

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.
synthesis. However, the expression of Ist2p in mother cells does not require the function of the She machinery; therefore, She-mediated mRNA transport is not a general prerequisite for Ist2p synthesis.

Ist2p is predicted to have eight TM segments with NH₂ and COOH termini oriented to the cytosol. In this study, we have identified the segment encoding the COOH-terminal domain as the sorting determinant, which is able to direct Ist2p and other membrane proteins via a novel pathway through the cortical ER to the plasma membrane. We suggest that this pathway involves a spatial control of IST2 translation, a local insertion of the newly synthesized protein into specific domains of the ER membrane, and the transport of Ist2p by a novel (SEC independent) mechanism to the plasma membrane.

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-length GFP-Ist2 localized to the plasma membrane of mother and daughter cells (Fig. 1 a; Jüschke et al., 2004). The removal of the COOH-terminal cytosolic domain of Ist2p prevented its localization to the plasma membrane and caused the accumulation of the truncated protein in the perinuclear ER (Fig. 1 b). The expression of the COOH-terminal part of Ist2p, together with the two TM segments closest to the COOH terminus, resulted in a peripheral localization that was indistinguishable from full-length GFP-Ist2 (Fig. 1, compare a with c). When the COOH-terminal domain of Ist2p (designated as Ist2C) was expressed without any TM segment, it was soluble in the cytoplasm (Fig. 1 d), indicating that Ist2C itself cannot interact with membranes. Together, these results show that the COOH-terminal domain of Ist2p is required to target Ist2p to the plasma membrane.

The COOH-terminal domain of Ist2p redirects the ER/Golgi-located Sac1p to the plasma membrane

We went on and asked if Ist2C can direct other membrane-spanning proteins to the plasma membrane as well. For this purpose, we chose the yeast phosphatidylinositol phosphatase Sac1p. Sac1p is a membrane protein with the NH₂ and COOH termini facing the cytosol, which localizes to both ER and Golgi membranes (Faulhammer et al., 2005). It has previously been demonstrated that GFP-Sac1, as well as Sac1-GFP, is functional (Foti et al., 2001; Konrad et al., 2002). We found that in a sac1Δ background, GFP-Sac1 mainly localizes to ER membranes (Fig. 2 A, a), whereas GFP-Sac1-Ist2C was exclusively located at the cell periphery (Fig. 2 A, b). Because we were not able to distinguish the cortical ER from the plasma 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 extracellular loop (Fig. 2 A, arrow), which in wild-type GFP-Sac1 faces the ER or Golgi lumen and, therefore, is not accessible to external proteases. These results clearly demonstrate that when Ist2C is fused to Sac1p, the resulting chimera localizes to the plasma membrane. This suggests that Ist2C contains a dominant signal that is able to override the endogenous localization signals of Sac1p and redirect this membrane protein from the ER to the plasma membrane.

Trafficking of Ist2p through the ER

To gain more insight into the trafficking route of Ist2p, namely if it enters the ER, we fused Ist2C to the COOH terminus of the pheromone-regulated, multispansing membrane protein Prm1p. We chose this protein because it becomes heavily glycosylated during its transport to the plasma membrane, and, like Ist2p, its COOH terminus is oriented toward the cytosol (Heiman and Walter, 2000). Because the PRM1 gene is selectively expressed during mating, we induced its expression by mixing cells of opposite mating types that expressed either Prm1-CFP or Prm1-GFP-Ist2C under the endogenous promoter. It has been previously reported that GFP-tagged Prm1p was located at the perinuclear ER 40 min after induction (Heiman and Walter, 2000). From there, the protein is transported to the cell periphery, where it accumulates at sites in the plasma membrane that are involved in cell fusion. From these sites, Prm1p is rapidly internalized and finally ac-
cumulates in the vacuole (Heiman and Walter, 2000). 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-
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 (Cossen 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). This processing 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, Gef1KR>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 and Gef1KR>AA-GFP-Ist2C migrated as bands of identical size (Fig. 5 B, third and fourth lanes).
GFP-Ist2C. In a situation that led to the homodimerization of cleavage of a protein C (PC) epitope–tagged version of Gef1p trans as a dominant plasma membrane sorting signal for the Ist2C-tagged Gef1p. Diploids that coexpressed Gef1-RFP and same size as Gef1(KR4PC was shifted into a slower migrating species of the tion of Ist2C in trans by coexpressing a wild-type as well as an Gef1p targets the resulting chimera to the plasma membrane. GFP-Ist2C at the cell periphery (Fig. 5 C). These observations indicate that both subunits assemble and that Ist2C functions in the untagged version and, thereby, can act as a dominant sorting signal to the plasma membrane. Dimerization of Gef1p and Gef1-GFP-Ist2C should most likely occur at the ER. Diploid gef1Δ yeast cells that expressed only RFP-tagged Gef1p showed dotlike structures representing late or post-Golgi vesicles and the prevacuole (Fig. 5 A, a; Schwappach et al., 1998). As with Sac1p and Prm1p chimeric proteins, the fusion of Ist2C to Gef1-GFP caused a shift to a peripheral 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(KR4AA)-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 proteins independently of COPII-mediated vesicular transport 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-Ste6TM1) and expressed this protein under the control of the GALI promoter (Fig. 6). According to the topology of Ste6p, the YFP-Ste6TM1 chimera should result in a membrane protein with both NH2 and COOH termini facing the cytosol (Geller et al., 1996). The expression of YFP-Ste6TM1 without an additional moiety at its COOH terminus resulted in the accumulation in an ER-associated compartment (unpublished data). This compartment has recently been described as a quality control subcompartment of the ER (Huyer et al., 2004).

