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 *she*Δ 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, *she*Δ 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

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Abbreviations used in this paper: COP, coat protein complex; Hxt, hexose transporter; PC, protein C; *sec*, secretory; TM, transmembrane; UTR, untranslated region.

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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 NH_2 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 NH_2- and COOH-terminally truncated versions of Ist2p. All constructs were tagged with GFP at the NH_2 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 $Ist2^C$) was expressed without any TM segment, it was soluble in the cytoplasm (Fig. 1 d), indicating that $Ist2^C$ 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.

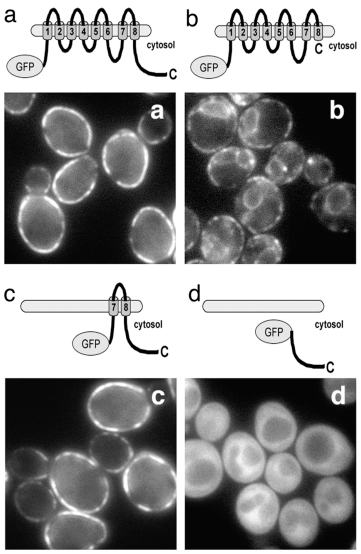

Figure 1. **Trafficking of Ist2p to the plasma membrane requires its COOH-terminal cytoplasmic domain.** Fluorescence of *ist2Δ* yeast cells, expressing different EGFP-tagged Ist2p variants under the control of their endogenous UTRs. (a) Full-length Ist2p (aa 946). (b) A COOH-terminal truncated variant, lacking the cytoplasmic domain (aa 592–946 are deleted). (c) An NH_2-terminal truncated variant lacking the NH_2 terminus, and TM segments one to six (aa 1–490 are deleted). (d) An NH_2-terminal truncated variant lacking the NH_2 terminus, and TM segments one to eight (aa 1–588 are deleted).

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

We went on and asked if $Ist2^C$ 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 NH_2 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-$Ist2^C$ 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-$Ist2^C$ 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 $Ist2^C$ is fused to Sac1p, the resulting chimera localizes to the plasma membrane. This suggests that $Ist2^C$ 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 $Ist2^C$ to the COOH terminus of the pheromone-regulated, multispanning 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 orientated 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-$Ist2^C$ 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-

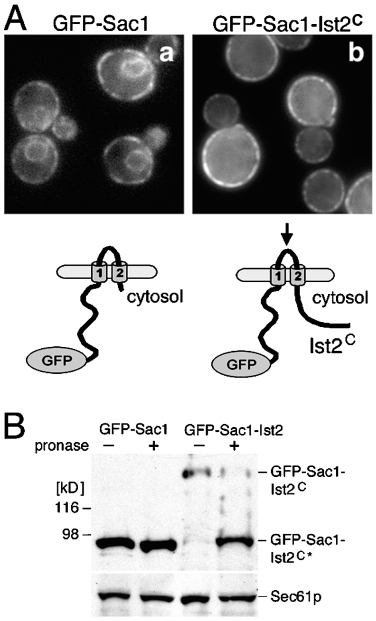

Figure 2. **The COOH-terminal domain of Ist2p redirects the ER- and Golgi-located protein Sac1p to the plasma membrane.** (A) Fluorescence of *sac1Δ* cells expressing EGFP-tagged Sac1p (a, GFP-Sac1) or EGFP-tagged Sac1p with the COOH-terminal domain of Ist2p (aa 589–946) at its COOH terminus (b, GFP-Sac1-Ist2^C). Both proteins were expressed under the control of the *SAC1* promoter and the *SAC1* and *IST2* 3′UTRs, respectively. The arrow approximates the position of the cleavage within the extracellular loop of Sac1p. (B) Intact yeast cells expressing GFP-Sac1 or GFP-Sac1-Ist2^C were treated with pronase, lysed, separated on SDS-PAGE, and analyzed by immunodetection with GFP- or Sec61p-specific antibodies. GFP-Sac1-Ist2^C* marks a cleavage product of GFP-Sac1-Ist2^C.

