COPII machinery cooperates with ER-localized Hsp40 to sequester misfolded membrane proteins into ER-associated compartments

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ABSTRACT Proteins that fail to fold in the endoplasmic reticulum (ER) are subjected to ER-associated degradation (ERAD). Certain transmembrane ERAD substrates are segregated into specialized ER subdomains, termed ER-associated compartments (ERACs), before targeting to ubiquitin–proteasome degradation. The traffic-independent function of several proteins involved in COPII-mediated ER-to-Golgi transport have been implicated in the segregation of exogenously expressed human cystic fibrosis transmembrane conductance regulator (CFTR) into ERACs in *Saccharomyces cerevisiae*. Here we focus on the properties of COPII components in the sequestration of enhanced green fluorescent protein (EGFP)–CFTR into ERACs. It has been demonstrated that the temperature-sensitive growth defects in many COPII mutants can be suppressed by overexpressing other genes involved in COPII vesicle formation. However, we show that these suppression abilities are not always correlated with the ability to rescue the ERAC formation defect, suggesting that COPII-mediated EGFP-CFTR entry into ERACs is independent of its ER-to-Golgi trafficking function. In addition to COPII machinery, we find that ER-associated Hsp40s are also involved in the sequestration process by directly interacting with EGFP-CFTR. COPII components and ER-associated Hsp40, Hlj1p, act in the same pathway to sequester EGFP-CFTR into ERACs. Our findings point to an as-yet-undefined role of COPII proteins in the formation of ERACs.

INTRODUCTION

The endoplasmic reticulum (ER) is responsible for the synthesis of secretory, transmembrane, and ER-resident proteins. Newly synthesized proteins are translocated into the ER lumen or ER membrane through the translocation channel, where they undergo folding, assembly, and posttranslational modifications with the aid of a variety of ER chaperones. During the folding process, a cellular mechanism

referred to as "ER quality control" continually monitors the protein folding status, and only correctly folded and assembled proteins are segregated from the ER-resident proteins and transported to the Golgi apparatus via membrane-bound vesicles or carrier intermediates for further processing and secretion. At the same time, the ER quality control system also plays important roles in preventing the accumulation of unfolded proteins in the ER. Misfolded or incompletely assembled proteins are retained within the ER to either complete the folding process or be targeted for retrotranslocation into the cytosol and subjected to ER-associated protein degradation (ERAD) by the ubiquitin–proteasome system (Nakatsukasa and Brodsky, 2008; Vembar and Brodsky, 2008; Brodsky and Skach, 2011).

Studies using both yeast and mammalian cells demonstrated that certain transmembrane ERAD substrates are physically segregated into specialized ER subdomains before degradation (Valetti *et al.*, 1991; Kamhi-Nesher *et al.*, 2001; Kiser *et al.*, 2001; Zhang

et al., 2001). The human cystic fibrosis transmembrane conductance regulator (CFTR), which normally resides at the plasma membrane, serves as an integral membrane ERAD substrate when expressed in the yeast *Saccharomyces cerevisiae* (Kiser *et al.*, 2001; Zhang *et al.*, 2001). Human CFTR expressed in yeast was shown to be sequestered into specialized ER subdomains called ER-associated compartments (ERACs), and such sequestration seemed to be a prerequisite for ER retrotranslocation and the subsequent proteasomal degradation (Fu and Sztul, 2003; Huyer *et al.*, 2004). However, the underlying mechanisms that drive the sequestration of transmembrane ERAD substrates into ERACs are unknown.

On the other hand, successfully folded and assembled proteins are exported from the ER in COPII-coated vesicular carriers (Sato and Nakano, 2007; Dancourt and Barlowe, 2010; Schmidt and Stephens, 2010; Gillon *et al.*, 2012). COPII vesicle–mediated protein export is believed to occur at specialized regions of the ER termed ER exit sites (ERES; Orci *et al.*, 1991; Bannykh *et al.*, 1996). In these distinct zones of the ER, a set of cytoplasmic proteins, collectively known as the COPII coat, generates COPII vesicles through a sequence of events under the control of multiple regulatory mechanisms (Aridor and Balch, 2000; Lee and Linstedt, 2000; Blumental-Perry *et al.*, 2006; Yamasaki *et al.*, 2006; Higashio *et al.*, 2008; Rismanchi *et al.*, 2009; Kodera *et al.*, 2011; Yorimitsu and Sato, 2012; Yoshibori *et al.*, 2012). The COPII coat is responsible for the direct or indirect capture of cargo proteins and for the physical deformation of the ER membrane that drives the COPII vesicle formation. In brief, the assembly of the COPII coat is triggered by GDP–GTP exchange on the small GTPase Sar1p, which is catalyzed by the ER-resident Sec12p guanine nucleotide exchange factor (Nakano and Muramatsu, 1989; Barlowe and Schekman, 1993). The activated Sar1p-GTP in turn binds to the ER membranes and recruits the Sec23/24p heterodimer by binding to the Sec23p portion, and the Sec24p subunit captures the cargo protein to form a prebudding complex (Barlowe *et al.*, 1994; Kuehn *et al.*, 1998; Miller *et al.*, 2003; Mossessova *et al.*, 2003). The membrane association of Sec23/24p is stabilized through the interactions with transmembrane cargo proteins and repeated cycles of Sec12p-dependent GTP loading of Sar1p, which facilitate proper and efficient cargo sorting into COPII vesicles (Sato and Nakano, 2005; Tabata *et al.*, 2009). Subsequently the prebudding complex recruits the Sec13/31p heterotetramer onto Sec23/24p, which polymerizes and drives membrane deformation and vesicle budding.

Although much of the attention to COPII function has focused on events associated with vesicular trafficking, an earlier study showed that the sequestration of enhanced green fluorescent protein (EGFP)–CFTR into ERACs in *S. cerevisiae* requires the COPII components (Fu and Sztul, 2003). In the absence of COPII function, EGFP-CFTR remains distributed throughout the ER rather than being segregated into ERACs, indicating the possibility that COPII machinery participates in the sequestration of transmembrane ERAD substrates to the ERACs by a noncanonical function. However, the exact mode of action of individual COPII components during these processes is very unclear. Here we investigate in detail the contribution of COPII components to the sequestration of EGFP-CFTR into ERACs in yeast cells. In addition to COPII components, we find that the ER-associated Hsp40 cochaperones are also involved in the sequestration process. Our results show that COPII components, along with Hsp40s, participate in the targeting of EGFP-CFTR to ERACs, independent of their role in ER-to-Golgi transport.
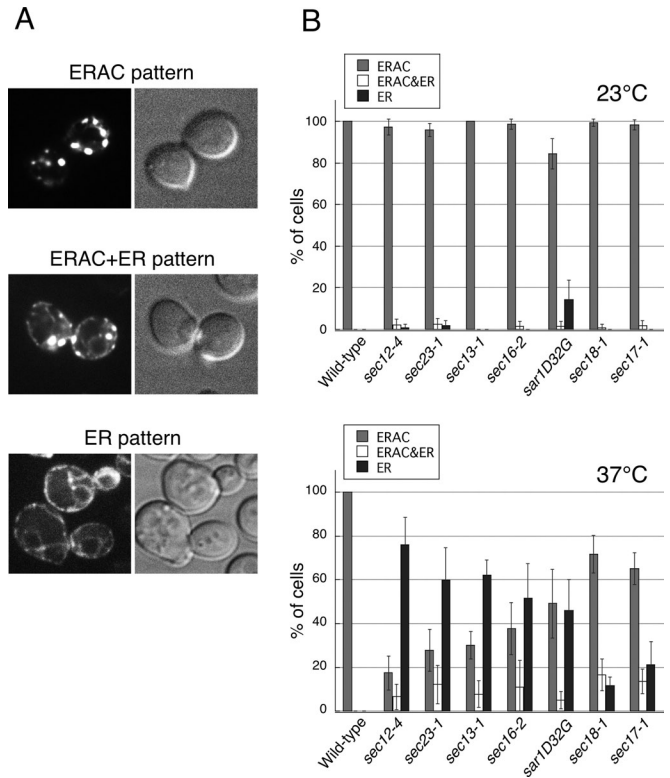

