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Human ARF4 Expression Rescues *sec7* Mutant Yeast Cells

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Vesicle-mediated traffic between compartments of the yeast secretory pathway involves recruitment of multiple cytosolic proteins for budding, targeting, and membrane fusion events. The *SEC7* gene product (Sec7p) is a constituent of coat structures on transport vesicles en route to the Golgi complex in the yeast *Saccharomyces cerevisiae*. To identify mammalian homologs of Sec7p and its interacting proteins, we used a genetic selection strategy in which a human HepG2 cDNA library was transformed into conditional-lethal yeast *sec7* mutants. We isolated several clones capable of rescuing *sec7* mutant growth at the restrictive temperature. The cDNA encoding the most effective suppressor was identified as human ADP ribosylation factor 4 (hARF4), a member of the GTPase family proposed to regulate recruitment of vesicle coat proteins in mammalian cells. Having identified a Sec7p-interacting protein rather than the mammalian Sec7p homolog, we provide evidence that hARF4 suppressed the *sec7* mutation by restoring secretory pathway function. Shifting *sec7* strains to the restrictive temperature results in the disappearance of the mutant Sec7p cytosolic pool without apparent changes in the membrane-associated fraction. The introduction of hARF4 to the cells maintained the balance between cytosolic and membrane-associated Sec7p pools. These results suggest a requirement for Sec7p cycling on and off of the membranes for cell growth and vesicular traffic. In addition, overexpression of the yeast GTPase-encoding genes *ARF1* and *ARF2*, but not that of *YPT1*, suppressed the *sec7* mutant growth phenotype in an allele-specific manner. This allele specificity indicates that individual ARFs are recruited to perform two different Sec7p-related functions in vesicle coat dynamics.

Membrane traffic between compartments of the secretory and endocytic pathways is mediated by transport vesicles whose formation is dependent on the assembly of specific protein coats (for reviews, see references 13, 17, 53, 56, and 59). Clathrin coat assembly, and possibly other vesicle coat complexes, also play a role in the sorting of molecules into the nascent vesicle bud. Some coat proteins may carry targeting information for transit to other intracellular locations. At least four distinct membrane coat structures have been described: clathrin-coated vesicles for endocytosis and for lysosomal targeting from the *trans*-Golgi network (32), coatomer or COPI-coated vesicles for traffic from the endoplasmic reticulum (ER) and between cisternae of the Golgi complex (5, 56), COPII-coated vesicles budding from the ER (3, 5, 33), and lace-like coated exocytic vesicles from the mammalian *trans*-Golgi network (34). Other proteins, such as Sec7p, Ypt1p, and Sec16p (15, 18, 38), have been found to coat yeast ER-to-Golgi transport vesicles, but it is not clear whether these molecules form distinct coat structures or if they contribute to COPI or COPII vesicle coat structures.

Both biochemical and genetic approaches have been used to identify proteins required for vesicular transport. The biochemical requirements for vesicular traffic have been studied in cell-free assays that reconstitute intercompartmental protein traffic, and several essential protein components have been characterized (for reviews, see references 2, 6, 41, 56, 58, and 59). Numerous gene products required for yeast secretory pathway function have also been identified by genetic selections for temperature-sensitive secretory (*sec*, *bet*, *bos*, etc.) mutants (for a review, see reference 53). These studies established that the molecular mechanisms in yeast and mammalian secretory pathways are highly similar and that several yeast and mammalian transport components function in analogous or interchangeable ways (2, 6, 14, 17, 29, 37, 50, 63, 65, 79). Because of this, complementation of yeast conditional-lethal mutants with mammalian cDNA has been successfully used to identify and characterize mammalian homologs of yeast gene products (4, 24, 36, 45, 60, 71). In this study, we used this genetic approach in an effort to isolate mammalian homologs of ySec7p and to identify proteins with which it interacts.

Sec7p is an abundant, 230-kDa acidic phosphoprotein required for vesicular traffic at multiple stages of the yeast secretory pathway (1, 21). Growth of *sec7* mutant yeast cells at 37°C results in the accumulation of cargo proteins at each stage where Sec7p function is required, with morphological exaggeration of both ER and Golgi organelles (21, 46, 54, 80). Sec7p is evenly distributed at steady state between cytosolic and membrane fractions (1, 19). Sec7p was proposed to function as a vesicle coat protein, cycling from the cytosol onto membranes during the assembly of transport vesicles (18, 19, 80). The tenets of this hypothesis were strengthened by the observation that antibodies to Sec7p blocked cell-free assays that reconstitute ER-to-Golgi transport, thereby causing the accumulation of coated transport vesicles. Sec7p-coated transport vesicles were efficiently immunoisolated from these cell-free reactions (18). The presence of core-glycosylated α-factor cargo in the immunoisolated vesicles suggests that Sec7p functions coordinately with COPII vesicle coat proteins in ER-to-Golgi traffic (5, 18, 39).

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ADP ribosylation factors (ARFs) are small GTPases involved in vesicular transport (7, 48). ARFs were first isolated as cytosolic cofactors required for cholera toxin-dependent ADP ribosylation of the heterotrimeric G protein, G_s (30). They are distinct from other small GTPases in their GTP-dependent binding to phospholipid micelles or membranes and the absence of intrinsic GTPase activity in purified protein fractions (7). In the yeast *Saccharomyces cerevisiae*, two *ARF* and four *ARL* (ARF-like) gene products have been identified, while six mammalian *ARF* gene products and several additional *ARL* gene products (of unknown function) have been identified (7, 35, 48). The ARF proteins play an important role in cell physiology, since disruption of both yeast *ARF1* (*yARF1*) and *yARF2* genes is lethal (68). The double-deletion mutant (*arf1*Δ *arf2*Δ) can be rescued by expression of any human *ARF* (*hARF*) gene product, each possessing high sequence identity to each other and to yARFs (7, 29, 69). Deletion of the *yARF1* gene, encoding the more abundant *yARF* gene product (90% of total ARF protein), results in cold- and fluoride-sensitive cell growth and aberrant patterns of protein glycosylation, which was interpreted as resulting from protein traffic missorting within the pathway (28, 69). Hence, the yARF proteins were predicted to participate in membrane traffic.