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-Ist2^C, 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 Ist2^C as a specific plasma membrane sorting signal. The presence of the Ist2^C 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-Ist2^C passes through the ER, we compared the apparent molecular mass of Prm1-CFP and Prm1-GFP-Ist2^C 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-Ist2^C 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-Ist2^C 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 Ist2^C 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-

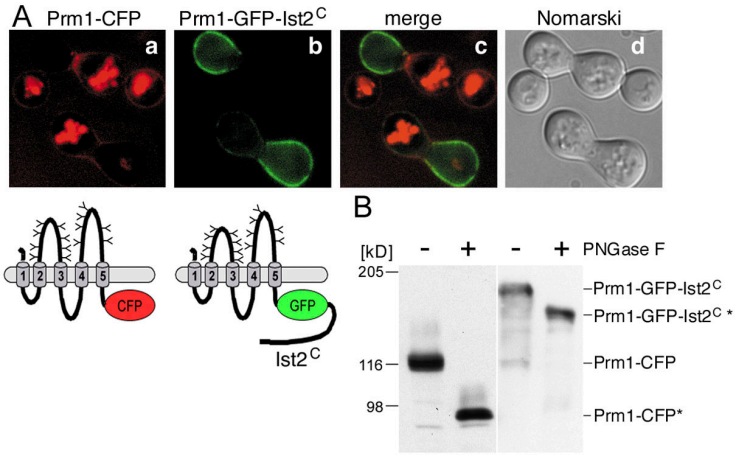

Figure 3. **The trafficking of Ist2p is initiated by insertion into ER membranes.** Wild-type cells of opposite mating types were mixed and incubated at 25°C for 90 min. (A) Schematic topology and the predicted position of N-linked glycosylation sites of Prm1p (Heiman and Walter, 2000) as well as fluorescence of mating cells expressing either CFP-tagged Prm1p (Prm1-CFP in red) or GFP-tagged Prm1-Ist2^C (Prm1-GFP-Ist2^C in green). (B) Membranes corresponding to 1 OD_{600} cells, expressing Prm1-CFP or Prm1-GFP-Ist2^C, 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-Ist2^C* mark the deglycosylated products of Prm1-CFP and Prm1-GFP-Ist2^C, respectively.

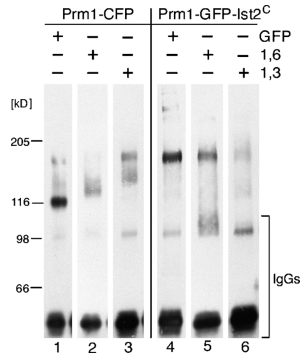

Figure 4. **Ist2^C-tagged Prm1p passes the cis- but not the trans-Golgi.** MAT**a** and MATα wild-type strains expressing Prm1-CFP or Prm1-GFP-Ist2^C were combined for 75 min. Prm1-CFP (lanes 1–3) and Prm1-GFP-Ist2^C (lanes 4–6) from 100 OD_{600} cells were immunoprecipitated with GFP-specific antibodies, separated on 6% SDS-PAGE, transferred to membranes, and probed with antibodies recognizing either GFP (lanes 1 and 4), α-1,6- (lanes 2 and 5), or α-1,3- (lanes 3 and 6) mannose residues. The samples were denatured at 50°C, which led to the accumulation of incompletely reduced antibody chains (designated as IgGs).