FIGURE 1: The formation of EGFP-CFTR–induced ERACs requires COPII function. (A) Wild-type (ANY21) cells containing the EGFP-CFTR expression plasmid were grown to mid–log phase at 23°C and incubated with copper-containing medium for 2 h to induce expression, followed by observation with confocal microscopy. (B) Wild-type and *sec12-4*, *sec23-1*, *sec13-1*, *sec16-2*, *sar1D32G*, *sec18-1*, and *sec17-1* strains were grown to mid–log phase and induced with copper at 23°C (top) or 37°C (bottom) for 2 h to induce EGFP-CFTR expression. ERAC formation is quantified using the three categories that were defined in A, and the data are presented as the percentage of cells expressing EGFP-CFTR. The experiments were repeated at least three times, and >100 cells were analyzed for each condition.

RESULTS

EGFP-CFTR–induced ERAC formation in COPII mutant strains

Fu and Sztul (2003) observed that the sequestration of the membrane ERAD substrate EGFP-CFTR into degradative ERACs in *S. cerevisiae* was impaired by temperature-sensitive mutations in key COPII components, including *SEC12*, *SEC13*, and *SEC23*, but not by a mutation in *SEC18* (mutant in transport vesicle fusion). These findings led to the idea that the segregation of EGFP-CFTR into ERACs required COPII function, but the function was independent of ER-to-Golgi trafficking. To further examine the potential involvement of the COPII machinery in the sequestration of EGFP-CFTR in the ER, we quantitatively analyzed the localization of EGFP-CFTR expressed under the control of a copper-inducible promoter in various temperature-sensitive mutants defective in COPII-mediated transport to the Golgi. We examined the mutant alleles *sar1D32G*, *sec12-4*, *sec23-1*, *sec13-1*, *sec16-2*, *sec17-1*, and *sec18-1*, all of which block ER-to-Golgi transport at restrictive conditions (37°C). As shown in Figure 1A, 2 h of EGFP-CFTR induction in yeast cells yielded three different localization patterns: the ERAC pattern, the ER pattern, or both the ERAC and ER patterns. We found that >95% of the wild-type cells expressing EGFP-CFTR and

>80% of the mutant cells expressing EGFP-CFTR displayed the ERAC pattern at the permissive temperature (23°C; Figure 1B, top). In contrast, when EGFP-CFTR was expressed at the restrictive temperature in the sar1D32G, sec12-4, sec23-1, sec13-1, and sec16-2 mutant strains, all of which were defective in COPII vesicle formation near the point of exit from the ER, the percentage of cells with the ERAC pattern was reduced, and the percentage with the ER pattern increased concomitantly (Figure 1B, bottom). The ability to form ERACs at the restrictive temperature varied from $17.4 \pm 7.67\%$ (for sec12-4) to $49.1 \pm 15.8\%$ (for sar1D32G), depending on the mutant used. The ERAC or ERAC and ER pattern of EGFP-CFTR localization was still observed in >70% of the vesicle targeting and fusion mutants sec17-1 and sec18-1 at the restrictive temperature, indicating that a block in the late step of ER-to-Golgi transport does not significantly influence EGFP-CFTR sequestration. These results confirm and extend earlier observations of the participation of COPII components in EGFP-CFTR–induced ERAC formation that is independent of their vesicular trafficking function.