We fused either Ist2C or the mature part of invertase (Suc2p) to the COOH terminus of YFP-Ste6TM1 and coexpressed the chimeric proteins together with the GALI-driven, CFP-tagged hexose transporter (Hxt) 1 (Hxt1-CFP) in sec12-4 mutants, which are defective in the formation of COPII vesicles (Barlowe and Schekman, 1993). This strategy allowed us to switch on the expression of the tagged proteins by shifting from a raffinose- to a galactose-containing medium and to follow the fate of the newly synthesized proteins under restrictive conditions by concomitantly shifting the growth temperature from 25 to 37°C (Jüschke et al., 2004). Under nonrestrictive
growth conditions, YFP-Ste6\textsuperscript{TM1} + 2-Ist2\textsuperscript{C} and Hxt1-CFP localized to the plasma membrane (Fig. 6A, a and b). Shifting the growth temperature to 37°C caused the accumulation of Hxt1-CFP in the ER (Fig. 6A, f). More important, under these restrictive conditions, YFP-Ste6\textsuperscript{TM1} + 2-Ist2\textsuperscript{C} still localized to the plasma membrane (Fig. 6A, e). The YFP-Ste6\textsuperscript{TM1} + 2-invertase 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. 6B, a and b). These data show that the COOH-terminal domain of Ist2p, which is located at the cytosolic site, can direct an NH\textsubscript{2}-terminal fragment of Ste6p to the plasma membrane independently of the COPII-mediated formation 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 membrane fusion to occur continuously, all of these reactions depend 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 trafficking of newly synthesized Prm1-GFP-Ist2\textsuperscript{C}, we induced its expression in a sec18-1 MAT\textsuperscript{a} cells by adding prewarmed media containing α-factor. These cells were incubated for another 60 min at 37°C. Although the expression of Prm1-GFP-Ist2\textsuperscript{C} was low, some of the protein appeared in a peripheral patchlike staining (Fig. 7A), which suggests sorting to the plasma membrane. To further test whether the newly synthesized Prm1-GFP-Ist2\textsuperscript{C} had reached the plasma membrane, we investigated its accessibility for protease digestion from the outside. We used the protease trypsin instead of pronase because 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 helix bundles and, thereby, induce membrane fusion. For membrane fusion to occur continuously, all of these reactions depend on Sec18p function.

**Figure 7. The COOH-terminal domain of Ist2p mediates SEC18-independent trafficking to the plasma membrane.** (A) Fluorescence of Prm1-GFP-Ist2\textsuperscript{C} in sec18-1 MAT\textsuperscript{a} cells, which were incubated for 60 min at 37°C with α-factor. (B) sec18-1 MAT\textsuperscript{a} 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 OD\textsubscript{600} 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-Ist2\textsuperscript{C} 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\textsuperscript{*} indicates the cleavage product of Dpm1-CFP, seen in lane 1, and Prm1-GFP-Ist2\textsuperscript{C}\textsuperscript{*} indicates the cleavage product of Prm1-GFP-Ist2\textsuperscript{C}, seen in lane 9.