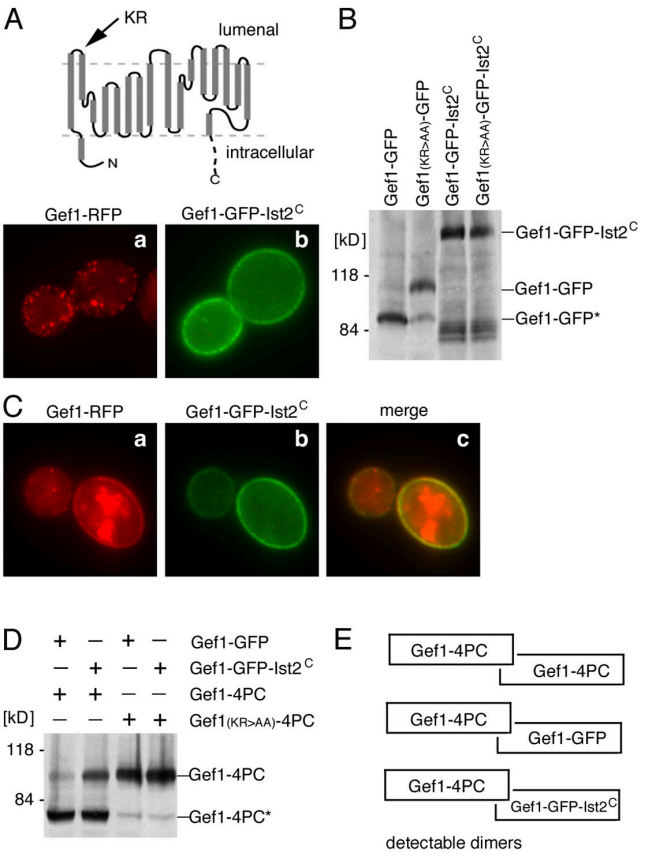

Figure 5. **Ist2^C-tagged Gef1p chloride channel redirects native Gef1p in trans to the plasma membrane, circumventing passage through the TGN.** All experiments were performed with diploid yeast cells transformed with plasmids that contain variants of *GEF1* under the control of the *MET25* promoter and the *CYC1* 3′UTR. Cells were grown at 30°C in synthetic dextrose minimal medium without methionine for induction. (A) Topology of Gef1p as deduced from the crystal structure of a bacterial homologue (Dutzler et al., 2002). KR indicates a Kex2p protease recognition site that is located at amino acid residues 136 and 137, which are positioned between the first and second TM segments. The bottom panel shows the fluorescence of cells that either express RFP-tagged Gef1p (Gef1-RFP in red) or a GFP-tagged fusion of Gef1p and Ist2^C (Gef1-GFP-Ist2^C in green). (B) Total extracts of cells expressing GFP-tagged versions of Gef1p (Gef1-GFP), Gef1p containing a KR to AA substitution fused to GFP (Gef1$_{(KR>AA)}$-GFP), a fusion of Gef1p and Ist2^C (Gef1-GFP-Ist2^C), or a fusion of Gef1p$_{(KR>AA)}$ and Ist2^C (Gef1$_{(KR>AA)}$-GFP-Ist2^C) were separated on SDS-PAGE and analyzed by immunodetection with GFP-specific antibodies. (C) Fluorescence of a cell coexpressing Gef1-RFP and Gef1-GFP-Ist2^C. (D) Total extract of cells that coexpressed Gef1p, which is tagged with four PC epitopes (Gef1-4PC) and Gef1-GFP or Gef1-GFP-Ist2^C (first and second lanes), and cells that coexpressed Gef1p$_{(KR>AA)}$ tagged with four PC epitopes (Gef1$_{(KR>AA)}$-4PC) and Gef1-GFP or Gef1-GFP-Ist2^C (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.

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-Ist2^C and Prm1-CFP in MAT**a** 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-Ist2^C 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-Ist2^C 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-Ist2^C 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-Ist2^C was not transported to the trans-Golgi. This retention of Prm1-GFP-Ist2^C in the early Golgi could be explained by the retrograde transport of Ist2p. The extreme COOH terminus of Ist2p, KKKL, contains a strong KKXX ER-retrieval signal (Cosson 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-Ist2^C 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 Ist2^C-tagged Gef1-GFP. Gef1-GFP-Ist2^C and Gef1$_{(KR>AA)}$-GFP-Ist2^C migrated as bands of identical size (Fig. 5 B, third and fourth

