996). We created a fusion protein of yEmCitrine, an improved YFP variant, with the first two TM segments of Ste6p (YFP-St6[Thr1-M2]-Suc2) and the mature part (aa 20–352) of invertase (YFP-St6[Thr1-M2]-Suc2 in red) under the control of the GAL1 promoter. All cells were shifted for 120 min from a raffinose- into a 2% galactose-containing medium. (A) Cells were shifted to galactose at 25°C (a–d) or at 37°C (e–h). Different cells from one picture were combined (e–h, insets). (B) Cells were shifted to galactose at 25°C (a–d).

Figure 6. The COOH-terminal domain of Ist2p mediates COPII-independent trafficking to the plasma membrane. Fluorescence of sec12-4 cells coexpressing CFP-tagged Hxt1p [Hxt1-CFP in green], yEmCitrine-tagged fusion proteins containing the NH2-terminal fragment of Ste6p [NH2 terminus followed by two TM segments, aa 1–109; Ste6[Thr1-M2]) and Ist2C (YFP-St6[Thr1-M2]-Ist2C in red), or the mature part (aa 20–352) of invertase (YFP-St6[Thr1-M2]-Suc2 in red) under the control of the GAL1 promoter. All cells were shifted for 120 min from a raffinose- into a 2% galactose-containing medium. (A) Cells were shifted to galactose at 25°C (a–d) or at 37°C (e–h). Different cells from one picture were combined (e–h, insets). (B) Cells were shifted to galactose at 25°C (a–d).
growth to the plasma membrane (Fig. 6 A, a and b). Shifting the
growth temperature to 37°C caused the accumulation of Hxt1-CFP in
the ER (Fig. 6 A, f). More important, under these re-
strictive conditions, YFP-St6(TM1 + 2)-Ist2C still localized to
the plasma membrane (Fig. 6 A, e). The YFP-St6(TM1 + 2)-inver-
tase fusion, however, was localized at the ER even under permissive
conditions, whereas Hxt1-CFP was mostly at the plasma
membrane (with some additional staining in endocytic vesicles
and in the vacuole; Fig. 6 B, a and b). These data show that the
COOH-terminal domain of Ist2p, which is located at the cyto-
solic site, can direct an NH2-terminal fragment of Ste6p to the plasma membrane independently of the COPII-mediated for-
mation of vesicles.

SEC18-dependent vesicular fusion is not required for the sorting of Ist2p to the plasma membrane

The vesicle-mediated transport steps of the SEC pathway are
mediated by the SNARE-dependent fusion of donor and target
membranes (Rothman and Wieland, 1996). SNARE molecules,
which are located on opposite membranes, form stable four-
helix bundles and, thereby, induce membrane fusion. For mem-
brane fusion to occur continuously, all of these reactions dep-
and on the regeneration of separate SNARE molecules, a
process that is catalyzed by the activity of an AAA-ATPase. In
yeast, this enzyme is encoded by SEC18, the orthologue of
NSF in mammalian cells (Sollner et al., 1993). In the yeast
sec18-1 mutant protein, transport ceases almost immediately
after shifting the cells to the nonpermissive growth temperature
of 37°C (Graham and Emr, 1991). Therefore, this mutant
could be used to analyze whether trafficking on the Ist2 pathway
involves classic membrane fusion events. To investigate the traf-
ficking of newly synthesized Prm1-GFP-Ist2C, we induced its
expression in a sec18-1 MATa strain by adding prewarmed
media containing α-factor. These cells were incubated for
another 60 min at 37°C. Although the expression of Prm1-GFP-
Ist2C was low, some of the protein appeared in a peripheral
patchlike staining (Fig. 7 A), which suggests sorting to the
plasma membrane. To further test whether the newly synthe-
sized Prm1-GFP-Ist2C had reached the plasma membrane, we
investigated its accessibility for protease digestion from the
outside. We used the protease trypsin instead of pronase be-
cause sec18 mutants have a weak cell wall at nonpermissive
conditions that is even further weakened by the initiation of the
mating response. We also coexpressed Dpm1-CFP to test the
intactness of the plasma membrane after protease addition.
This ER membrane protein has one COOH-terminally located
TM segment and a large NH2-terminus, which is exposed to the
cytoplasm (Faulhammer et al., 2005) and would be digested in the
case of protease entering the cytosol. The trypsin resistance of
Dpm1-CFP (Fig. 7 B, lanes 6–9) indicates that the plasma
membrane has remained intact during the protease treatment.
The occurrence of a 28-kD breakdown product of Dpm1-CFP
after the mechanical disruption of the plasma membrane con-
ﬁrmed the trypsin sensitivity of Dpm1-CFP (Fig. 7 B, lane 1).
Adding increasing amounts of trypsin protease resulted in the
cleavage of the 180-kD band of Prm1-GFP-Ist2C into faster mi-
grating bands (Fig. 7 B, lane 9). Under the same conditions,
Prm1-CFP remained intact (Fig. 7 B, lanes 2–5). These results
indicate that Ist2C–tagged Prm1p was located at the plasma
membrane under conditions in which the forward transport of
Prm1-CFP was abolished. Altogether, these results show that
the transport of newly synthesized Prm1-GFP-Ist2C to the
plasma membrane does not require vesicular fusion events that
depend on Sec18p function.