ARF proteins have been localized to the cytosolic surface of the mammalian Golgi membrane (69, 77) and on COPI-coated vesicles (64). ARFs are involved in the regulation of clathrin- and COPI-coat assembly in mammalian cells by recruiting coat complexes onto the donor membrane (48, 56, 67, 76). This view is supported by observations that treatment of mammalian cell-free transport reactions with GTPγS results in the enhanced binding of ARFs to membranes (25, 43, 73); that purified ARFs stimulate the in vitro binding of β-COP (as part of the coatomer COPI protein complex) with Golgi membranes (12); that the addition of mutant ARFs locked into the GTP-bound conformation stabilized coatomer binding to membranes, with the accumulation of coated vesicles and/or exaggeration of Golgi organelles (11, 72, 75, 81); and that depletion of ARFs from mammalian cytosol restricts vesicle budding from the Golgi apparatus (74). Another role recently assigned to ARF is the stimulation of phospholipase D activity (8, 10, 31). This enzyme hydrolyzes phosphatidylcholine, releasing phosphatidic acid and choline. If this activity were restricted to specific sites within the donor membrane, the triggering of phospholipase D by ARFs could initiate vesicle budding through second-messenger cascades and/or through local remodeling of the membrane. The identification of ARFs as suppressors of *sec7* mutant phenotypes reveals insights into their role in vesicle coat dynamics.

MATERIALS AND METHODS

Media, strains, reagents, and antibodies. Cultures were grown as previously described (1) in YP medium (1% yeast extract, 2% peptone, 4% glucose; Difco Laboratories) or minimal medium. Minimal medium plates contained 0.67% yeast nitrogen base, 1 M sorbitol, 2% glucose, and 2% agar with the required amino acid supplements. Strains were grown in 0.67% yeast nitrogen base, 1 M sorbitol, and 4% glucose with the required amino acid supplements. One unit (OD_{600}) of optical density at 600 nm was equivalent to approximately 10^7 yeast cells.

The yeast strains used for genetic tests were AFY89 (*Mat*α *his3-11,15 leu2-3,112 ura3-1 sec7-4*) and AFY73 (*Mat*α *ade2 ura3-1 his3-11,15 sec7-4*). Yeast strains tested for hARF4 suppression were AFY71 (*Mat*α *ade2 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*), AFY281 (*Mat*α *ura3-52 his4-619 sec18-1*), AFY80 (*Mat*α *ade2 his3-11,15 leu2-3,112 trp1-1 ura3-1 sec7-1*), and AFY89. The *Escherichia coli* strain used was HB101 (*hsdR hsdM leu pro recA*).

Analytical-grade reagents were mostly obtained from Sigma (St. Louis, Mo.). Protein A-Sepharose CL4B was from Pharmacia (Piscataway, N.J.). Oxalyticase was obtained from Enzogenetics (Corvallis, Oreg.). Tran^{35}S-label was from ICN (Costa Mesa, Calif.). Glass beads (0.5-mm diameter) were purchased from Biospec (Bartlesville, Okla.) and were acid washed prior to use. ARF-specific antibodies were generated against synthetic peptides unique to each ARF protein (9, 29). R-23 and R-29 were rabbit polyclonal sera against yArf1p and yArf2p, respectively. R-891 and R-1525 were rabbit polyclonal antisera against hArf4p and hArf5p, respectively. Anti-α1,6-mannose, anti-α1,3-mannose, anti-invertase, anti-carboxypeptidase Y (CPY), and anti-Sec7p antisera were rabbit polyclonal antisera described previously (19, 21, 70, 80).

TABLE 1. Growth of *sec7* transformants at 37°C[a]

Plasmid	Growth	
	sec7-1	*sec7-4*
Vector	−	−
SEC7-CEN	++++	++++
hARF4-CEN	−	−
hARF4-2μm	+/−	+++
yARF1-CEN	++	−
yARF2-CEN	−	+++
hARF5-2μm	−	−
YPT1-2μm	−	−

[a] The indicated plasmids were transformed into AFY80 (*sec7-1*) and AFY89 (*sec7-4*), and growth was evaluated on plates as in Fig. 1. Growth of wild-type yeast cells corresponds to ++++ and was indicated by the complementation of the *sec7* mutations with the authentic *SEC7* gene on a centromeric vector.

Genetic selection by human cDNA complementation. A mammalian HepG2 cDNA library (kindly provided by Helen Gibson and Phil Barr, Chiron Corp., Emeryville, Calif.) was inserted into the yeast expression vector pAB23BXN (generously provided by Anthony Brake, Chiron Corp.) under the control of the constitutive glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter (60). The library was amplified in *E. coli* and transformed into AFY89 (*sec7-4*) by spheroplast transformation as described previously (1). Techniques for DNA transformation of *S. cerevisiae* have previously been described (80). After growth at 25°C for 2 days, transformed cells were shifted to 37°C for 2 to 3 days. The transformation efficiency was about 1,000 colonies per μg of DNA, and 56 of 10^5 transformants obtained grew at 37°C. These transformants were first streaked onto rich medium plates, then transferred to minimal medium plates containing 5-fluoroorotic acid to chase out the plasmid at 25°C, and finally cultured at 37°C. Transformed colonies that grew at 37°C in the absence of suppressor plasmids were probably intragenic revertants of the *sec7* mutation and were discarded. Plasmids corresponding to bona fide extragenic suppressors of the *sec7* mutation were isolated from the transformed yeast cells, amplified in *E. coli*, and used to transform another *sec7-4* yeast strain, AFY73, to verify that the suppressor activity resided on a single vector. The cDNA inserts were sequenced by using a Sequenase kit (Amersham, Arlington Heights, Ill.). The sequence of the cDNA suppressor in pAF372 revealed identity to the hARF4 sequence in the GenBank/EMBL data bank (accession number M36341; listed as HUMARF2A in the database), first described by Kahn and colleagues (29).

Genetic suppressor analysis. The following plasmids were transformed into strains AFY71 (wild type), AFY80 (*sec7-1*), and AFY89 (*sec7-4*) for comparison of suppressor effects on growth at the restrictive temperature (summarized in Table 1): SEC7-CEN (pTAYc66) is the authentic *SEC7* gene and promoter in a low-copy-number, centromeric vector (1). The hARF4-2μm (pAF372) and hARF5-2μm (pAF680) plasmids are the hARF4 and hARF5 cDNAs in the high-copy-number vector pAB23BXN under control of the yeast *GAP* promoter (60). The hARF5 cDNA was cloned by PCR amplification from a HeLa cDNA library (6a). The cDNA sequence was confirmed to be identical to that of hARF5. This cDNA was competent for hARF5 protein production in bacteria (41a). The hARF4-CEN plasmid (pCW7) is the hARF4 cDNA under control of the *GAP* promoter from pAF372 subcloned into the low-copy-number, centromeric vector pRS316 (66). The yARF1-CEN (pRB1297) and yARF2-CEN (pRB1306) plasmids express the *yARF1* and *yARF2* genes from their own respective promoters in a centromeric, low-copy-number vector (69). The pGAL-*yARF1*-low-copy-number plasmid (pRB1302) is the *yARF1* gene under the control of the *GAL1* promoter in a centromeric vector (69). The YPT1-2μm (pAAH5) plasmid is the yeast *YPT1* gene under control of the yeast *ADH1* promoter in a 2μm high-copy-number vector (61).

