Characterization of a Novel ADP-ribosylation Factor-like Protein (yARL3) in Saccharomyces cerevisiae*

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ADP-ribosylation factors (ARFs) are highly conserved, ~20-kDa guanine nucleotide-binding proteins that enhance the ADP-ribosyltransferase activity of cholera toxin and have an important role in vesicular transport. Several cDNAs for ARF-like proteins (ARLs) have been cloned from human, Drosophila, rat, and yeast, although the biological function(s) of ARLs is unknown. We have identified a yeast gene (yARL3) encoding a protein that is structurally related (>43% identical) to the mammalian ARF-like protein ARP. Biochemical studies of purified recombinant yARL3 protein revealed properties similar to those of ARF and ARL proteins, including the ability to bind and hydrolyze GTP. Like other ARLs, recombinant yARL3 did not stimulate cholera toxin-catalyzed auto-ADP-ribosylation. Anti-yARL3 antibodies did not cross-react with yARFs or yARL1. yARL3 was not essential for cell viability, but disruption of yARL3 resulted in cold-sensitive cell growth. At the nonpermissive temperature, processing of alkaline phosphatase and carboxypeptidase Y in arl3 mutant was slowed. yARL3 might be required for protein transport from endoplasmic reticulum to Golgi or from Golgi to vacuole at nonpermissive temperatures. On subcellular fractionation, unlike its mammalian homologue ARP, yARL3 was detected in the soluble fraction but not in the plasma membrane. Indirect immunofluorescence analysis revealed that yARL3 when overexpressed was associated in part with the endoplasmic reticulum-nuclear envelope. Thus, the structural and functional characteristics of yARL3 indicate that it may have a unique role(s) in vesicular trafficking.

ADP-ribosylation factors (ARFs) are a family of ~20-kDa GTP-binding proteins (or GTPases) that includes both ARFs and ARF-like proteins (ARLs) (for recent reviews, see Refs. 1 and 2). ARFs were originally identified and purified on the basis of their ability to increase the ADP-ribosyltransferase activity of cholera toxin (3, 4). Members of the ARF family have been identified in every eukaryotic cell examined; at least six ARFs have been identified in mammalian tissues. They share 65–96% overall amino acid identity and include identical consensus sequences involved in guanine nucleotide binding and GTP hydrolysis.

The physiological roles of at least some ARF proteins involve the regulation of vesicular transport in the endoplasmic reticulum, Golgi, endosomes, or nuclear membranes. The best characterized is ARF1, which is required for the assembly of coat proteins on Golgi membranes (5, 6) and of AP-1 adaptor particles on the trans-Golgi network (7, 8). In in vitro assays, ARFs 1, 3, and 5 differed in their binding to Golgi (9), as well as in their dependence on accessory proteins for interaction with Golgi and, perhaps, other cellular membranes (10). In addition, recombinant human ARF6 was localized to the plasma membrane (11) and might, like the Rho-related GTPases, regulate plasma membrane architecture and participate in endocytosis by mediating cytoskeletal reorganization (12).

ARF function depends on its alternation between inactive GDP-bound and active GTP-bound conformations. As ARF has no detectable GTPase activity and exchanges bound nucleotide very slowly at physiological concentrations of Mg2+, its cycling between active and inactive forms is controlled by GTPase-activating proteins and guanine nucleotide-exchange proteins. In in vitro assays, ARFs also have been shown to stimulate the activity of phospholipase D, an enzyme found in Golgi membranes (13, 14), raising the speculation that phospholipase D may mediate ARF signals to initiate coated vesicle formation (15, 16).

cDNAs encoding proteins with similarity to the ARF isoforms, designated ARF-like or ARL, have been cloned from several species, including human, rat, mouse (17, 18), and yeast (17–22). The products of these genes appear to lack ADP-ribosyltransferase-enhancing activity, and they differ in GTP-binding requirements and GTPase activity from ARF isoforms. Although some of these proteins exhibit tissue- and/or differentiation-specific expression, the biological functions of ARLs are unknown.