Some Ist2p-tagged Prm1p enters the classical SEC pathway

To investigate whether the transport of Prm1-GFP-Ist2C from
the ER to the cis-Golgi occurs via the classical SEC pathway or
by a SEC18-independent route, we introduced constructs encod-
ing Prm1-GFP-Ist2C and Prm1-CFP in sec18-1 MATa and
MATα strains and induced expression by mixing cell cultures
under permissive and nonpermissive conditions. To achieve bet-
er induction of Prm1 proteins, we reduced the nonpermissive
temperature to 23°C. Immunoprecipitated Prm1-CFP and Prm1-
GFP-Ist2C were separated by SDS-PAGE and were probed with
an antibody recognizing GFP. Prm1-CFP from cells grown at
25°C was seen as a major 115-kD band with additional diffuse
bands that had reduced mobility (Fig. 8, lane 1). A comparison of
the ratio between the core glycosylated 115-kD, Prm1-CFP band (Fig. 4, lane 1) and the α-1,6- and α-1,3-mannose–reactive

Figure 7. The COOH-terminal domain of Ist2p mediates SEC18-independent trafficking to the plasma membrane. (A) Fluorescence of Prm1-GFP-Ist2C in
sec18-1 MATa cells, which were incubated for 60 min at 37°C with α-factor. (B) sec18-1 MATa cells were incubated for 60 min at 37°C with α-factor and
incubated with 0, 0.02, 0.2, or 2 mg/ml trypsin. Membranes corresponding
to 2 OD600 cells were separated on 7.5% SDS-PAGE and were analyzed by
immunodetection with GFP-specific antibodies. Cells shown in lanes 2–5
express Prm1-CFP. Cells shown in lanes 1 and 6–9 express Prm1-GFP-Ist2C
and coexpress CFP-tagged Dpm1p (Dpm1-CFP). Membranes of cells shown
in lane 1 were disrupted by vortexing with glass beads immediately after
the addition of trypsin. Dpm1-CFP* indicates the cleavage product of
Dpm1-CFP, seen in lane 1, and Prm1-GFP-Ist2C* indicates the cleavage
product of Prm1-GFP-Ist2C, seen in lane 9.
Figure 8. A portion of Ist2-tagged Prm1p passes through the cis-Golgi in a SEC18-dependent fashion. sec18-1 strains of opposite mating types, expressing either CFP-Prm1 (lanes 1, 2, 5, 6, 9, and 10) or Prm1-GFP-Ist2C (lanes 3, 4, 7, 8, 11, and 12), were shifted to the indicated temperatures and were combined for 60 min to induce the expression of Prm1 proteins. After immunoprecipitation with GFP-specific antibodies, proteins were separated by SDS-PAGE, transferred to membranes, and probed with antibodies recognizing either GFP, α-1,6-, or α-1,3-mannose.

bands (Fig. 8, lanes 5 and 9) suggests that the majority of Prm1-CFP is still present in its ER core glycosylated form 60 min after induction. A temperature shift to 33°C abolished the accumulation of the slower migrating bands. Probing with α-1,6- or α-1,3-mannose–specific antibodies confirmed that the trafficking of Prm1-CFP from the ER to the Golgi was blocked under these conditions (Fig. 8, lanes 6 and 10).

At permissive conditions, Prm1-GFP-Ist2C was seen as a 180-kD band (Fig. 8, lane 3). This band was immunoreactive with α-1,6-mannose–specific antibodies (Fig. 8, lane 7), but was only to a very low extent with α-1,3-mannose–specific antibodies (Fig. 8, lane 11). These findings are consistent with the idea that Prm1-GFP-Ist2C enters the cis-Golgi and is then recycled to the ER or is brought directly to the plasma membrane, but not to trans-Golgi compartments. At the nonpermissive temperature, the α-1,6-mannose–specific signal of Prm1-GFP-Ist2C was strongly reduced, suggesting that the transport of Prm1-GFP-Ist2C from the ER to the cis-Golgi requires the function of Sec18p.