Cell fractionation and immunoblotting. ARF protein immunoblotting was performed by growing transformants in minimal medium containing 1 M sorbitol and appropriate nutrients to 1 OD_{600} per ml. Cells were harvested, lysed with glass beads in 2× sodium dodecyl sulfate (SDS) sample buffer, and heated to 98°C for 5 min. Two hundred micrograms of protein per lane was resolved by 12.5% SDS-polyacrylamide gel electrophoresis and transferred to 0.22-μm-pore-size nitrocellulose (MSI, Westboro, Mass.) overnight at 0.05 to 0.075 A. The nitrocellulose membranes were blocked for 1 h and incubated with primary antibody overnight at room temperature in phosphate-buffered saline–4% bovine serum albumin (BSA)–0.05% Tween 20. Secondary antibodies (peroxidase-coupled, goat anti-rabbit immunoglobulin G [Boehringer Mannheim, Indianapolis, Ind.]) were applied, and then blots were visualized by chemiluminescence with a Dupont-NEN (Boston, Mass.) Renaissance kit.

Fractionation of wild-type and *sec7* mutant yeast cells was performed by growing cells at 25°C in minimal medium containing 1 M sorbitol, 2% glucose, and the appropriate nutrients to 1 OD_{600} per ml and either kept at 25°C or shifted to 37°C for 1 h. Cells were harvested and washed in ice-cold buffer 88 (B88; 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 6.8], 150 mM potassium acetate, 250 mM sorbitol, 5 mM magnesium acetate) and resuspended at 100 OD_{600} ml of B88. The cells were then vortexed 10 times with glass beads for 30 s each time, with chilling on ice for at least 1 min between periods of vortexing. Lysates were precleared by centrifugation at 500 × *g* for 5 min at 4°C, and the supernatants from the low-speed spin were centrifuged at 200,000 × *g* for 30 min at 4°C. The supernatants were saved, and the pellets were resuspended in B88 and recentrifuged at 200,000 × *g* for 20 min. The pellets from the second spin were resuspended in B88 in a volume equivalent to the original lysis volume. The protein concentrations in the samples were measured by the Bradford assay (Bio-Rad, Richmond, Calif.).

Anti-Sec7p immunoblotting was performed by resolving 150 μg of protein from supernatants and pellets of fractionated cells per lane on SDS–7.5% polyacrylamide gels and transferring the blots to nitrocellulose overnight. After blocking, the blots were probed with primary antibody for 3 to 4 h at room temperature. The anti-Sec7p blots were visualized by using [^{125}I]protein A as the secondary label.

Pulse-chase and immunoprecipitation. Strains were grown overnight at 25°C to <1 OD_{600}/ml in minimal medium containing 2% glucose, 1 M sorbitol, and appropriate nutrient supplements. Cells were resuspended in prewarmed medium containing 2% raffinose, 0.1% BSA, 0.1% glucose, 1 M sorbitol, and amino acid supplements. After 30 min at 37°C, Tran^{35}S-label was added at 30 μCi/OD_{600} of cells for 12 min. The chase solution [0.3% L-cysteine and 0.4% L-methionine in 100 mM $(NH_4)_2SO_4$] was diluted 100-fold into the cultures, and aliquots were removed at various time points and diluted with 1 volume of ice-cold 10 mM NaN_3. Cells were pelleted, and cell lysates were prepared by glass bead beating in 2× SDS sample buffer. Lysates were boiled for 5 min and diluted to 1 ml (final volume) with IP-I buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 3.3 mM NaN_3, 50 mM Tris-HCl [pH 7.5]). Extracts were sedimented 13,000 × *g* for 1 min to remove insoluble debris. The supernatants were used for immunoprecipitations.

Immunoprecipitations were carried out as described before (20). Four microliters of anti-invertase serum, 2 μl of anti-CPY serum, 6 μl of anti-α1,6-mannose serum, and 4 μl of anti-α1,3-mannose serum were used to immunoprecipitate their respective antigens per OD_{600} cell equivalent of labeled extract. Five microliters of 20% protein A-Sepharose (Pharmacia) in IP-I buffer per μl of antiserum was added, and immunoprecipitation mixtures were rotated 16 h at 4°C. Samples were washed, resuspended in 2× SDS sample buffer, and boiled for 5 min. Samples were resolved on SDS–8% polyacrylamide gels (33:0.8, acrylamide/bisacrylamide) and subjected to autoradiography.

Invertase induction and assay. Invertase synthesis was induced by transferring log-phase cells from minimal medium containing 4% glucose and 1 M sorbitol to prewarmed minimal medium containing 1% raffinose, 0.1% glucose, 1 M sorbitol, and 0.1% BSA for 1 h at 37°C. Cells were incubated for the indicated times, and then sodium azide was added to prevent further protein secretion (47). Spheroplasts were prepared (20) and then centrifuged through a 3% Ficoll cushion to separate the secreted invertase from internal invertase. Invertase activity was assayed as previously described (47). Secreted invertase activity detected for each strain tested was plotted as the mean of three independent determinations with the standard deviation. Invertase activity was measured in units of activity (10^2) per milligram of protein, where 1 U = 1 μg of glucose released per min at 37°C. Glucose-6-phosphate dehydrogenase was assayed as a marker for cytoplasmic proteins released during the spheroplast preparation as previously described (22).

RESULTS

Human cDNA clones rescued the *sec7* mutant growth phenotype. A human HepG2 cDNA library constructed in pAB23BXN, a high-copy-number yeast expression vector under control of the constitutive *GAP* promoter (60), was used to transform AFY89, a *sec7-4* mutant yeast strain. From ~10^5 transformants, 56 colonies grew at the 37°C restrictive temperature. Twelve of the 56 strains were unable to grow at 37°C in the absence of their human cDNA-containing plasmids. The plasmids isolated from these 12 transformants were cycled through *E. coli* and used to retransform *sec7* mutant yeast cells. All 12 cDNAs retained the ability to suppress the temperature-sensitive *sec7-4* growth defects. Each of the 12 cDNA inserts was distinct by restriction enzyme analysis (data not shown), implying that the selection of cDNA suppressors by complementation had not been saturated. The sizes of the cDNA inserts ranged from 1.4 to 4 kb. Eleven of the cDNA clones weakly supported growth, yielding only very small colonies on plates, while one suppressor cDNA supported *sec7-4* mutant growth to near wild-type levels. The strong suppressor cDNA was sequenced, revealing a 1.4-kb insert encoding hARF4 (29).