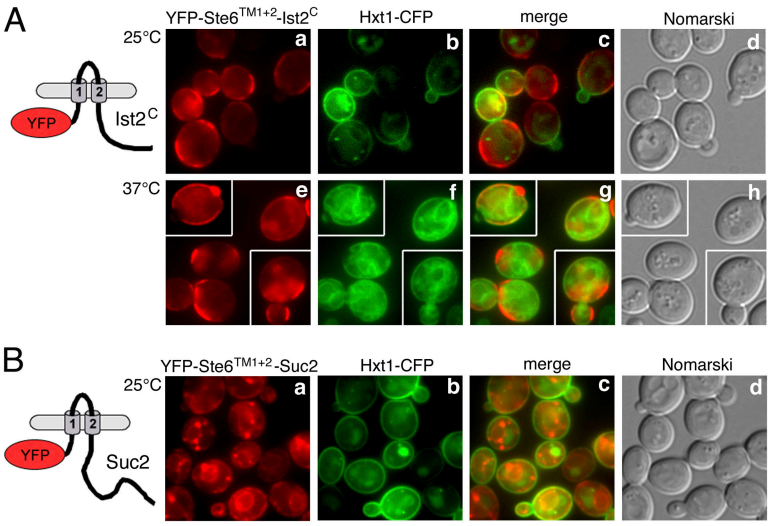

Figure 6. **The COOH-terminal domain of Ist2p mediates COPII-independent trafficking to the plasma membrane.** Fluorescence of *sec12-4* cells coexpressing CFP-tagged Hxt1p (Hxt1-CFP in green), yEmCitrine-tagged fusion proteins containing the NH_2-terminal fragment of Ste6p (NH_2 terminus followed by two TM segments, aa 1–109; Ste6^{TM1+2}) and Ist2^C (YFP-Ste6^{TM1+2}-Ist2^C in red), or the mature part (aa 20–532) of invertase (YFP-Ste6^{TM1+2}-Suc 2 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).

lanes). These data indicate that the COOH-terminal domain of Ist2p prevents the transport of fusion proteins through the TGN and is consistent with the bypassing of the medial- and trans-Golgi, as shown in Fig. 4 (lanes 4–6).

An additional characteristic of Gef1p is that it functions as a dimer (Middleton et al., 1996; Dutzler et al., 2002). Therefore, we could tag one subunit with the COOH-terminal domain of Ist2p and determine whether this subunit heterodimerizes with the untagged version and, thereby, can act as a dominant sorting signal to the plasma membrane. Dimerization of Gef1p and Gef1-GFP-Ist2^C 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 Ist2^C to Gef1-GFP caused a shift to a peripheral localization (Fig. 5 A, b), indicating that the addition of Ist2^C to Gef1p targets the resulting chimera to the plasma membrane.

The dimerization allowed us to study the sorting function of Ist2^C in trans by coexpressing a wild-type as well as an Ist2^C-tagged Gef1p. Diploids that coexpressed Gef1-RFP and Gef1-GFP-Ist2^C showed a diminished dotlike, intracellular localization of Gef1-RFP, which partially overlapped with Gef1-GFP-Ist2^C at the cell periphery (Fig. 5 C). These observations indicate that both subunits assemble and that Ist2^C 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-Ist2^C. 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-Ist2^C, a significant portion of the Gef1-4PC was shifted into a slower migrating species of the same size as Gef1$_{(KR>AA)}$-4PC (Fig. 5 D, second and fourth lanes). This means that the presence of one copy of Ist2^C 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 directs 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 Ist2^C to membrane proteins is sufficient to bypass the classical SEC pathway to the plasma membrane. To investigate this question, we chose Ste6p, the **a**-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-Ste6^{TM1+2}) and expressed this protein under the control of the *GAL1* promoter (Fig. 6). According to the topology of Ste6p, the YFP-Ste6^{TM1+2} chimera should result in a membrane protein with both NH_2 and COOH termini facing the cytosol (Geller et al., 1996). The expression of YFP-Ste6^{TM1+2} 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 Ist2^C or the mature part of invertase (Suc2p) to the COOH terminus of YFP-Ste6^{TM1+2} and coexpressed the chimeric proteins together with the *GAL1*-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^{TM1+2}-Ist2^{C} and Hxt1-CFP localized 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 restrictive conditions, YFP-Ste6^{TM1+2}-Ist2^{C} still localized to the plasma membrane (Fig. 6 A, e). The YFP-Ste6^{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. 6 B, a and b). These data show that the COOH-terminal domain of Ist2p, which is located at the cytosolic site, can direct an NH_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^{C}, we induced its expression in a *sec18-1* MAT**a** 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-Ist2^{C} 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 synthesized Prm1-GFP-Ist2^{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 TM segment and a large NH_2 terminus, which is exposed to the cytosol (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 confirmed 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-Ist2^{C} into faster migrating bands (Fig. 7 B, lane 9). Under the same conditions, Prm1-CFP remained intact (Fig. 7 B, lanes 2–5). These results indicate that Ist2^{C}-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-Ist2^{C} to the plasma membrane does not require vesicular fusion events that depend on Sec18p function.