Together, these results demonstrate that Ist2C-tagged Prm1p at the ER can enter two different routes: a SEC-independent transport, which delivers the protein to the plasma membrane, or the ER to Golgi step of the classical SEC-dependent transport route, which results in trafficking to the cis-Golgi.

Discussion

We have shown in this study that the COOH-terminal domain of Ist2p comprises all the necessary information for targeting Ist2p and other different integral membrane proteins to the plasma membrane. Positioned at the COOH terminus of integral membrane proteins and facing the cytosol, this domain confers the efficient targeting of membrane proteins to the plasma membrane in a SEC-independent manner, defining it as a novel sorting determinant. The only requirements for sorting to the plasma membrane are the presence of upstream hydrophobic domains, which mediate the integration of the polypeptide into the ER membrane, and a topology, which confers a cytosolic orientation of this domain. As demonstrated by the redirection to the plasma membrane of ER/Golgi-located Sac1p and TGN/prevacuolar-located Gef1p, the COOH terminus of Ist2p functions as a dominant sorting determinant. Ist2C-tagged proteins remain in a domain of the plasma membrane, colocalizing with the underlying cortical ER (Jüschke et al., 2004). Polypeptides that are substrates for degradation (e.g., the COOH-terminally truncated plasma membrane protein Ste2p, which is degraded at the ER [Huyer et al., 2004] and Prm1p, which is rapidly delivered to the vacuole) are redirected to these plasma membrane domains. The COOH terminus of Ist2p could locate Prm1p in a new microenvironment with a lipid composition that abolishes the internalization step (Munn et al., 1999; Heese-Peck et al., 2002). Alternatively, the disruption of an internalization signal in Prm1p could explain the stable plasma membrane localization. However, the signal for the transport of Prm1p to the vacuole is still unknown (Heiman and Walter, 2000).

Ist2C-tagged proteins pass through the ER on their way to the plasma membrane, as shown by the ER core glycosylation of Ist2C-tagged Prm1p. After insertion into the ER membrane, Ist2p can enter two different routes. One leads independently of the function of Sec12p and Sec18p to the plasma membrane, indicating that this pathway operates without the function of the COPII coat and SNARE-mediated vesicular fusion events. To our knowledge, this is the first example of such a pathway for an integral membrane protein. The selective secretion in the absence of the COPII components Sec13p and Sec24p has recently been observed for Hsp150p (Fatal et al., 2002, 2004). However, in contrast to the transport of Ist2p, the secretion of Hsp150p depends on Sec23p and Sec12p function (Fatal et al., 2002). The other route leads to SEC18-dependent transport Ist2p to the cis-Golgi. Although our immunoprecipitation assays are not quantitative, we suggest that only a small fraction of Ist2C-tagged Prm1p reporter protein reaches the cis-Golgi. The extreme COOH terminus of Ist2p, KKKL, comprises a strong KXXK ER-retention signal that could very well initiate the retrograde transport of Ist2p back to the ER. From there, the protein might get another chance to enter ER domains, which are capable of SEC-independent sorting to the plasma membrane. Whether this transport through the classical SEC pathway to the cis-Golgi and subsequent modification by the addition of sugar side chains are necessary for the function of proteins on this route is still unknown. The transport via the classical SEC pathway could simply represent the misincorporation of Ist2p into COPII-coated vesicles at the ER. The bypassing of late Golgi compartments has been shown by the lack of α-1,3-mannose modification of Prm1-GFP-Ist2C and is further supported by the observed lack of Kex2p cleavage in the Ist2C-tagged Gef1 reporter protein.

The simplest model that explains how the sorting of Ist2p could operate would be a mechanism that includes a local translation of IST2 mRNA at cortical ER sites, which are competent to initiate the SEC18-independent transport to the plasma membrane. Information within the mRNA, which en-
codes the COOH-terminal domain of Ist2p, could spatially restrict the translation and, thereby, direct the insertion of the nascent polypeptide chain into the cortical ER. As shown for many localized mRNAs, IST2 mRNA is present as an RNP particle, which is exported from the nucleus into the cytosol. According to the current model of RNA transport in yeast, the translation of the transported mRNAs is repressed by cis-acting, RNA localization elements, which have been predicted to form stem loops (Chartrand et al., 1999, 2002). In the right environment and at the cortical ER, the translational repression is released, and the newly synthesized protein is inserted into the cortical ER membrane. The localization of IST2 mRNA to the cortex of daughter cells by the She machinery is not necessary for its translation, indicating that Ist2p could be synthesized at the cortical ER in daughter and mother cells (Takizawa et al., 2000; Jüschke et al., 2004). Other components that are distinct from the She proteins, which are present in the IST2 mRNA particle, might regulate this local translation. The candidates are RNA-binding proteins (e.g., Khd1p or Scp160p), which repress the translation of ASHI mRNA (Irie et al., 2002). The postulated local translation of Ist2C-tagged proteins at the cortical ER does not lead to a spatial restriction of trafficking through the confined areas of the ER. Ist2C-tagged proteins have access to other proteins that are sorted via the classical SEC pathway, as shown by the function of Ist2C as a sorting determinant in trans and by the cis-Golgi modification of Ist2C-tagged Prm1p.