Genetic analysis of ERAC formation

To ensure that the foregoing results with the sec mutants were not a consequence of indirect effects of traffic inactivation, we next used genetic analysis to provide additional evidence for the role of COPII components in the formation of ERACs. The temperature-sensitive growth phenotype of certain ER budding mutants can be suppressed by overexpressing other genes involved in the ER-to-Golgi transport (Nakano and Muramatsu, 1989; d'Enfert et al., 1991; Oka and Nakano, 1994; Gimeno et al., 1995; Saito et al., 1999; Kurihara et al., 2000; Buchanan et al., 2010). To study whether the sequestration of EGFP-CFTR into ERACs and COPII-mediated vesicle formation function in parallel or as part of an integrated system, we tested whether the ERAC formation defect of the COPII vesicle budding mutants also could be suppressed by overexpressing other genes that are required for COPII vesicle formation. A multicopy plasmid containing the gene required for the biogenesis of COPII vesicles or an empty vector control was introduced into several temperature-sensitive mutants that were defective at the stage of ER vesicle budding, and the suppression activity was monitored by growth at 37°C (Figure 2A). Among them, the high-copy expression of SAR1 suppressed the temperature-sensitive growth defect of the sec12-4 and sec16-2 mutants, which was also reported previously (Nakano and Muramatsu, 1989; d'Enfert et al., 1991). As expected, the ERAC formation defect of the sec12-4 mutant was almost fully corrected by SAR1 overexpression (Figure 3B). In contrast, the high gene dosage of SAR1 could not rescue the failure of EGFP-CFTR to be sequestered into ERACs in sec16-2 cells (Figure 3C). As controls, we verified that adding back each gene (SEC23, SEC12, or SEC16) on a high-copy plasmid reversed both the temperature sensitivity and the ERAC formation defect (Figures 2 and 3). Furthermore, we found that although overexpression of SEC16 did not suppress the temperature-sensitive growth defect of the sec23-1 mutant (Figure 2A), it significantly rescued the ERAC formation defect at the restrictive temperature (Figure 3A). A similar effect was observed with SEC13, although the extent of the suppression was less pronounced. To confirm that these suppressions were not due to the incomplete block of the ER-to-Golgi transport, we followed the transport of the vacuolar protease carboxypeptidase Y (CPY) as a marker for early events in the secretory pathway under the same conditions as described. As shown in Figure 2B, the ER form of CPY (p1) accumulated within the sec23-1 cells overexpressing SEC16, confirming that the foregoing conditions indeed caused a severe block in the ER-to-Golgi transport of secretory proteins. The mutant lacking
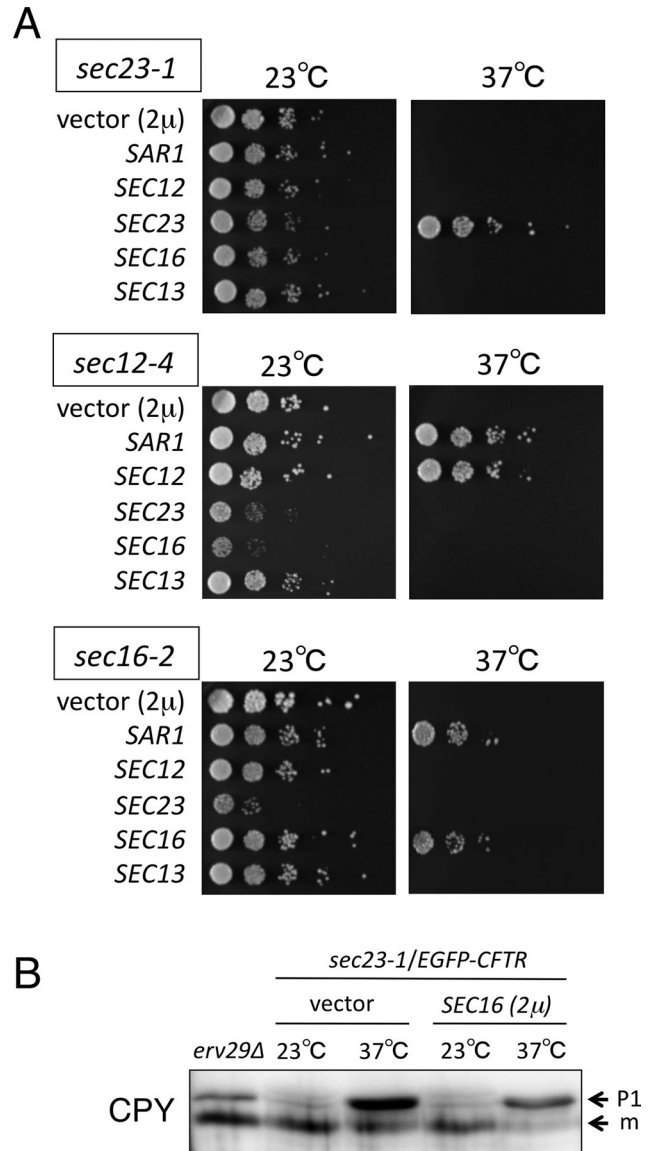
FIGURE 2: The effect of overexpressing individual COPII components on the growth of temperature-sensitive mutants defective in COPII vesicle formation. (A) sec23-1 (MBY8-20C), sec12-4 (MBY10-7A), and sec16-2 (MBY4-1A) cells harboring pEGFP-CFTR were transformed with pYO324 (2 μ vector), pSAR1 (2 μ SAR1), pSEC12 (2 μ SEC12), pSEC23 (2 μ SEC23), pSEC16 (2 μ SEC16), or pSEC13 (2 μ SEC13), and the transformants were grown to saturation at 23°C in MCD medium lacking uracil and tryptophan (MCD-Ura-Trp). The cells were diluted to a final concentration of 0.3 at OD_{600}, and 5 μl of 10-fold dilutions was spotted onto MCD-Ura-Trp plates. The plates were incubated at 23 or 37°C as indicated. (B) sec23-1 (MBY8-20C) cells transformed with pYO324 (2 μ vector) or pSEC16(2 μ SEC16) were grown at 23 or 37°C in selective media. Equal amounts of whole-cell extracts were subjected to immunoblotting with anti-CPY antibody. The erv29Δ strain was used as a control. The ER (p1) and mature (m) forms of CPY are indicated.

ERV29, which encodes a sorting receptor for CPY export from the ER (Belden and Barlowe, 2001), was used as a control for transport inhibition. These results clearly indicate that the suppression of the temperature-sensitive growth defects of the COPII mutants is not necessarily correlated with the ability to rescue the ERAC formation defect.
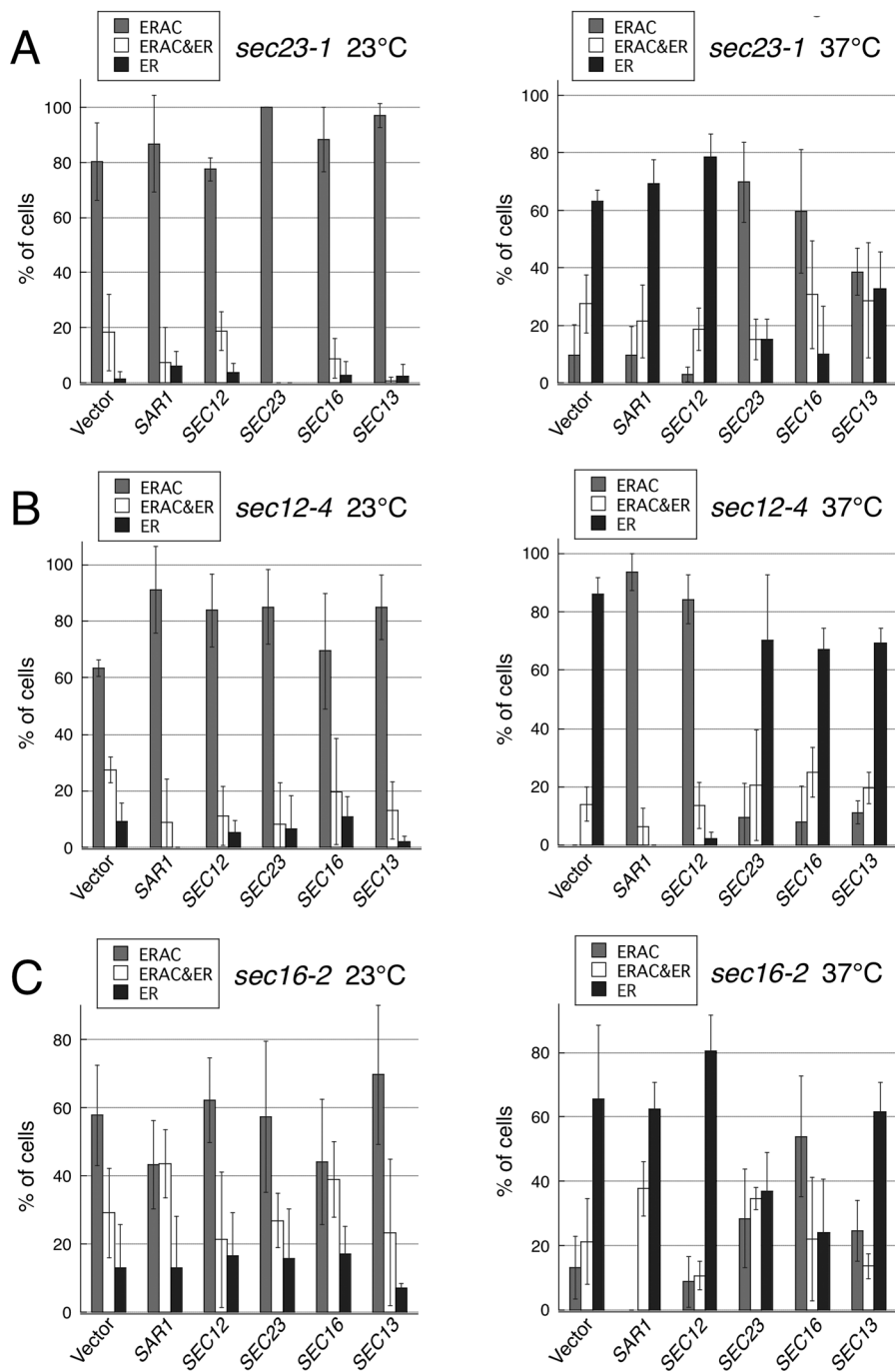
FIGURE 3: The effect of overexpressing individual COPII components on ERAC formation in temperature-sensitive mutants defective in COPII vesicle formation. (A–C) The transformants used in Figure 2 were grown to mid–log phase and treated with copper at 23°C (left) or 37°C (right) for 2 h to induce EGFP-CFTR expression. ERAC formation was quantified as described in Figure 1B. The experiments were repeated at least three times, and >70 cells were analyzed for each condition.