hARF4 suppressed *sec7* yeast growth defects at the restrictive temperature. Suppression of the *sec7-4* temperature-sensitive growth phenotype by hARF4 overexpression prompted testing of the other well-characterized allele, *sec7-1*. By using standard methods for mutation site mapping by plasmid gap repair in *S. cerevisiae* (57) and by generating hybrid chimeras between wild-type and mutant *SEC7*, we have determined that the mutations in *sec7-1* and *sec7-4* alleles reside in different regions of the *SEC7* gene (11a). Both mutant yeast strains were transformed with vectors lacking or harboring hARF4, and their growth was comparable to that of similarly transformed wild-type yeast cells. All of the transformed strains were able to grow at 25°C. The hARF4 suppression phenotype was more effective for the *sec7-4* yeast cells than for *sec7-1* cells on solid plates (Fig. 1 and Table 1). In liquid cultures, hARF4 rescued the growth of both mutant strains at the restrictive temperature. The immunoblots in Fig. 2 indicated that hARF4 protein levels from the high-copy-number plasmid were significant, and quantitation of these blots verified that hARF4 was expressed to similar levels in both *sec7-1* and *sec7-4* yeast cells. However, comparisons between hARF4 and yARF protein levels cannot be assessed with these data because of differences in the antibody sensitivities toward the different ARFs (9). It is also important to recognize that ARF protein must be N myristylated for functional activity in vivo (28) and that, in contrast to the endogenous ARFs, N myristylation of hARF4 in yeast cells was not as efficient (27a). Hence, simple analysis of ARF abundance does not necessarily reflect the size of the active protein pool.

The dosage requirement for hARF4 suppression of the *sec7* phenotype was tested by subcloning the hARF4 cDNA into a low-copy-number centromeric vector. Expression of hARF4 from this vector did not suppress *sec7* phenotypes, despite the fact that the same strong, constitutive *GAP* promoter was present in both plasmids (Table 1). Although hARF4 expression is required for suppression of the *sec7* phenotype, high levels of hARF4 protein appear to be detrimental to cell growth. Each strain transformed with the high-copy-number hARF4 plasmid typically yielded ~10-fold-fewer colonies than strains transfected with the vector alone or with the vector expressing other genes. This observation is in agreement with published reports that overexpression of either yeast or mammalian *ARF* genes from the more potent, inducible *GAL1* and *GAL10* promoters caused cell death in wild-type yeast cells (28, 29, 68). Since the lethal dose of ARF expression has not been determined, our observations suggest that either an acceptable level of hARF4 expression was achieved in the *sec7* mutants serendipitously or that the *sec7* mutant background can more easily tolerate ARF overexpression.

Growth suppression by hARF4 was specific to *sec7*^{ts} mutant yeast cells. To determine whether hARF4 suppression was specific for *sec7* mutants, the hARF4 plasmid (pAF372) was transformed into 11 other *sec* mutants (*sec1*, *sec13*, *sec14*, *sec16*, *sec17*, *sec18*, *sec19*, *sec20*, *sec21*, *sec22*, *sec23* mutants) whose defects affect a variety of steps in the yeast secretory pathway from the ER to the cell surface. Several of the genes tested encode known vesicle coat proteins, such as Sec13p and Sec23p (COPII) and Sec21p (γ-COPI) (53). hARF4 expression was unable to suppress the mutant phenotypes of any of the other *sec* mutant strains either on solid media or in liquid culture, demonstrating that the suppressor effect of hARF4 is specific for *sec7* mutations.

The specificity of hARF4 effects was further examined by

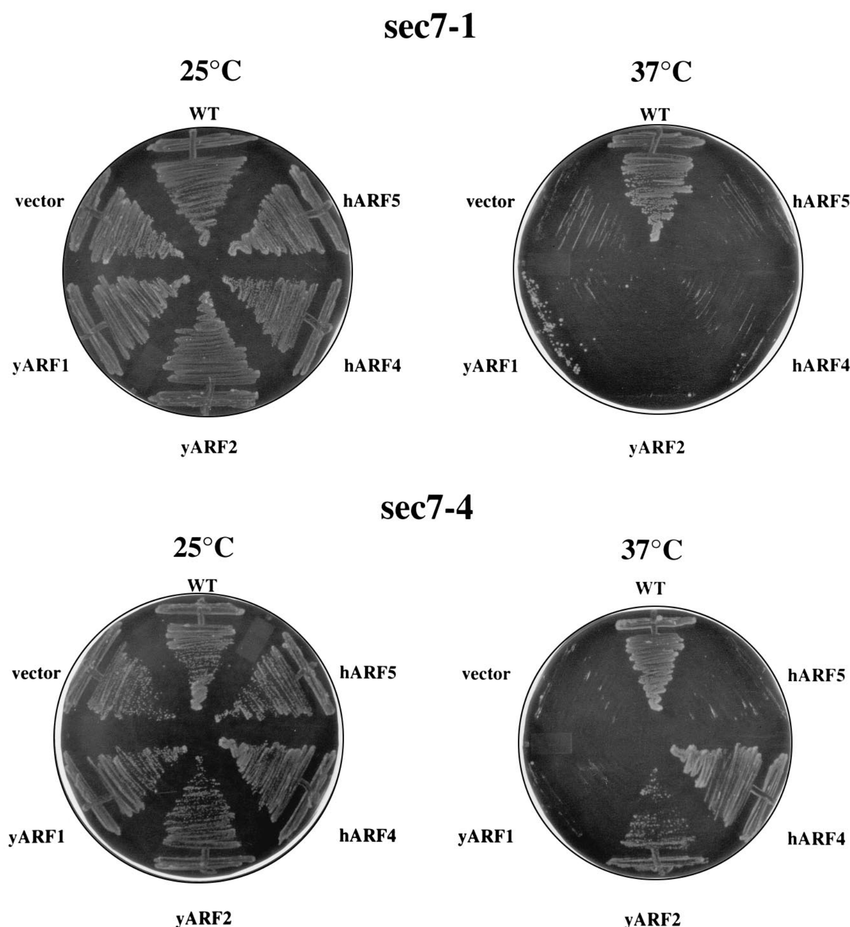

FIG. 1. ARF overexpression rescues *sec7* growth at the restrictive temperature. Wild-type (wt) or *sec7* mutant yeast cells were transformed with vectors harboring no insert (pAB23BXN), with hARF4 on a high-copy-number plasmid under the control of the *GAP* promoter (pAF372), with *yARF1* on a low-copy-number plasmid under the control of its own promoter (pRB1297), with *yARF2* on a low-copy-number plasmid under the control of its own promoter (pRB1306), or with hARF5 in the same high-copy-number expression plasmid as for hARF4 (pAF680). The transformants were grown on selective medium for 2 days at 25 or 37°C.

overexpressing *YPT1*, a gene encoding another GTPase known to function in vesicular traffic. The *YPT1* gene under the control of the *ADH1* promoter in a high-copy-number vector was transformed into both *sec7-1* and *sec7-4* yeast cells. Although the construct overproduced Ypt1p (data not shown and reference 61), it clearly failed to suppress either *sec7* allele (Table 1). We also transformed *sec7* yeast cells with the hARF5 cDNA in the same high-copy-number plasmid construct used for hARF4. The gene encoding hARF5 was chosen because the sequence of its product is closest to that of hARF4 (89% identical) among mammalian ARFs, thereby testing the specificity of the hARF4 effect. The hARF5 construct failed to suppress either *sec7* allele (Fig. 1 and Table 1). Since we were not able to examine hARF5 expression in yeast cells by immunoblot analysis, this result cannot be unambiguously interpreted at this time. However, the inability of Ypt1p overproduction to mimic the hARF4 effects was further evidence for the specificity of *sec7* mutant suppression.