In contrast to a model based on a locally restricted translation of IST2 mRNA, the translation and insertion of the polypeptide could occur randomly at ER membranes. In this case, strong proteinacious sorting signals in Ist2C would confer an efficient, posttranslational recruitment of Ist2p into COPII-independent, ER exit sites. Because of the time required for the folding of GFP, we cannot exclude this possibility. The observed function of Ist2C as a sorting determinant in trans rules out a third mechanism; namely, that the protein would be extracted from the ER into the cytosol before insertion into the plasma membrane.

To explain the transport from the cortical ER to the neighboring plasma membrane, we suggest two possibilities: a local, transient fusion of part of the cortical ER with the plasma membrane or a fission and fusion mechanism between the cortical ER and the plasma membrane with a novel type of Ist2p containers. The fusion of parts of the ER with the plasma membrane has been suggested to play a role in the process of rapid membrane expansion in macrophages during the formation of phagocytic cups, when macrophages engulf large pathogens (Gagnon et al., 2002). It has been proposed that the exocyst complex provides a direct contact between parts of the ER and the plasma membrane (Lipschutz et al., 2003; Toikkanen et al., 2003). This is supported by findings in yeast, in which a direct contact between translocon and exocyst components has been reported (Toikkanen et al., 2003), and by contacts between these membranes in neurons during the trafficking of N-methyl-D-aspartate receptors in synapses (Sans et al., 2003). The coupling of Ca2+ signaling between the plasma membrane and the sarcoplasmic reticulum in muscle cells (for review see Blaustein et al., 2002) and the transport of lipids from the cortical ER to the yeast plasma membrane (Pichler et al., 2001) are further examples of a close contact between the domains of the ER and plasma membrane.

To summarize, our data suggest that trafficking of an integral membrane protein by a novel pathway through the cortical ER operates independently of Sec12p- and Sec18p-mediated vesicle formation and fusion. Furthermore, we have identified a novel dominant sorting determinant that redirects membrane proteins on this route to the plasma membrane and that could, in this respect, serve as a tool for investigating intracellular membrane proteins.

Materials and methods

Media and yeast strains

Media were prepared as described previously (Sherman, 2002). Yeast transformation was performed according to the method of Gietz and Woods (2002). The strains CJY3 (ist2::HIS3MX) and CJY70 (sec12-4) are isogenic derivatives of W303 (Thomas and Rothstein, 1989). MSY325 (sec11-1), MSY549 (gef1::KANMX4), and the wildtype MATa and MATα strains for the localization of Prm1p are isogenic derivatives of BY4741/2 (Brachmann et al., 1998). The strains JY39 and 40 were created by crossing the sec18-1 allele (provided by P. Novick, Yale University, New Haven, CT) into BY4741/2 expressing Prm1-GFP-Ist2C; and JY43 by crossing into BY4741/2 expressing Prm1-GFP-Ist2C- and JY43 by crossing into BY4741/2 a strain expressing Dpm1-CFP (Jüschke et al., 2004).