EGFP-CFTR–enriched ERACs. When coexpressed with EGFP-CFTR, we found that Sar1p-mCherry colocalized well with EGFP-CFTR ERACs in addition to its well-established ER localization (Figure 4A). However, it is not certain whether Sar1p is specifically targeted to ERACs or arrives there simply because of its association with the ER. Therefore we examined whether the inactive GDP form of mutant Sar1p (Sar1p-D32G) (Saito et al., 1998) was also present in ERACs. Similar to the wild-type Sar1p, Sar1p-D32G-mCherry was also present both in ERACs and in the ER at the restrictive temperature (Figure 4B), suggesting that Sar1p localization to ERACs was not directly regulated in response to Sar1p activation. A common feature of most ERAC-like structures is the colocalization of Kar2p/BiP (Valetti et al., 1991; Kiser et al., 2001; Zhang et al., 2001; Fu and Sztul, 2003; Sullivan et al., 2003; Huyer et al., 2004). However, we found that another ER-resident protein, a subunit of the ER translocon, Sec71p-mCherry, which had nonchaperone functions, was also clearly present in ERACs (Figure 4C). These results suggest that components of the ER are also localized within EGFP-CFTR ERACs.

ERES are also subdomains of the ER that are detected as small punctate structures scattered throughout the ER and colocalize with COPII coat subunits Sec23/24p and Sec13/31p (Connerly et al., 2005; Castillon et al., 2009; Shindiapina and Barlowe, 2010; Yorimitsu and Sato, 2012). We used mCherry-fused Sec23p and Sec13p to explore the relationship between ERES and EGFP-CFTR–induced ERACs. In cells expressing EGFP-CFTR, several ERES-like punctate structures were often observed within large foci of the EGFP-CFTR–containing ERACs in addition to the typical ERES punctate pattern distributed throughout the ER (Figure 4, D and E). Therefore ERES appear morphologically distinct from ERACs, and punctate structures visualized by Sec23p-mCherry and Sec13p-mCherry within ERACs most likely represent ERES that have been incidentally engulfed into ERACs. Taken together, these findings led to the conclusion that there was no specific accumulation of COPII components in the already formed ERACs.

Localization of COPII components relative to ERAC

We next examined the cellular localization of COPII components relative to EGFP-CFTR–induced ERACs. Previous studies reported that CFTR-induced ERACs were subdomains of ER and that they consisted of a tubular network of ER membranes (Fu and Sztul, 2003; Huyer et al., 2004). We first examined the localization of Sar1p (which normally resides throughout the entire ER) relative to the

A diacidic ER exit code of CFTR is dispensable for its entry into ERACs

An earlier study showed that the COPII machinery was required for CFTR to be exported out of the ER in mammalian cells (Yoo et al., 2002). A diacidic ER exit code was identified in the CFTR molecule that directly interacted with the COPII coat subunit Sec23/24. It was demonstrated that mutation of the diacidic motif DAD (residues
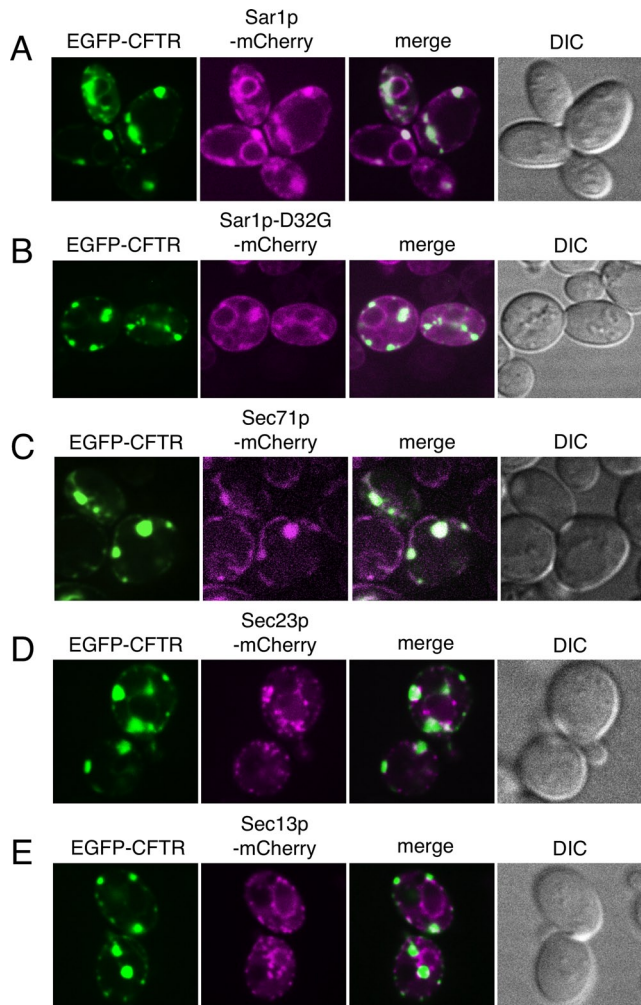
FIGURE 4: The relationship between the components of the endoplasmic reticulum and ERAC structures. Log-phase cultures of wild-type cells (ANY21) coexpressing EGFP-CFTR (induced as described in Figure 1A) and Sar1p-mCherry (A), Sar1p-D32G-mCherry (B), Sec71p-mCherry (C), Sec23p-mCherry (D), or Sec13p-mCherry (E) were visualized by confocal microscopy at 23°C, except B, which was observed at 37°C.
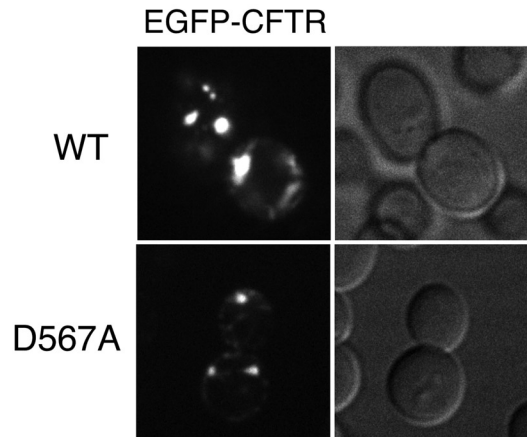

FIGURE 5: The diacidic ER export motif of CFTR is dispensable for its entry into ERACs. Wild-type (ANY21) cells containing the EGFP-CFTR plasmid (top) or EGFP-CFTR-D567A plasmid (bottom) were grown to mid–log phase at 23°C and incubated with copper-containing medium for 2 h to induce expression. The cells were then visualized by confocal microscopy.