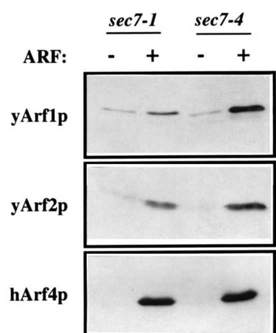

FIG. 2. Overexpression of hARF4 and yARFs in *sec7* transformants. The *sec7-1* and *sec7-4* yeast strains containing vector alone (−) or a vector containing hARF4 (pAF372), *yARF1* (pRB1297), or *yARF2* (pRB1306) (+) were shifted to 37°C for 1.5 h before lysis. Equal amounts of protein were resolved in all lanes. The blots were probed with antibodies specific for each ARF protein and then detected by chemiluminescence.

We used two complementary approaches to test whether hARF4 overexpression bypassed Sec7p function. First, disruption of *SEC7* gene expression in haploid cells by marker gene replacement (1, 57) failed to yield transformants even in the presence of the hARF4 plasmid, suggesting that hARF4 overexpression does not allow cell growth in the absence of the *SEC7* gene product. Further demonstration that hARF4 overexpression does not cause bypass of Sec7p function was obtained with pGAL-SEC7 yeast strains in which *SEC7* gene expression is solely under the control of the *GAL* promoter. Transferring these strains from galactose- to glucose-containing cultures causes Sec7p depletion, arrests growth, and stops

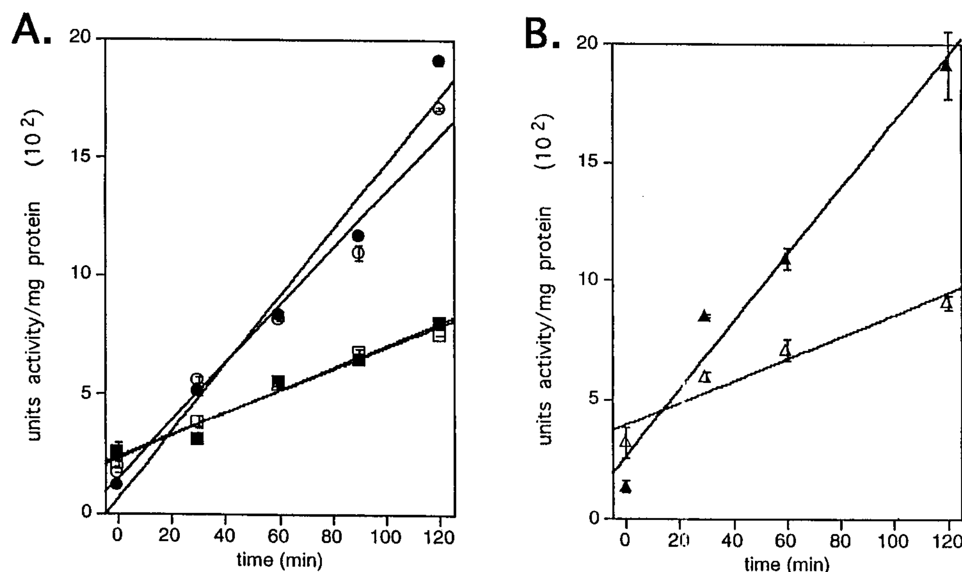

FIG. 3. Invertase secretion was restored in hARF4-expressing *sec7* mutants. Spheroplasts were prepared from strains grown at 37°C for 1 h under low-glucose conditions and then assayed for rate of secreted invertase activity as described in Materials and Methods. The data were plotted as the means of three determinations, with the error bars representing standard deviations. Open and filled symbols represent strains transformed with vectors lacking and harboring the hARF4 cDNA, respectively. (A) Comparison of invertase secretion from wild-type (circles) and *sec18* mutant (squares) yeast cells; (B) invertase secretion from *sec7-1* strains.

traffic through the secretory pathway (80). The presence of the hARF4 suppressor plasmid was unable to sustain cell viability upon Sec7p depletion. These two approaches confirmed that hARF4 overexpression does not eliminate cellular requirements for Sec7p function.

Allele-specific suppression of *sec7*^{ts} mutants by *yARF* genes. To test whether overexpression of the endogenous yARF proteins could also cause suppression, *yARF1* and *yARF2* genes on low-copy-number plasmids were transformed into *sec7* mutant yeast cells. Increasing yARF protein levels suppressed *sec7* mutations, but with surprising allele specificity (Fig. 1 and Table 1). The addition of a second copy of the *yARF2* gene on a plasmid was sufficient to restore growth to *sec7-4* but not *sec7-1* mutants. In contrast, increasing the dosage of the *yARF1* gene product, either from its own promoter (Fig. 1 and Table 1) or from the *GAL1* promoter (not shown), suppressed *sec7-1* but not *sec7-4* yeast growth defects. Quantitation of immunoblots specific for yArf1p (Fig. 2) revealed that protein levels were three- to sixfold higher than endogenous yArf1p levels in wild-type strains. Similar analysis with yArf2p-specific antibodies established that introduction of the low-copy-number *yARF2*-containing plasmid led to a 4.5- to 9-fold increase in yArf2p levels. Again, differential antibody sensitivities preclude direct comparison of the relative levels of Arf1p and Arf2p in the transformants. However, since yArf1p is normally 10-fold more abundant than yArf2p in wild-type cells (29), the 4.5- to 9-fold increase in yArf2p from the plasmid would imply that the total ARF protein concentration (Arf1p plus Arf2p) was at best doubled, yet that increase was sufficient to specifically suppress *sec7-4* but not *sec7-1* mutations. Conversely, these data suggest that Arf1p overproduction led to a sixfold increase in total ARF protein pools, yet the additional yArf1p restored *sec7-1* but not *sec7-4* mutant growth. This unambiguous allele-specific suppression of *sec7*^{ts} mutants strongly points to differential activities for ARFs in vesicular traffic.