Construction of plasmids

The plasmid pCJ083 encoding GFP-Ist2C, which is under the control of its own promoter for integration into the LEU2 locus, and the plasmid pCJO70 encoding the GAL1-CPF, which is under the control of the GAL1 promoter for integration into the TRP1 locus, have been described previously (Jüschke et al., 2004). The plasmids pCJO97, pCJO99, pCJ100, and pCJ102 were derived from pCJ083 by replacing the full-length IST2 ORF with different versions: the sequence that encodes the COOH-termina l aas 455 of Ist2p (including the last two TM segments) and the COOH-termina l aas 358 of Ist2p, together with 995 nucleotides of the IST2 3'-untranslated region (UTR), were amplified and subcloned into the pCRTOPO vector (Invitrogen). Each fragment was introduced between the BamH1 and XhoI sites of pCJO83, resulting in pCJO97 and pCJO99, respectively. The plasmid pCJ100 was created by ligating full-length IST2 together with 995 nucleotides of the IST2 3'-UTR into the BamH1 and XhoI sites of pCJO83. The plasmid pCJO102 was made by introducing the sequence coding for the first 591 NH2-terminal amino acids of Ist2p together with 995 nucleotides of the IST2 3'-UTR into the BamH1 and XhoI sites of pCJO83. The plasmid pCJ113 encoded yEmCitrine (Griesbeck et al., 2001) under the control of the GAL1 promoter for integration into the URA3 locus and was constructed by subcloning the GAL1 promoter and the yEmCitrine fragment of pKT211 into the SacI and BamH1 sites of pRS506 (Sikorski and Hieter, 1989). The plasmids pCJ115 and pCJ119 were generated by introducing full-length IST2 or the sequence coding for the COOH-terminal aas 455, including the last two TM segments, into the BamH1 and XhoI sites of pCJ113. The plasmids pCJ116 and pCJ124 were created by introducing sequences, which encode either an NH2-terminal fragment of Ste6p (aa 1–109) fused to a COOH-Hemical fragment of Ist2p (aa 592–948) or an NH2-terminal fragment of Ste6p (aa 1–109) fused to the mature part of Sux2p (aa 20–532), into the BamH1 and XhoI sites of pCJ113. The plasmid pCJ137 encoding Prm1-GFP-Ist2C under the control of the endogenous PRM1 promoter for integration into the HIS3 locus was constructed by amplifying the ~387 to 1983 nucleotide region of PRM1, which introduced a SacI and an Xmal restriction site. GFP-Ist2C was amplified from pCJO99, introducing an Xmal and an XhoI site. Both fragments were immediately ligated into the SacI and XhoI sites of pRS506 (Sikorski and Hieter, 1989).

The construction of plasmids encoding the GEF1 gene, a GFP-tagged version, and a four time PC epitope-tagged version of the GEF1 gene (which are under the control of the MET25 promoter) and the mutagenesis of KR at position aa 136 and 137 of GEF1p to AA have been described previously (Wachter and Schwappach, 2003). The plasmid pMS470 was generated by exchanging GFP with RFP (tdimer[2][12];
Campbell et al., 2002. The plasmid pMS471 was created by exchanging a fragment encoding GFP with a NotI-Xhol fragment encoding GFP and the COOH-terminal domain of lst2p (aa 592–946).

Fluorescence microscopy

Yeast cells expressing GFP fusion proteins were analyzed as previously described (Giesbeck et al., 2004). The cells were mounted in growth medium at room temperature and were examined live using an inverted microscope (model DM RE2; Leica) with a 100×/1.40 oil immersion objective (model HCX PL APO CS; Leica). Images were acquired using a camera (model ORCA-ER CCD; Hamamatsu) controlled by the OpenLab software package (Improvision) and were processed with Adobe Photoshop.

Western blotting and susceptibility to external proteases

Expression of Prm1-GFP-Ist2C in sec18Δ cells (JY43) was induced at 37°C by the addition of 1/500 vol of 5 mg/ml α-factor (T6901; Sigma-Aldrich) in DMSO. Western blotting using GFP- (1:20,000 diluted; provided by A. Spang, Friedrich Miescher Lab der Max Planck Gesellschaft, Tübingen, Germany) or 250 ng/ml PC-specific antibodies (Roche) was performed as described previously, as was the susceptibility of plasma membrane proteins to external proteases (Jüschke et al., 2004). As an alternative to pronase, we used trypsin for sec18Δ-1 strains.

Immunoprecipitation

Strains of opposite mating types that expressed Prm1-CFP and Prm1-GFP-lst2Δ were grown at 25°C in YEPD media (1% wt/vol yeast extract, 2% wt/vol bacto-peptone, and 2% wt/vol dextrose) to 1 OD600, and an equal volume of media with a temperature of 25 or 40°C was added. The cells were incubated for an additional 5 min at 25 or 33°C before cells of opposite mating types were mixed to induce the expression of Prm1p. 100 OD600 cells were harvested, and the resulting cell pellet was disrupted by vortexing for 5 min with 1 vol of glass beads and 2 vol of low salt buffer (20 mM Heps-KOH, pH 7.6, 100 mM KCl, 5 mM Mg(OAC)2, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and complete protease inhibitor mix (Roche) was performed as described previously, as was the susceptibility of plasma membrane proteins to external proteases (Jüschke et al., 2004).

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125–137; discussion 137–141, 221–227.