565–567) to DAA disrupted Sec23/24 binding (Wang et al., 2004). Because the human homologue of the yeast COPII component, Sec23A, complements the temperature-sensitive growth of the yeast sec23-1 mutant at the restrictive temperature (37°C; Paccaud et al., 1996), it is possible that the yeast COPII coat can bind to the diacidic sequence of EGFP-CFTR expressed in yeast cells and may have some role in coordinating the sequestration process. Therefore we examined the ability of the diacidic EGFP-CFTR-D567A mutant to be sequestered into ERACs, but this did not significantly affect its entry into the ERAC (Figure 5). Thus the diacidic ER export motif of CFTR, although necessary for ER export in mammalian cells, does not participate in the ERAC formation process in yeast.

Involvement of Hsp40s in ERAC formation

Although much less is known about how misfolded membrane proteins are sorted to the ERAD pathway, it has been reported that the cytosolic Hsp70–Hsp40 chaperone system affects the degradation of several integral membrane ERAD substrates (Strickland et al., 1997; Meacham et al., 1999; Hill and Cooper, 2000; Zhang et al.,

2001; Youker et al., 2004; Ahner et al., 2007), although precisely how these chaperones facilitate the turnover of integral membrane ERAD substrates is not completely understood. CFTR degradation was slowed in yeast mutated for either SSA1-4 (cytosolic Hsp70s) or YDJ1/HLJ1 (ER-associated Hsp40; Zhang et al., 2001; Youker et al., 2004). S. cerevisiae has four Ssa Hsp70 proteins (Ssa1–4p), and a temperature-sensitive ssa1-45 mutant strain lacking functional SSA2/3/4 was shown to retain the ability to form CFTR-induced ER-ACs at the restrictive temperature (Zhang et al., 2001). To test whether Hsp40 cochaperones Ydj1p and Hlj1p are involved in the sequestration of EGFP-CFTR into ERACs, we assessed ERAC formation in ydj1Δ or hlj1Δ mutants and in an isogenic wild-type strain (Figure 6). We found that ydj1Δ and hlj1Δ mutant strains were moderately defective in ERAC formation relative to the wild-type strain; nearly 60% of the mutant cells displayed the ER pattern of localization. Because Ydj1p and Hlj1p have been demonstrated to function redundantly to facilitate the degradation of CFTR in conjunction with Hsp70 Ssa proteins (Youker et al., 2004), it is possible that the loss of one protein may not completely impair ERAC formation. In contrast to ydj1Δ and hlj1Δ mutants, the cells with a deletion of the other cytosolic Hsp40 chaperone, APJ1 (Kryndushkin et al., 2002), showed no effect on EGFP-CFTR ERAC formation under the same conditions (Figure 6B), implying a direct and specific effect of Ydj1p/Hlj1p. We also examined the stability of EGFP-CFTR in ydj1Δ or hlj1Δ mutants by cycloheximide chase analysis. We observed that deletion of either HLJ1 or YDJ1 was sufficient to slow degradation of EGFP-CFTR relative to the isogenic wild-type strain (Figure 6C). Taken together, these results demonstrate that ER-associated Hsp40 cochaperones Ydj1p and Hlj1p participate in the sequestration of EGFP-CFTR into ERACs.

In general, Hsp40 proteins can bind to abnormally folded polypeptides and deliver them to specific Hsp70 partners to prevent aggregation and facilitate refolding (Craig et al., 2006; Buck et al., 2007). In addition, in some cases, Hsp40 protein itself can act as a chaperone, avoiding protein aggregation by forming stable complexes with folded and unfolded polypeptides (Szabo et al., 1994; Rudiger et al., 2001). Therefore, to determine whether a direct
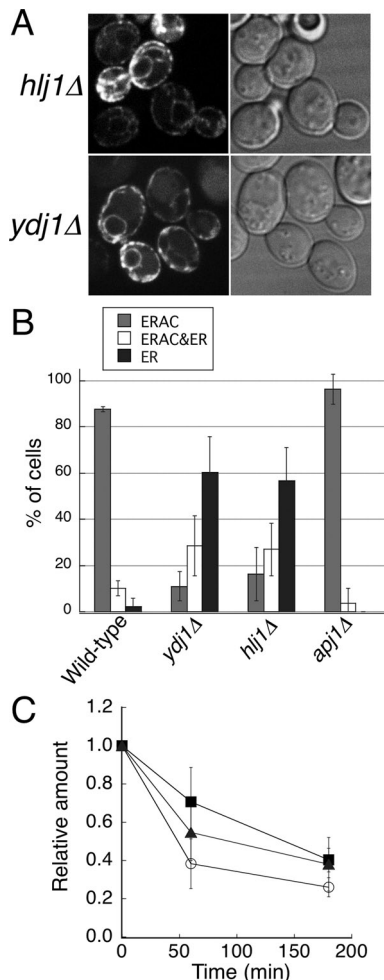
FIGURE 7: EGFP-CFTR forms a complex with Hlj1p in the ER. Microsomes (100 μg) expressing EGFP-CFTR (induced for 2 h) with (+) or without (–) GST-Hlj1p were solubilized with 1% Triton X-100. The resulting extract was cleared by centrifugation, and the supernatant was incubated with glutathione–Sepharose beads. The beads were washed, and proteins were released in the presence of 10 mM glutathione. The released proteins were analyzed by SDS–PAGE, followed by immunoblotting with antibodies to GFP, Sec23p, Sar1p, Sec12p, Sec61p, and GST. The total lane (T) represents 10% of the starting microsomal extract used in the lane labeled (+).