hARF4 expression in *sec7* mutants restored protein secretion. To test whether hARF4 expression in *sec7* yeast cells suppressed the mutant growth defects by altering the efficiency or fidelity of protein transport through the secretory pathway, three methods were used to monitor protein traffic. The first approach involved quantitation of invertase secretion at the restrictive temperature. Wild-type yeast cells transformed with vectors lacking or harboring hARF4 secreted invertase at ~15 U of activity per mg of protein per min (Fig. 3A). This rate was fivefold higher than that measured for *sec18* strains with or without hARF4 or for *sec7* mutants transformed with vector alone. However, transformation of *sec7* yeast with the hARF4 plasmid restored secretion to wild-type levels (Fig. 3B). Control experiments established that invertase in the supernatant did not result from leakage from spheroplasts during cell lysis, as negligible amounts of a cytosolic marker protein, glucose-6-phosphate dehydrogenase, were released (data not shown). Therefore, the rate of invertase secretion in *sec7* but not *sec18* mutants at 37°C was restored to wild-type levels by hARF4 expression.

hARF4 expression in *sec7* mutants restored CPY delivery to the vacuole. Previous analysis of *sec7* mutants has established that Sec7p is involved not only in protein secretion but also in vacuolar biogenesis (21, 70, 80). For the second approach, we examined the processing of the well-characterized CPY in *sec7* cells expressing hARF4. CPY is a soluble enzyme that undergoes several covalent protein modifications in transit from the ER through the Golgi apparatus to the vacuole. The core-glycosylated 67-kDa ER form (p1) receives outer-chain mannoses in the Golgi to become the fully glycosylated 69-kDa (p2) species. The mature 61-kDa polypeptide is formed by proteolytic processing in the vacuole (70).

Pulse-chase experiments were performed, and CPY was immunoprecipitated from whole yeast lysates (Fig. 4). At 25°C, wild-type, *sec18*, and *sec7-1* yeast cells showed similar kinetics of CPY transit from the ER to the vacuole (half-time of ~7.5 min) without hARF4 (not shown) or with hARF4 (Fig. 4A to C, lanes 1 to 4). At 37°C, CPY accumulated as the p1 ER form in *sec18* mutants, regardless of hARF4 presence (Fig. 4B, lanes 5 to 12). In *sec7* yeast cells at 37°C, CPY was recovered mostly as the p1 form, with some accumulation of an aberrant p1*

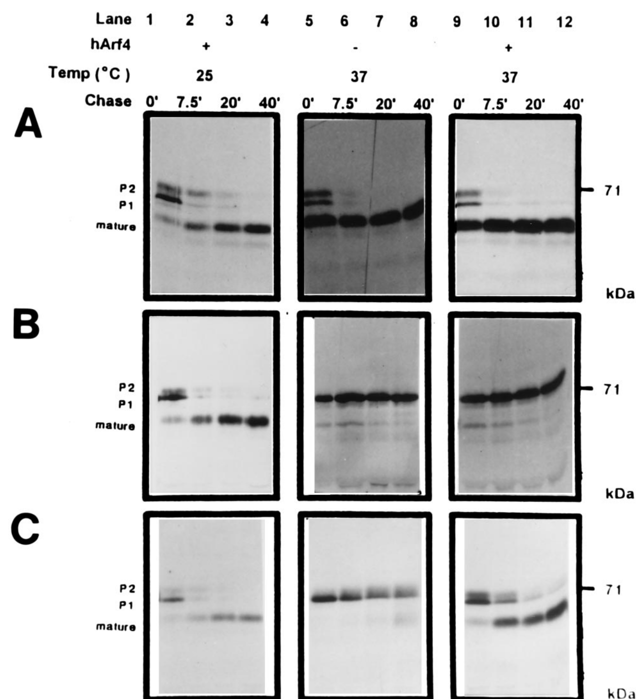

FIG. 4. CPY transit to the vacuole resumed in *sec7* yeast cells expressing hARF4. Cells were grown to log phase and then shifted to 37°C for 30 min. Cells were pulse-labeled for 12 min with Tran^{35}S-label, and then the metabolic labeling was chased with excess unlabeled methionine and cysteine for the indicated times. CPY was immunoprecipitated from total cell lysates as described in Materials and Methods from wild-type (AFY71) (A), *sec18-1* (AFY281) (B), and *sec7-1* (AFY80) (C) strains transformed with vectors either lacking (−) or harboring (+) the hARF4 cDNA. CPY undergoes several covalent modifications in transit from the ER (p1 form; 67 kDa) through the yeast Golgi apparatus (p2 form; 69 kDa) to the vacuolar compartment (mature CPY; 61 kDa).

species, as reported previously (21) (Fig. 4C, lanes 5 to 8). In contrast, addition of hARF4-expression restored CPY transit through the secretory pathway, albeit with slightly reduced kinetics (Fig. 4C, lanes 9 to 12). The kinetic differences of CPY transport in wild-type and hARF4-expressing *sec7* mutant cells at 37°C were consistent with the generation times for these strains.

Outer-chain carbohydrates with distinct linkages are transferred to nascent glycoproteins in transit through early and late compartments of the Golgi apparatus (21, 23). The availability of specific antibodies that recognize the proximal Golgi outer-chain α1,6-mannose or distal Golgi α1,3-mannose glycosyl modifications was used to test whether the normal sequential transit of CPY was reconstituted with proper fidelity in *sec7* yeast cells suppressed by hARF4 expression. CPY from [^{35}S]methionine-labeled cell lysates was incubated with antibodies specific to α1,6-mannose or to α1,3-mannose linkages of yeast outer-chain carbohydrates. We observed that the mature CPY species from *sec7* mutants expressing hARF4 exhibited both Golgi modifications (data not shown). These results indicated that pathway traffic fidelity from the ER to the vacuole was restored in *sec7* yeast cells expressing hARF4.

hARF4 expression affected Sec7p distribution in *sec7* mutant yeast cells. Because Sec7p is found in both cytosolic complexes and membrane-associated fractions (19, 39), we examined its distribution in wild-type and *sec7* yeast cells without or with hARF4 expression. In wild-type yeast cells, Sec7p was found in both high-speed supernatant (cytosol) and pellet (membrane-associated) fractions. This distribution was not altered by either temperature or hARF4 expression (Fig. 5). In *sec7-4* mutants at the permissive growth temperature, Sec7p distribution resembled that in wild-type yeast cells, although the total Sec7p pool was considerably reduced (Fig. 5). The ability of reduced Sec7p levels to support growth was not surprising, as we have observed that yeast cells can grow with wild-type kinetics even if the Sec7p concentration is reduced eightfold (80).