ARP, a mammalian ARF-like protein, was identified and characterized by cDNA cloning (23). It is 33–39% identical to members of the ARF family and contains the characteristic sequence motifs involved in nucleotide binding (DVG, NKQD, and CAT sequences) and GTP hydrolysis (GLDXAGK) (1). ARP differs, however, from other ARF family proteins by the absence of a myristoylation site, an insertion of eight amino acids between the GLDXAGK and DVGG consensus sequences, and the capacity to hydrolyze bound GTP in the absence of other

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1 The abbreviations used are: ARF, ADP-ribosylation factor; yARF, recombinant ARF from Saccharomyces cerevisiae; dARF, recombinant ARF from Drosophila melanogaster; ARL, ARF-like protein; ER, endoplasmic reticulum; PGR, polymerase chain reaction; kDa, kilodaltons; PAGE, polyacrylamide gel electrophoresis; LY, Lucifer Yellow; ALP, alkaline phosphatase; CPY, carboxypeptidase Y; GTPγS, guanosine 5′-O-(thiotriphosphate).

2 F.-J. S. Lee, C.-Y. Lin, C.-F. Huang, and L.-M. Buu, unpublished data.
proteins. Most of the ARP protein, unlike ARFs, is associated with plasma membrane instead of the cytosol.

Four members of the ARF family have been reported in yeast Saccharomyces cerevisiae. Those include three ARFs (yARF1 (24), yARF2 (25), and yARF3 (26)) and an ARF-like protein (yARL1 (22)). yARF1 and yARF2 are thought to act in ER-to-Golgi secretory protein sorting; yARF3 is most similar in amino acid sequence to human ARF6, which has been implicated in the regulation of early endocytic transport. yARL1 is a recently characterized ~20-kDa GTPase, which may in part reside in the Golgi and which has a function distinct from those of yARF1 and yARF2.

Sequence analysis of the S. cerevisiae genome identified a ARF-like gene, yARL3, the product of which is homologous to mammalian ARP (23). This report describes the genetic, molecular, and biochemical characterization of yARL3. Like yARL1 and yARF3, yARL3 is not essential for cell viability; however, arl3 mutants appear to be cold-sensitive. yARL3 is required for vacuolar protein transport at nonpermissive temperatures. Unlike mammalian ARP, which was exclusively detected on the plasma membrane, yARL3 was found in the soluble fraction and associated in part with the ER-nuclear envelope structures. Thus, yARL3 may function in a novel ER-associated vesicular trafficking pathway.

**MATERIALS AND METHODS**

**Strains, Media, and Microbiological Techniques**—The S. cerevisiae strains used in this study are listed in Table I. Yeast culture media were prepared as described by Sherman et al. (27).YPD and YPGal contained 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose or 2% galactose, respectively; SD contained 0.7% Difco yeast nitrogen base (without amino acids) and 2% glucose. Nutrients essential for auxotrophic strains were supplied at specified concentrations (27). Sporulation, growth, and mating were carried out as described (28). Yeast strains were transformed by the lithium acetate method (29). Plasmids were constructed by standard protocols (30).