FIGURE 6: The formation of EGFP-CFTR–induced ERACs is ER associated and Hsp40 dependent. (A) *ydj1Δ* and *hlj1Δ* cells containing the EGFP-CFTR plasmid were grown to mid–log phase at 23°C and incubated with copper-containing medium for 2 h to induce EGFP-CFTR expression, followed by observation with confocal microscopy. (B) Wild-type, *hlj1Δ*, *ydj1Δ*, and *apj1Δ* cells harboring pEGFP-CFTR were grown to mid–log phase and induced with copper at 23°C for 2 h to induce EGFP-CFTR expression. ERAC formation was quantified as described in Figure 1B. The experiments were repeated at least three times, and >50 cells were analyzed for each condition. (C) Wild-type, *hlj1Δ*, and *ydj1Δ* cells expressing EGFP-CFTR were subjected to cycloheximide chase analysis as described in *Materials and Methods*, and the amount of EGFP-CFTR at time point 0 was set to 1.0. The relative amounts of EGFP-CFTR remaining in wild-type (open circles), *hlj1Δ* (closed squares), and *ydj1Δ* (closed triangles) strains over time were plotted.

interaction could be observed between EGFP-CFTR and the Hsp40 chaperone in the ER, we constructed cells expressing both glutathione S-transferase–tagged Hlj1p (GST-Hlj1p) and EGFP-CFTR. GST-Hlj1p complemented the EGFP-CFTR–induced ERAC formation defect of the *hlj1Δ* strain (unpublished data). ER-enriched microsomes from this strain were solubilized with a detergent, and then a portion of the GST-Hlj1p was pulled down by glutathione beads. The amounts of adsorbed proteins were determined by immunoblotting. The results in Figure 7 show that GST-Hlj1p pulled down a significant amount of EGFP-CFTR but not the ER-resident proteins Sec61p and Sec12p, indicating a specific interaction between Hlj1p and EGFP-CFTR in the ER. We further
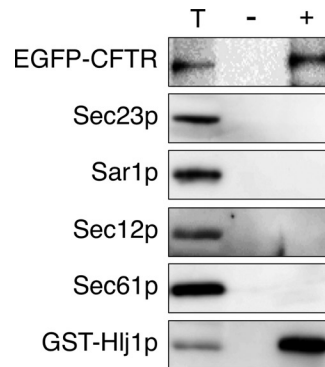
examined whether COPII components were also recovered in this complex, but no appreciable amount of Sar1p or Sec23p was detected, implying that Hlj1p and COPII components do not function in the same stable complex. We also characterized the effects of EGFP-CFTR expression on mCherry-Hlj1p distribution in the ER (Figure 8A). GFP-Hlj1p has been reported to localize throughout the ER at a steady state (Youker *et al.*, 2004). With EGFP-CFTR expression, mCherry-Hlj1p, in addition to its typical ER localization, also localized to EGFP-CFTR–induced ERACs, although the particular action of Hlj1p after entry into the ERAC is not yet defined. Taken together, our data support the notion that Hlj1p plays a role in the sequestration of EGFP-CFTR into ERACs by directly binding to EGFP-CFTR in the ER.

Hsp40 and COPII components function in the same pathway to drive ERAC formation

Given the role of both Hlj1p and COPII components in ERAC formation, a crucial question is whether these components participate in the same functional pathway. To explore this, we examined whether overexpression of a protein involved in COPII vesicle formation could rescue the ERAC formation defect of *hlj1Δ* cells. We forced *hlj1Δ* cells to overexpress one of the COPII proteins from a multi-copy plasmid used in Figure 2A and monitored the EGFP-CFTR–induced ERAC formation by confocal microscopy (Figure 8B). Of the five COPII components (Sar1p, Sec12p, Sec23p, Sec13p, Sec16p) tested, overexpression of Sar1p and Sec12p significantly increased the number of ERAC patterns of localization by ~2.2-fold (44.2 ± 5.98% for Sar1p, 44.4 ± 6.40% for Sec12p) compared with the vector control (19.8 ± 12.5%). No obvious increase in ERAC pattern was detected with Sec23p, Sec16p, Sec13p, or Sar1p-D32G. These genetic relationships indicate that Hlj1p acts upstream or in conjunction with COPII components during ERAC formation. The ability of high-copy expression of Sar1p or Sec12p to suppress defects in ERAC formation is consistent with the fact that these two components act the most upstream in COPII assembly of the investigated COPII components. Collectively these findings suggest that overexpression of some of the COPII genes can, at least partially, bypass the ERAC formation defect of *hlj1Δ* cells, which confirms that Hlj1p and COPII components act in the same ERAC formation pathway.

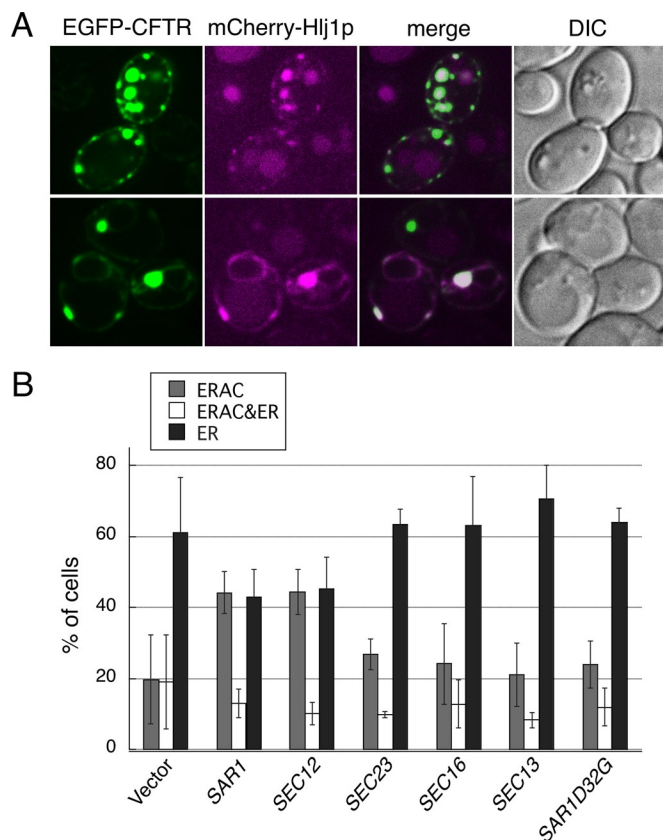**A** EGFP-CFTR mCherry-Hlj1p merge DIC

B

FIGURE 8: Hsp40s and COPII components act in the same pathway to drive ERAC formation. (A) *hlj1Δ* cells coexpressing EGFP-CFTR (induced as described in Figure 1A) and mCherry-Hlj1p were observed by confocal microscopy. (B) *hlj1Δ* cells harboring pEGFP-CFTR were transformed with pYO324 (*2 μ vector*), pSAR1 (*2 μ SAR1*), pSEC12 (*2 μ SEC12*), pSEC23 (*2 μ SEC23*), pSEC16 (*2 μ SEC16*), pSEC13 (*2 μ SEC13*), or pSAR1D32G (*2 μ SAR1D32G*), and the transformants were grown to mid–log phase and induced with copper at 23°C for 2 h to induce EGFP-CFTR expression. ERAC formation was quantified as described in Figure 1B. The experiments were repeated at least three times, and >150 cells were analyzed for each condition.