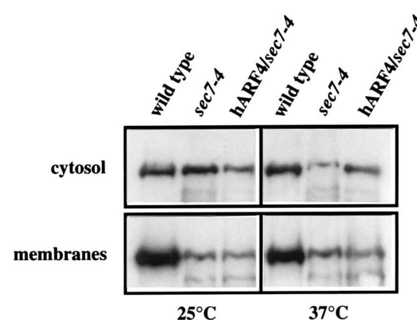

FIG. 5. Sec7p distribution in *sec7-4* yeast cells with and without hARF4. Lysates were prepared from wild-type, *sec7-4*, and *sec7-4* yeast cells expressing hARF4 grown at 25°C or shifted to the restrictive temperatures (37°C) for 90 min. Supernatants and pellets were collected from high-speed centrifugation of total yeast lysates (see Materials and Methods), and equivalent amounts of the two fractions were probed for Sec7p by immunoblot and then detected by [^{125}I]protein A.

At the restrictive temperature without hARF4, the soluble Sec7p pool was greatly reduced (Fig. 5) or in some experiments eliminated, depending on the length of incubation at the restrictive temperature (11a). In the presence of hARF4, higher levels of mutant Sec7p were observed in the soluble fraction (Fig. 5), and the relative Sec7p distribution in membrane and soluble fractions again resembled that in the mutants at the permissive growth temperature. Of further note, the amount of Sec7p in the membrane fractions was unchanged in the *sec7* mutants without or with hARF4 (Fig. 5). These results suggested that the soluble pool and membrane-associated pools of mutant Sec7p no longer exchanged at the restrictive temperature unless hARF4 was present.

DISCUSSION

The present work provides several principal observations about ARF function in *S. cerevisiae*, with implications for all eukaryotes. First, a genetic search for human homologs of ySec7p and its associated proteins unexpectedly revealed that hARF4 expression rescues the *sec7* growth defect. Expression of hARF4 on high-copy-number but not low-copy-number vectors restores near-normal rates of growth and protein transport through each compartment of the secretory pathway in *sec7* yeast cells at the restrictive temperature. The effects of hARF4 expression appear to be specific to *sec7* mutants, since it neither rescues the lethality of *SEC7* gene disruptions nor suppresses the temperature-sensitive growth of 11 other *sec* mutants tested. Second, overexpression of the *yARF1* and *yARF2* genes, but not *YPT1*, also suppresses the *sec7* growth defects. Expression of the endogenous yARFs from their own promoters on low-copy-number plasmids is sufficient for suppression. Third, the observation of allele-specific suppression by yARFs provides strong evidence that Sec7p and the two endogenous ARFs functionally interact in distinct ways. This genetic evidence is supported by the demonstration that hARF4 expres-

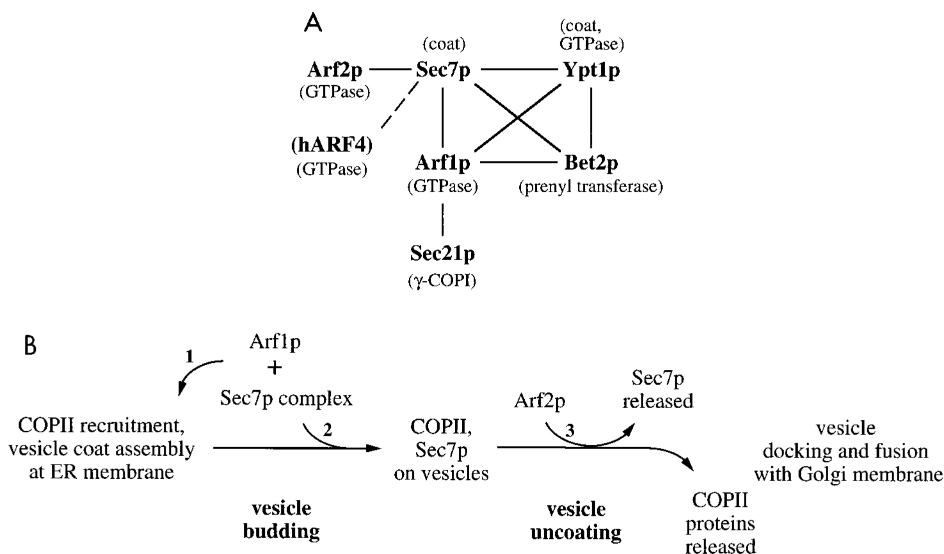

FIG. 6. Molecular interactions involving *ARF* genes and *SEC7*. (A) Cartoon representing a summary of genetic interactions surrounding *ARF* genes and *SEC7*. The lines indicate evidence for genetic interactions, namely, synthetic lethality, synthetic defects, and mutant suppression (taken from references 39 and 69 and this study). (B) Model describing the predicted involvement of ARFs and Sec7p in vesicle coat assembly and disassembly. Arf1p is predicted to support Sec7p binding to the membrane, either during vesicle assembly (arrow 1) or after vesicle budding (arrow 2). In contrast, Arf2p is required to trigger Sec7p release from the vesicle coat, possibly prior to release of COPII coat proteins. From the available data, we cannot predict when Arf2p associates with the vesicle coat structure. However, the 10-fold-lower abundance of Arf2p than of Arf1p suggests that its association will not be stoichiometric with the other coat proteins and may initiate the uncoating process.

sion altered the distribution of mutant Sec7p at the restrictive temperature. Finally, this biochemical result is predicted to reflect a functional relationship between ARFs and Sec7p in vesicular traffic and suggests that ARFs trigger vesicle coat disassembly, in addition to functioning in the recruitment of coat proteins to the vesicle membrane.

Genetic evidence for ARF-Sec7 protein interactions. Analysis of double-mutant combinations giving rise to synthetic defects and synthetic lethality is often predictive of protein-protein interactions (27). Genetic evidence linking ARFs and Sec7p was first observed when yeast cells harboring both *arf1*-null (*arf1*Δ) and *sec7-1* mutations exhibited synthetic defects. The temperature sensitivity of double mutants was exacerbated such that the cells cease growth at 30°C instead of 37°C (69).

Double-mutant combinations of *arf1*Δ with either *sec21*, *ypt1*, or *bet2* mutation were found to be synthetically lethal (69) (Fig. 6A). In the case of *sec21*, the genetic result is explainable in biochemical terms, since it was subsequently shown that Sec21p is related to the γ subunit of the mammalian COPI coatomer complex (26) and that mammalian coatomer binding to membranes requires hARF1 function (see below). None of the core COPII coat components were identified by the double-mutant genetic analysis with *arf1*Δ mutants.