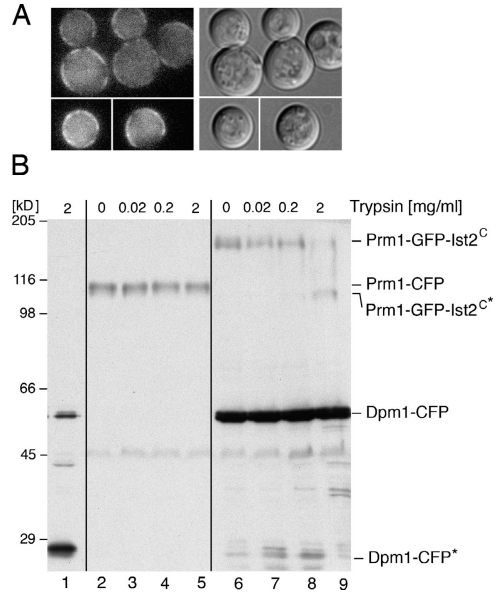

Figure 7. **The COOH-terminal domain of Ist2p mediates *SEC18*-independent trafficking to the plasma membrane.** (A) Fluorescence of Prm1-GFP-Ist2^{C} in *sec18-1* MAT**a** cells, which were incubated for 60 min at 37°C with α-factor. (B) *sec18-1* MAT**a** cells were incubated for 60 min at 37°C with α-factor and incubated with 0, 0.02, 0.2, or 2 mg/ml trypsin. Membranes corresponding to 2 OD_{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^{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* indicates the cleavage product of Dpm1-CFP, seen in lane 1, and Prm1-GFP-Ist2^{C}* indicates the cleavage product of Prm1-GFP-Ist2^{C}, seen in lane 9.

Some Ist2-tagged Prm1p enters the classical SEC pathway

To investigate whether the transport of Prm1-GFP-Ist2^{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^{C} and Prm1-CFP in *sec18-1* MAT**a** and MATα 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^{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

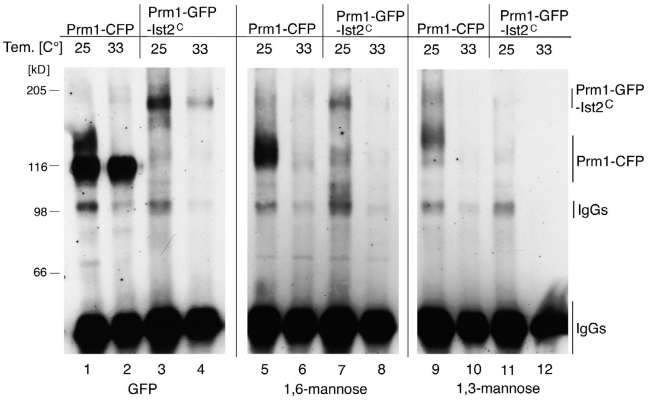

Figure 8. **A portion of Ist2-tagged Prm1p passes through the cis-Golgi in a *SEC*18-dependent fashion.** *sec18-1* strains of opposite mating types, expressing either CFP-Prm1 (lanes 1, 2, 5, 6, 9, and 10) or Prm1-GFP-Ist2^C (lanes 3, 4, 7, 8, 11, and 12), were shifted to the indicated temperatures 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-Ist2^C 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-Ist2^C 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-Ist2^C was strongly reduced, suggesting that the transport of Prm1-GFP-Ist2^C from the ER to the cis-Golgi requires the function of Sec18p.

Together, these results demonstrate that Ist2^C-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. Ist2^C-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).