**Some Ist2-tagged Prm1p enters the classical SEC pathway**

To investigate whether the transport of Prm1-GFP-Ist2\textsuperscript{C} from the ER to the cis-Golgi occurs via the classical SEC pathway or by a SEC18-independent route, we introduced constructs encoding Prm1-GFP-Ist2\textsuperscript{C} and Prm1-CFP in sec18-1 MAT\textsuperscript{a} and MAT\textsuperscript{a} strains and induced expression by mixing cell cultures under permissive and nonpermissive conditions. To achieve better induction of Prm1 proteins, we reduced the nonpermissive temperature to 33°C. Immunoprecipitated Prm1-CFP and Prm1-GFP-Ist2\textsuperscript{C} 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
brane, or the ER to Golgi step of the classical transport route, which results in trafficking to the cis-Golgi.

Prm1p at the ER can enter two different routes: a function of Sec18p. Together, these results demonstrate that Ist2C-tagged Prm1 reporter protein reaches the cis-Golgi. Although our immunoprecipitation assays are not quantitative, we suggest that only a small fraction of Ist2C-tagged Prm1 reporter protein reaches the cis-Golgi. The extreme COOH terminus of Ist2p, KKKL, comprises a strong KKXX 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 Scl160p), which repress the translation of ASH1 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 proteinaceous 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 Rothe, 1989). MSY325 (sec11-1, sec13-1, sec34-1, sec101-1, sec10-1) and the wildtype MATa and MATa strains for the localization of Prm1p are isogenic derivatives of BY4741/2 (Brauchmann 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-CFP; JY41 and 42 by crossing into BY4741/2 expressing Prm1-GFP-Ist2C; and JY43 by crossing into JY41 into a strain expressing Dpm1-CFP (Jüschke et al., 2004).

Construction of plasmids

The plasmid pCJ083 encoding GFP-Ist2, which is under the control of its own promoter for integration into the LEU2 locus, and the plasmid pCJ070 encoding Prm1-CFP, 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 pCJ097, pCJ099, pCJ100, and pCJ102 were derived from pCJ083 by replacing the full-length IST2 ORF with different versions: the sequence that encodes the COOH-termin al 455 of Ist2p (including the last two TM segments) and the COOH-termin al 358 of Ist2p, together with 995 nucleotides of the IST2 3’-untranslated region (UTR), were amplified and subcloned into the pCR8-TOPO vector (Invitrogen). Each fragment was introduced between the BamHI and Xhol sites of pCJ083, resulting in pCJ097 and pCJ099, respectively. The plasmid pCJ100 was created by ligating full-length IST2 together with 995 nucleotides of the IST2 3’-UTR into the BamHI and Xhol sites of pCJ083. The plasmid pCJ102 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 BamHI and Xhol sites of pCJ083. 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 BamHI sites of pRS306 (Sikorski and Hieter, 1989). The plasmids pCJ115 and pCJ119 were generated by introducing full-length IST2 or the sequence coding for the COOH-termin al 455, including the last two TM segments, into the BamHI and Xhol 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-termin al fragment of Ist2p (aa 592–946) or an NH2-terminal fragment of Ste6p (aa 1–109) fused to the mature part of Suc2p (aa 20–532), into the BamHI and Xhol 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 pCJ099, introducing an Xmal and an Xhol site. Both fragments were immediately ligated into the SacI and Xhol sites of pRS303 (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 Gei1p to AA have been described previously (Wachter and Schwappach, 2003). The plasmid pMS470 was generated by exchanging GFP with RTF (tdimer[2][12];
Fluorescence microscopy

Yeast cells expressing GFP fusion proteins were analyzed as previously described by Jüschke 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-lst2 in sec18-1 cells (YJ43) 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 Laboratorium 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 sec18-1 strains.

Immunoprecipitation

Strains of opposite mating types that expressed Prm1-CFP and Prm1-GFP-lst2 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 medium 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. GFP-lst2 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 HEPES-KOH, pH 7.6, 100 mM KCl, 0.5 mM MgOAC, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and complete protease inhibitor mix) according to the manufacturer's instructions (Roche). The lysate was then cleared of unbroken cells by centrifugation (12100 g at 4°C for 2 min) and was subjected to centrifugation at 25,000 g at 4°C for 20 min. Membranes were resuspended in 1% (vol/vol) Triton X-100, 400 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM K2HPO4, and complete protease inhibitor mix (Roche) and were subjected to centrifugation at 25,000 g at 4°C for 20 min. The supernatant was incubated for 90 min at 4°C before cells of opposite mating types that expressed Prm1-CFP and Prm1-GFP-lst2 were separated by 6% SDS-PAGE.