Figure 6A summarizes the available evidence for genetic interactions involving Sec7p and ARFs. Significantly, at least four of these molecules, Sec7p, Arf1p, Sec21p, and Ypt1p (5, 18, 62), have been found coating transport vesicles, and the results from this work would predict the vesicle association of yArf2p as well. Bet2p, a prenyltransferase, is required to confer membrane attachment competence to both Ypt1p and Sec4p (55). The symmetric genetic interactions of *BET2* and *YPT1* with both *SEC7* and *ARF1* strengthens the proposal for functional interactions between ARFs and Sec7p.

Our observations that overexpression of the human and yeast *ARF* gene products rescues *sec7* defects in an allele-specific manner significantly extends the genetic evidence for interactions between ARFs and Sec7p. It is important to note that the fidelity of the allele specificity was not compromised by further increasing the production of yARF protein, since even the high expression of *yARF1* from the strong *GAL* promoter, which was able to suppress the *sec7-1* mutation, did not rescue *sec7-4* yeast cells. The allele-specific effects predict that the two mutant alleles affect different domains of Sec7p function that interact differentially with the two endogenous yARFs.

Furthermore, the importance of our results is not limited to vesicular traffic in *S. cerevisiae*. Despite the lack of tangible evidence for a mammalian homolog of Sec7p, our genetic results suggest the existence of a human Sec7p homolog that should exhibit similar interactions with hARF4 in vesicular traffic.

Sec7p and ARFs function at multiple stages of the secretory pathway. The fact that both Sec7p and ARFs are associated with vesicle coat structures suggests a biochemical explanation for our observations. Genetic and biochemical evidence shows requirements for Sec7p function in protein traffic from the ER and through different compartments of the yeast Golgi apparatus (18, 21, 39, 46, 80). Anti-Sec7p antibodies efficiently immunoisolate α-factor containing vesicular intermediates from an in vitro ER-to-Golgi transport assay, demonstrating that Sec7p is a constituent of the transport vesicle protein coat (18). At the time these studies were performed, we proposed that Sec7p may play a structural rather than informational role in the formation of transport vesicles, analogous to that of clathrin (42). However, more recent studies using purified proteins and enriched membrane fractions have demonstrated that neither COPI nor COPII vesicle budding requires the presence of soluble Sec7p (5). As shown in Fig. 6B, the addition of soluble Sec7p is required for targeting the COPII-dependent, ER-derived vesicles to the *cis*-Golgi compartment (39). In this model, Sec7p is recruited in vitro onto vesicles after budding from the organelle membrane (Fig. 6B, arrow 2). This observation does not preclude the possibility that in vivo, Sec7p is recruited to vesicles during their formation

at the organelle membrane (Fig. 6B, arrow 1) yet acts after budding is completed.

ARFs regulate both COPI- and clathrin-coated vesicle formation in mammalian cells (for reviews, see references 16 and 48; also see references 40, 43, and 78). Evidence to date provides no indication that ARFs are involved in the formation or consumption of COPII-coated vesicles. The most widely accepted model for ARF function is that the association of ARFs with a saturable binding site on membranes directly stabilizes coat protein binding to membranes until completion of vesicle coat assembly. Alternatively, it has been proposed that ARFs function indirectly by promoting remodeling of the Golgi membrane through its activation of phospholipase D (8, 10, 31). It is unclear why multiple ARFs are present in the cell and whether ARFs operate with distinct, overlapping or redundant activities. The high degree of sequence similarity among ARFs may allow for limited interchangeability, thus explaining how ARF1 could be implicated in multiple functions at different intracellular locations (for reviews, see references 44 and 48). Deletion of either yARF1 or yARF2 is not lethal in *S. cerevisiae*, presumably because the two ARFs are able to substitute for one another (68). However, the allele-specific suppression of *sec7* mutants by different ARFs provides an opportunity to dissect the distinct roles of the different ARFs in *S. cerevisiae*.

ARF regulation of Sec7p membrane association. Clues toward a biochemical rationale for our genetic results with ARFs and Sec7p are provided by examining the effects of the elevated ARF protein levels on the behavior of the mutant Sec7p. Because Sec7p is found in both cytosolic complexes and membrane-associated fractions (19, 39), we examined its distribution without or with hARF4 expression. At the restrictive temperature without hARF4, the soluble Sec7p pool is greatly reduced or eliminated, while the amount associated with the pellet fractions appears unchanged. The disappearance of soluble Sec7p may result from increased degradation of the mutant protein. On the other hand, the mutant Sec7p is locked onto its membrane anchor, protecting that fraction from degradation, which also would explain the traffic block and exaggerated organelle morphology at the restrictive temperature. In the presence of hARF4, the relative Sec7p distribution in supernatant and pellet fractions once again resembles that observed in the permissive growth state. These results suggest that ARF proteins regulate Sec7p cycling on and off the membranes and that both soluble and membrane-associated Sec7p pools participate in vesicular traffic. A decreased cycling rate at the restrictive temperature would result in stagnation of the cytosolic Sec7p pool, increasing the probability for degradation of the misfolded mutant protein. The addition of ARFs to the mutant drives the Sec7p membrane on-off cycle, thereby promoting vesicular traffic, with the additional consequence of restraining degradation of the cytosolic pool.

How do ARFs govern Sec7p distribution? Either directly or indirectly, through a third protein, interactions between ARFs and Sec7p could be initiated in the cytosol or on the membrane surface. Evidence against cytosolic interactions come from observations that mammalian ARFs exist as monomeric proteins in cytosol (9, 74), and attempts to coimmunoprecipitate ARFs with Sec7p have proven unsuccessful (11a). Therefore, we favor the hypothesis that functional interactions between ARFs and Sec7p occur on the membrane surface during Sec7p recruitment events and/or during vesicle coat disassembly (Fig. 6B). Support for membrane surface interactions comes from findings that ARFs are required for COPI coatomer binding to membranes and that GTP hydrolysis by ARFs is required for coat disassembly (11, 12, 49, 51, 52, 72, 75, 81). Our data provide interesting new evidence for ARF involvement in releasing proteins from the membrane, thus highlighting a role for ARFs in disassembly in addition to the well-established recruitment of coat proteins to the membrane. Since multiple ARFs are present within yeast and mammalian cells, and multiple ARFs suppress different domains of Sec7p function, Sec7p may require one ARF for membrane recruitment (Fig. 6B, arrow 1 or 2) and another for driving disassembly from the vesicle coat (Fig. 6B, arrow 3). The 10-fold-greater abundance of Arf1p than Arf2p implies that Arf1p may function stoichiometrically for recruitment of the COPI and Sec7 coat proteins into vesicle coats throughout the secretory pathway. Uncoating may be initiated by a single molecule per vesicle and therefore could be accommodated by the less abundant Arf2p. Future studies will utilize proteins from heterologous and homologous systems to biochemically dissect the participation of individual ARFs in vesicle coat dynamics.

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