DISCUSSION

A previous study demonstrated a requirement for the COPII components in the sequestration of the transmembrane ERAD substrate CFTR into ERACs in *S. cerevisiae* (Fu and Sztul, 2003). However, the molecular mechanism by which the COPII machinery contributes to this process is unclear. To achieve a better understanding of the involvement of COPII proteins in ERAC formation, we expanded the previous study by examining the localization of EGFP-CFTR in various temperature-sensitive mutants with a deficiency in ER-to-Golgi transport. The pattern of EGFP-CFTR localization in the *sar1D32G*, *sec12-4*, *sec23-1*, *sec13-1*, and *sec16-2* strains, all of which were defective in the stage of COPII vesicle budding, showed significant changes at the restrictive temperature: EGFP-CFTR was homogeneously dispersed throughout the ER. In contrast, when the vesicle targeting and fusion mutants *sec17-1* and *sec18-1* were grown at the restrictive temperature, >70% of the mutant cells displayed the ERAC or ERAC and ER pattern of EGFP-CFTR localization, confirming that the sequestration of EGFP-CFTR is independent of ER-to-Golgi trafficking (Figure 1B).

These experiments require a temperature shift from the permissive temperature (23°C) to the restrictive temperature (37°C) to evaluate the effect of the mutant COPII component on ERAC formation. Thus one might expect that cytosolic and luminal chaperones that are induced in response to a rapid shift of yeast cells to the restrictive temperature prevent the formation of protein aggregates and/or promote protein refolding, which may act to limit ERAC formation. In fact, others have observed that an elevated temperature markedly stabilizes wild-type CFTR in mammalian cells and its temperature-sensitive folding mutant, ΔF508-CFTR, from ERAD in response to the induction of heat shock proteins (Strickland et al., 1997; Loo et al., 1998; Meacham et al., 1999; Choo-Kang and Zeitlin, 2001). However, this is certainly not the case for EGFP-CFTR in yeast, because ERAC formation was not significantly altered upon the temperature shift in either wild-type or *sec17-1* and *sec18-1* mutants (Figure 1B). Alternatively, the accumulation of aberrant proteins in the ER generally leads to the induction of the unfolded protein response (UPR; Walter and Ron, 2011), which also induces the expression of a variety of genes required for protein folding and ER export (Travers et al., 2000). Upregulation of COPII-pathway genes may be of significance in view of the data presented here and in previous work (Fu and Sztul, 2003). However, CFTR fails to induce the UPR (Zhang et al., 2001); other transmembrane ERAD substrates expressed in yeast also fail to induce the UPR (Ferreira et al., 2002; Huyer et al., 2004). Indeed, EGFP-CFTR was normally sequestered into ERACs in cells lacking *IRE1*, the master membrane kinase involved in transmitting the unfolded protein signal to activate UPR target genes (Supplemental Figure S1).

Additional evidence for the involvement of a traffic-independent function of COPII components in ERACs formation comes from multicopy suppression analyses. The temperature-sensitive growth defects in many mutants defective in COPII vesicle formation can be suppressed by high-copy expression of other genes involved in this process. However, we observed that such multicopy suppressors were not always able to rescue the ERAC formation defect. In addition, we also found that the overexpression of a number of genes involved in COPII vesicle formation that fail to suppress the ER-to-Golgi transport defect suppresses the ERAC formation defect (Figure 3). These collective genetic data provide circumstantial evidence that the COPII machinery has at least two different functions in the ER. One function involves ER-to-Golgi transport, and the other involves the sorting of integral membrane ERAD substrates into specialized ER subdomains.

ERAC-like structures that form in response to certain mutant or heterologously expressed proteins have been observed both in yeast and in mammalian cells (Valetti et al., 1991; Hobman et al., 1992, 1998; Supply et al., 1993; Nishikawa et al., 1994; Raposo et al., 1995; Kamhi-Nesher et al., 2001; Ferreira et al., 2002). Because certain misfolded proteins reside only transiently in the ERAC-like structures and eventually enter the secretory pathway, some of these structures have been proposed to represent expanded ER exit sites (Nishikawa et al., 1994; Hobman et al., 1998; Kamhi-Nesher et al., 2001; Ferreira et al., 2002). However, we could not observe the specific localization of ERES marker proteins within the ERACs induced by EGFP-CFTR. Furthermore, a constitutively inactive form of Sar1p (Sar1p-D32G), which fails to be targeted to ERES (Yorimitsu and Sato, 2012), was also found to localize to ERACs (Figure 4). Therefore it is likely that at least the EGFP-CFTR–induced ERACs are not expanded ER exit sites. Instead, some small punctate structures labeled by ERES markers, which closely resemble the structures characteristic of ERES, were often associated within ERACs. Because EGFP-CFTR–induced ERACs are accumulations of tubular membrane extensions connected to the ER (Fu and Sztul, 2003; Huyer et al., 2004) and a resident transmembrane ER protein (Sec71p) is

also present in ERACs (Figure 4), we assume that these small puncta represent ERES that had been coincidentally incorporated into the ERAC during its formation. However, we do not have data to indicate that these punctate signals are functionally equivalent to ERES observed within the ER.

The recognition of ERAD substrates is believed to be mediated primarily by molecular chaperones. Integral membrane ERAD substrates with large cytoplasmic domains have been proposed to bind to cytosolic Hsp70s and Hsp40s. In fact, CFTR includes two large cytosolic nucleotide-binding domains, and cytosolically localized Hsp70 and Hsp40s have been shown to facilitate the ERAD of CFTR in both yeast (Zhang *et al.*, 2001; Youker *et al.*, 2004) and mammals (Meacham *et al.*, 1999; Rubenstein and Zeitlin, 2000). The contributions of Hsp40 proteins in the process of ERAD have been observed to require their interaction with partner Hsp70 proteins. In general, Hsp40 binds directly to substrate polypeptides first and then, in nearly all cases, transfers the bound substrates to its cognate Hsp70 (Craig *et al.*, 2006; Buck *et al.*, 2007). We could indeed detect a direct interaction between ER-localized Hsp40, Hlj1p, and EGFP-CFTR (Figure 7). We demonstrated that the maximal formation of ERAC requires at least two ER-associated Hsp40s, Ydj1p and Hlj1p. However, the function of the Hlj1p/Ydj1p Hsp70 cognate, Ssa proteins, has been shown to be dispensable for CFTR-induced ERAC formation (Zhang *et al.*, 2001). Although it has been shown that Hlj1p and Ydj1p redundantly function to facilitate CFTR degradation (Youker *et al.*, 2004), our data showed that deletion of either one caused defects in both sequestration and degradation of EGFP-CFTR (Figure 6). One possible reason for this discrepancy could be due to a difference in expression level of CFTR: the previous report (Youker *et al.*, 2004) used the constitutive PGK promoter, whereas we used the inducible CUP1 promoter. When expressed from the relatively weak PGK promoter, the cells with the loss of either *HLJ1* or *YDJ1* may still manage to sequester CFTR into ERACs, whereas CFTR expressed from the strong inducible CUP1 promoter requires full Hsp40 activity. Our finding is the first example in which ER-associated Hsp40s are shown to play a role in a step upstream of ERAC formation.