References

Antony, B., and R. Schekman. 2001. ER export: public transportation by the COPII coach. Curr. Opin. Cell Biol. 13:438–443.
Barlowe, C., and R. Schekman. 1993. SEC12 encodes a guanine-nucleotide-exchange factor essential for transport vesicle budding from the ER. Nature. 365:347–349.
Blau, E.M., V.P. Golovina, H. Song, J. Choate, L. Lencsosova, S.W. Robinson, and W.G. Wier. 2002. Organization of Ca2+ stores in vascular smooth muscle: functional implications. Novartis Found. Symp. 246:
125–137; discussion 137–141, 221–227.
Brachmann, C.B., A. Davies, G.J. Cost, E. Caputo, J. Li, P. Hieter, and J.D. Boeke. 1998. Designer deletion strains derived from Saccharomyces cerevisiae S288C: A useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast. 14:115–132.
Brigance, W.T., C. Barlowe, and T.R. Graham. 2000. Organization of the yeast Golgi complex at post four functionally distinct compartments. Mol. Biol. Cell. 11:171–182.
Campbell, R.E., O. Tour, A.E. Palmer, P.A. Steinbach, G.S. Baird, D.A. Zacharias, and R.Y. Tsien. 2002. A monomeric red fluorescent protein. Proc. Natl. Acad. Sci. USA. 99:7877–7882.
Chartrand, P., X.H. Meng, R.H. Singer, and R.M. Long. 1999. Structural elements required for the localization of ASH1 mRNA and of a green fluorescent protein reporter particle in vivo. Curr. Biol. 9:333–336.
Chartrand, P., X.H. Meng, S. Huttelmaier, D. Donato, and R.H. Singer. 2002. Asymmetric sorting of ash1p in yeast results from inhibition of translation by localization elements in the mRNA. Mol. Cell. 10:139–1340.
Coxson, P., and F. Letourneau. 1994. Coatomer interaction with di-lysine endoplasmic reticulum retention motifs. Science. 263:1629–1631.
Dutzler, R., E.B. Campbell, M. Cadene, B.T. Chait, and R. MacKinnon. 2002. X-ray structure of a CIC chloride channel at 3.0 Å reveals the molecular basis of anion selectivity. Nature. 415:287–294.
Fatai, N., T. Suntio, and M. Makarow. 2002. Selective protein exit from yeast endoplasmic reticulum in absence of functional COPII coat component Sec13p. Mol. Biol. Cell. 13:4130–4140.
Fatai, N., L. Karhinon, E. Ylitalo, and M. Makarow. 2004. Active and specific recruitment of a soluble cargo protein for endoplasmic reticulum exit in the absence of functional COPII component Sec24p. J. Cell Sci. 117:1665–1673.
Faulhammer, F., G. Konrad, B. Brankatsch, S. Tahirovic, A. Knodler, and P. Mayinger. 2005. Cell growth-dependent coordination of lipid signaling and glycosylation is mediated by interactions between Sec1p and Dpm1p. J. Cell Biol. 168:185–191.
Foti, M., A. Audhya, and S.D. Emr. 2001. Sac1 lipid phosphatase and Stt4 phosphatidylinositol 4-kinase regulate a pool of phosphatidylinositol 4-phosphatase that functions in the control of the actin cytoskeleton and vacuole morphology. Mol. Biol. Cell. 12:2396–2411.
Gagnon, E., S. Duclos, C. Rondeau, E. Chevet, P.H. Cameron, O. Steele-Mortimer, J. Puiement, J.H. Bergeron, and M. Desjardins. 2002. Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. Cell. 110:119–131.
Geller, D., D. Taglicht, R. Edgar, A. Tam, O. Pines, S. Michaelis, and E. Bibi. 1996. Comparative topology studies in Saccharomyces cerevisiae and in Escherichia coli. The N-terminal half of the yeast ABC protein Ste9. J. Biol. Chem. 271:13746–13753.
Gietz, R.D., and R.A. Woods. 2002. Transformation of yeast by lithium acetate/polyethylene glycol method. Methods Enzymol. 350:87–96.
Gonsalvez, G.B., C.R. Urbiniati, and R.M. Long. 2005. RNA localization in yeast: moving towards a mechanism. Biol. Cell. 97:75–86.
Graham, T.R., and S.D. Emr. 1991. Compartmental organization of Golgi-specific protein modification and vacuolar protein sorting events defined in a yeast sec18 (NSF) mutant. J. Cell Biol. 114:207–218.
Griesbeck, O., G.S. Baird, R.E. Campbell, D.A. Zacharias, and R.Y. Tsien. 2001. Reducing the environmental sensitivity of yellow fluorescent protein. Mechanism and applications. J. Biol. Chem. 276:29188–29194.
Heiman, M.G., and P. Walter. 2000. Prm1p, a pheromone-regulated multispanning membrane protein, facilitates plasma membrane fusion during yeast mating. J. Cell Biol. 151:719–730.
Huyer, G.L., R.L. Wright, and S. Michaelis. 2004. A striking quality control subcomponent of the endoplasmic reticulum-associating complex. Mol. Biol. Cell. 15:908–921.
Irie, K., T. Tadauchi, P.A. Takizawa, R.D. Vale, K. Matsumoto, and I. Herkowitz. 2002. The Khd1 protein, which has three KH RNA-binding motifs, is required for proper localization of ASH1 mRNA in yeast. EMBO J. 21:1158–1167.
Jüschke, C., F. Ferring, R.P. Jansen, and M. Seedorf. 2004. A novel transport factor, sec18-independent sorting of ist2p.
Lipschutz, J.H., V.R. Lingappa, and K.E. Mostov. 2003. The exocyst affects protein synthesis by acting on the translocation machinery of the endoplasmic reticulum. J. Biol. Chem. 278:20954–20960.