Ist2^C-tagged proteins pass through the ER on their way to the plasma membrane, as shown by the ER core glycosylation of Ist2^C-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 Ist2^C-tagged Prm1 reporter protein reaches the cis-Golgi. The extreme COOH terminus of Ist2p, KKKL, comprises a strong KKXX ER-retrieval 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-IST2^C and is further supported by the observed lack of Kex2p cleavage in the Ist2^C-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 *ASH1* mRNA (Irie et al., 2002). The postulated local translation of $Ist2^C$-tagged proteins at the cortical ER does not lead to a spatial restriction of trafficking through the confined areas of the ER. $Ist2^C$-tagged proteins have access to other proteins that are sorted via the classical SEC pathway, as shown by the function of $Ist2^C$ as a sorting determinant in trans and by the cis-Golgi modification of $Ist2^C$-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 $Ist2^C$ 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 $Ist2^C$ 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 Ca^{2+} 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 (*ist2Δ::HIS3MX)* and CJY70 (*sec12-4)* are isogenic derivates of W303 (Thomas and Rothstein, 1989). MSY325 (*sac1Δ::URA3*), MSY349 (*gef1::KANMX4),* and the wild-type MAT**a** and MATα strains for the localization of Prm1p are isogenic derivates 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-CFP; JY41 and 42 by crossing into BY4741/2 expressing Prm1-GFP-$Ist2^C$; and JY43 by crossing 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 Hxt1-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-terminal aa 455 of Ist2p (including the last two TM segments) and the COOH-terminal aa 358 of Ist2p, together with 995 nucleotides of the *IST2* 3′-untranslated region (UTR), were amplified and subcloned into the pCRII-TOPO vector (Invitrogen). Each fragment was introduced between the BamHI and XhoI 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 XhoI sites of pCJ083. The plasmid pCJ102 was made by introducing the sequence coding for the first 591 NH_2-terminal amino acids of Ist2p together with 995 nucleotides of the *IST2* 3′-UTR into the BamHI and XhoI 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-terminal aa 455, including the two last TM segments, into the BamHI and XhoI sites of pCJ113. The plasmids pCJ116 and pCJ124 were created by introducing sequences, which encode either an NH_2-terminal fragment of Ste6p (aa 1–109) fused to a COOH-terminal fragment of Ist2p (aa 592–946) or an NH_2-terminal fragment of Ste6p (aa 1–109) fused to the mature part of Suc2p (aa 20–532), into the BamHI and XhoI sites of pCJ113.

The plasmid pCJ137 encoding Prm1-GFP-$Ist2^C$ 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 XmaI restriction site. GFP-$Ist2^C$ was amplified from pCJ099, introducing an XmaI and an XhoI site. Both fragments were immediately ligated into the SacI and XhoI 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 Gef1p to AA have been described previously (Wachter and Schwappach, 2005). The plasmid pMS470 was generated by exchanging GFP with RFP (tdimer2[12];

Campbell et al., 2002). The plasmid pMS471 was created by exchanging a fragment encoding GFP with a NotI–XhoI fragment encoding GFP and the COOH-terminal domain of Ist2p (aa 592–946).

Fluorescence microscopy

Yeast cells expressing GFP fusion proteins were analyzed as previously described (Jüschke et al., 2004). The cells were mounted in growth medium at room temperature and were examined live using an inverted microscope (model DM IRE2; Leica) with a 100×/1.4-0.7 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-Ist2^{C} in *sec18-1* 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 D. Görlich, University of Heidelberg, Heidelberg, Germany), Sec61p- (1:10,000 diluted), α-1,6-, and α-1,3-mannose (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-Ist2^{C} 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 OD_{600}, 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 OD_{600} 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 KOAc, 5 mM $Mg(OAc)_2$, 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 (1,200 *g* at 4°C for 2 min) and were 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 Na_2HPO_4, 2 mM KH_2PO_4, 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 with protA beads (Amersham Biosciences), which were preloaded with 5 μl of affinity-purified, GFP-specific antibody and were washed four times for 5 min with 1 ml of buffer, followed by one wash with PBS (150 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4). To remove bound protein, the beads were incubated for 10 min at 50°C in 100 μl of high urea buffer (8 M urea, 5% [wt/vol] SDS, 200 mM Tris-HCl, pH 6.8, 1 mM EDTA, 0.05% [wt/vol] bromphenol blue, 5% [vol/vol] β-mercaptoethanol, and 100 mM DTT), and 20 μl/lane were separated by 6% SDS-PAGE.

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