Several lines of genetic evidence presented here revealed partial overlaps in the functions of COPII proteins and ER-associated Hsp40s during ERAC formation. It is therefore likely that the substrate-binding activities of Hlj1p involved in ERAC formation have a close mechanistic link with COPII functions rather than simply delivering bound substrates to its partner Hsp70. However, we could not detect COPII components in the same Hlj1p-EGFP-CFTR protein complex under detergent-solubilized conditions (Figure 7). This might be due to several factors, including a breakdown of the interactions in the presence of detergent and/or an inability to maintain the complex under detergent-solubilized membrane-free conditions. Understanding this key step will be important to exploring how COPII might facilitate the sorting of EGFP-CFTR into specialized ER subdomains. Because ERACs are extensive networks of tubulovesicular structures, we speculate that the membrane-deforming ability of Sar1p and COPII coats (Bi *et al.*, 2002; Bielli *et al.*, 2005; Lee *et al.*, 2005; Stagg *et al.*, 2008; Long *et al.*, 2010; O'Donnell *et al.*, 2011) might contribute to drive ERAC formation. Moreover, there is a report that at least some ERACs are cleared from cells through the autophagy-dependent pathway (Fu and Sztul, 2009). Therefore, taking into account that the requirement for a subgroup of COPII proteins in autophagosome formation has been demonstrated in yeast (Ishihara *et al.*, 2001; Hamasaki *et al.*, 2003), it is possible to consider ERACs to be a kind of preautophagosomal-like structure accumulated by COPII components.

In summary, our results demonstrated that the inhibition of ERAC formation in the absence of COPII functions was not due to indirect effects of inactivating ER-to-Golgi trafficking; instead, COPII components played a certain role during ERAC formation. Furthermore, we found that the ER-associated Hsp40s were specifically involved in the substrate sequestration process. We propose that a continued characterization of the underlying mechanisms that govern substrate partitioning to ERACs will yield additional insights into the varied activities of COPII machinery.

MATERIALS AND METHODS

Yeast strains and plasmids

The *S. cerevisiae* strains used in this study are listed in Supplemental Table S1. The yeast strains were grown in MCD medium (0.67% yeast nitrogen base without amino acids [Difco Laboratories, Franklin Lakes, NJ], 2% glucose, and 0.5% casamino acids with auxotrophic supplements). The coding sequences of the *SAR1*, *SEC12*, *SEC23*, *SEC13*, and *SEC16* genes, together with upstream and downstream flanking regions, were amplified by PCR from *S. cerevisiae* genomic DNA and inserted into the *Sac*I and *Xho*I sites of pYO324 (*2 μ*, *TRP1*), yielding pSAR1, pSEC12, pSEC23, pSEC13, and pSEC16, respectively. The coding sequences of the *SEC71* and *SEC23* genes, together with upstream and downstream flanking regions, were amplified from genomic DNA by PCR and inserted into the *Bam*HI and *Xho*I sites (for *SEC71*) or *Sac*I and *Xho*I sites (for *SEC23*) of pRS314 (*CEN*, *TRP1*). For fusion of fluorescent proteins to the C-terminus, a *Sph*I site (for *SEC71*) or a *Bam*HI site (for *SEC23*) was created just before the stop codon of each gene, and the fragment encoding mCherry, which was PCR amplified from pmCherry (Clontech, Mountain View, CA), was inserted into these sites to yield pSEC71-mCherry or pSEC23-mCherry. The coding sequence of the *HLJ1* gene, together with upstream and downstream flanking regions, was amplified from the genomic DNA by PCR and inserted into the *Sac*I–*Xho*I sites of pYO324. A *Sph*I site was created just before the start codon, and the fragment encoding mCherry or GST was inserted into the *Sph*I site to yield pmCherry-HLJ1 or pGST-Hlj1, respectively. The generation of plasmids expressing Sar1p-mCherry, Sar1p-D32G-mCherry, and Sec13p-mCherry was described previously (Yorimitsu and Sato, 2012). The plasmid expressing EGFP-tagged human CFTR under a copper-inducible promoter (pCU426CUP1/EGFP-CFTR) was a generous gift from Elizabeth Sztul and Lianwu Fu (University of Alabama). The mutation to alanine at position 567 of CFTR was introduced into the gene of EGFP-CFTR in plasmid pEGFP-CFTR using primer-directed mutagenesis, yielding pEGFP-CFTR-D567A.

Fluorescence microscopy

Yeast cells were grown to an OD_{600} of 0.5 at 23°C, and EGFP-CFTR expression was induced with 100 μM $CuSO_4$ for 2 h. To analyze ERAC formation in temperature-sensitive mutants, yeast cells containing the EGFP-CFTR plasmid were grown to an OD_{600} of 0.5 at 23°C. Then each culture was separated into two aliquots, and the aliquots were incubated at 23 or 37°C for 10 min. Cells were then induced to express EGFP-CFTR in the presence of 100 μM $CuSO_4$ for 2 h. Fluorescence microscopy observation was carried out using an Olympus IX71 microscope equipped with a CSU10 spinning-disk confocal scanner (Yokogawa Electric Corporation, Tokyo, Japan) and an electron-multiplying charge-coupled device camera (iXon, DU897; Andor Technology, South Windsor, CT). The acquired images were analyzed by using Andor iQ (Andor Technology). In this setting, a 473-nm solid-state laser (Showa Optronics, Tokyo, Japan J050BS) was used to excite AcGFP and mCherry at 561 nm (Jive; Cobolt, Solna, Sweden).

Cycloheximide chase assay

Cells containing *CUP1* promoter regulated EGFP-CFTR were grown to an OD_{600} of 0.5 at 23°C, and EGFP-CFTR was induced with 100 µM $CuSO_4$ for 2 h. To begin the chase, protein synthesis was stopped by the addition of cycloheximide to a final concentration of 50 µg/ml with continued incubation at 23°C. At specified time points, 3.0 OD of cells were collected and 1/10 volume of ice-cold 100% trichloroacetic acid (TCA) was added to terminate the chase. The cells were washed with cold water and disrupted by vortexing with glass beads in lysis buffer (50 mM Tris-Cl, pH 7.5, 0.5% SDS). The cleared lysate was precipitated using TCA and analyzed by immunoblotting.

Pull-down assays

Microsomes were prepared from cells expressing EGFP-CFTR (induced for 2 h) and GST-Hlj1p as described previously (Sato and Nakano, 2003). For the pull-down assays, 100 µg of microsomes was solubilized with 1% Triton X-100 in B88-8 (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–KOH, pH 8.0, 150 mM KOAc, 5 mM MgOAc, and 250 mM sorbitol). After 30 min on ice, the insoluble material was removed by centrifugation, and then the supernatant was incubated with glutathione–Sepharose beads (GE Healthcare, Piscataway, NJ) at 4°C for 1 h. The beads were washed with the same buffer, and bound proteins were eluted using 10 mM glutathione in buffer B88-8. The samples were subjected to TCA precipitation, followed by SDS–PAGE and immunoblotting. The anti-GFP antibody (Living Colors A.v. Monoclonal Antibody) was obtained from Clontech. The anti-Sec23p polyclonal antibody was generated against glutathione-Sepharose–purified GST-Sec23p. The anti-Sec61p polyclonal antibody was raised against a synthetic peptide corresponding to residues 12–30. Antibodies directed against Sar1p (Saito-Nakano and Nakano, 2000) and Sec12p (Nishikawa and Nakano, 1993) were described previously.

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