Middleton, R.E., D.J. Pheasant, and C. Miller. 1996. Homodimeric architecture of a CIC-type chloride ion channel. Nature. 383:337–340.

Muniz, M., P. Morsomme, and H. Riezman. 2001. Protein sorting upon exit from the endoplasmic reticulum. Cell. 104:313–320.

Munn, A.L., A. Heese-Peck, B.I. Stevenson, H. Pichler, and H. Riezman. 1999. Specific sterols required for the internalization step of endocytosis in yeast. Mol. Biol. Cell. 10:3943–3957.

Munro, S. 2004. Organelle identity and the organization of membrane traffic. Nat. Cell Biol. 6:469–472.

Nakayama, K., T. Nagasu, Y. Shimma, J. Kuromitsu, and Y. Jigami. 1992. OCH1 encodes a novel membrane bound mannosyltransferase: outer chain elongation of asparagine-linked oligosaccharides. EMBO J. 11:2511–2519.

Pichler, H., B. Gaigg, C. Hrastnik, G. Achleitner, S.D. Kohlwein, G. Zellnig, A. Perkold, and G. Daum. 2001. A subfraction of the yeast endoplasmic reticulum associates with the plasma membrane and has a high capacity to synthesize lipids. Eur. J. Biochem. 268:2351–2361.

Raschke, W.C., K.A. Kern, C. Antalis, and C.E. Ballou. 1973. Genetic control of yeast mannan structure. Isolation and characterization of mannan mutants. J. Biol. Chem. 248:4660–4666.

Rayner, J.C., and H.R. Pelham. 1997. Transmembrane domain-dependent sorting of proteins to the ER and plasma membrane in yeast. EMBO J. 16:1832–1841.

Rossanese, O.W., J. Soderholm, B.J. Bevis, I.B. Sears, J. O’Connor, E.K. Williamson, and B.S. Glick. 1999. Golgi structure correlates with transi-}

Takizawa, P.A., J.L. DeRisi, J.E. Wilhelm, and R.D. Vale. 2000. Plasma membrane compartmentalization in yeast by messenger RNA transport and a septin diffusion barrier. Science. 290:341–344.

Sherman, F. 2002. Getting started with yeast. Methods Enzymol. 350:3–41.

Sikorski, R.S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics. 122:19–27.

Sollner, T., M.K. Bennett, S.W. Whiteheart, R.H. Scheller, and J.E. Rothman. 1993. A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. Cell. 75:409–418.

Takizawa, P.A., J.L. DeRisi, J.E. Wilhelm, and R.D. Vale. 2000. Plasma membrane compartmentalization in yeast by messenger RNA transport and a septin diffusion barrier. Science. 290:341–344.

Thomas, B.J., and R. Rothstein. 1989. Elevated recombination rates in transcriptionally active DNA. Cell. 56:619–630.

Toikkanen, J.H., K.J. Miller, H. Soderlund, J. Jantti, and S. Keranen. 2003. The beta subunit of the Sec61p endoplasmic reticulum translocon interacts with the exocyst complex in Saccharomyces cerevisiae. J. Biol. Chem. 278:20946–20953.

Wachter, A., and B. Schwappach. 2005. The yeast CLC chloride channel is proteolytically processed by the furin-like protease Kex2p in the first extracellular loop. FEBS Lett. 579:1149–1153.

Watanabe, R., and H. Riezman. 2004. Differential ER exit in yeast and mammalian cells. Curr. Opin. Cell Biol. 16:350–355.