**Polymerase Chain Reaction**—Unless otherwise specified, the protocol used for PCR amplification was 35 cycles of 1 min at 95 °C, 1 min at 52 °C, 1 min at 72 °C, followed by extension at 72 °C for 10 min, in a volume of 50 μL. PCR products were digested with NdeI and BamHI, purified, and annealed to expression vector pET15b (Novagen), yielding pET15byL3. For the non-fusion protein, PCR products were digested with NdeI and BamHI, purified, and annealed to expression vector pT77 (34), yielding pT7yARL3. BL21 (DE3) cells containing expression plasmids were grown to a density of 1.0 at which time the inducer, isopropyl-β-D-thiogalactopyranoside, was added to a final concentration of 1 mM. After 3 h, cells were harvested by centrifugation, washed once in 20 mM Tris, pH 7.4/1 mM EDTA, and stored at −80 °C until used. For large scale production of recombinant proteins, 5 mL of overnight culture were used to inoculate 1 liter of LB broth containing ampicillin (100 μg/mL), followed by shaking at 37 °C. When A600 reached 0.6–0.8, protein production was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 3 h, and bacteria were collected by centrifugation and stored at −20 °C. Cell pellets were suspended in 10 mL of phosphate-buffered saline (pH 7.4) containing lysozyme, 0.5 mg/mL, and disrupted by sonication. The lysate was centrifuged after addition of Triton X-100 to 1%, and His-tagged fusion protein was isolated on Ni2+-NTA resin (Qiagen, Chatsworth, CA) by standard methods. Purity was assessed by SDS-PAGE and staining with Coomassie Blue. Protein was assessed by SDS-PAGE and staining with Coomassie Blue.

**Polyclonal Antibody Production**—The recombinant N-terminal His-tagged fusion protein yARL3 was synthesized in Escherichia coli using a yeast genomic DNA as template and primers complementary to sequences upstream or downstream of the Lpe21p gene (Yeast Genome Project, accession number U39205). The nucleotide sequence of yARL3 has GenBank accession number U89332. DNA sequences were analyzed and multiple protein alignments were prepared using a GeneWorks software package (IntelliGenetics, Inc., Mountain View, CA). yARL3 Gene Disruption—yARL3 DNA generated by PCR was subcloned into pGEM-TZI plasmid resulting in pGyL3. The yeast URA3 gene was inserted at the single EcoRI site in the yARL3 gene as follows: The 3.8-kb DNA fragment containing the yeast URA3 gene and two hisG repeat sequences was excised from the plasmid pNY51 (32) by digestion with BglII and BamHI; the 5′ overhangs were filled in with Klenow fragment. Plasmid pGyL3, containing the yARL3 gene, was linearized at the internal EcoRI site; the overhang ends were filled in with Klenow fragment and the cDNA was ligated to the 3.8-kb hisG-URA3-hisG fragment, resulting in pGyL5U.

**Expression and Purification of Recombinant Proteins**—The open reading frame of yeast ARL3 was obtained by PCR amplification using a yeast genomic DNA as template and primers complementary to sequences upstream or downstream of the Lpe21p gene (Yeast Genome Project, accession number U39205). The nucleotide sequence of yARL3

**Table I: Yeast strains**

| Strain          | Genotype* | Source       |
|-----------------|-----------|--------------|
| SEY 6210.5      | MATa/MA Ta his3/13, trpl/trpl, ura3/ura3, leu2/1eu2 | S. Cheng    |
| MGD.1d          | MATa ade2, arg4, cyh4, his3, trpl, ura3, ARF1, ARF2, ARF3, ARL1, ARL3 | J. Sherman  |
| MGD.1drp        | MATa ade2, arg4, cyh4, his3, trpl, ura3, ARF1, ARF2, arf3–1, ARL1, ARL3 | Ref. 26     |
| MGD.1dr3p13     | MATa ade2, arg4, cyh4, his3, trpl, ura3, ARF1, ARF2, arf3–1, ARL1, ARL3 | This work   |
| MGD.1d1p        | MATa ade2, arg4, cyh4, his3, trpl, ura3, ARF1, ARF2, ARF3, arl1–1, ARL3 | This work   |
| MGD.1d1p13      | MATa ade2, arg4, cyh4, his3, trpl, ura3, ARF1, ARF2, ARF3, arl1–1, ARL3 | This work   |
| YPH250          | MATa ade2, his3, leu2, ly2, trpl, ura3–52, ARF1, ARF2, ARF3, arl1–1, ARL3 | Ref. 22     |
| YPH250dr3p      | MATa ade2, his3, leu2, ly2, trpl, ura3–52, ARF1, ARF2, ARF3, arl1–1, ARL3 | This work   |
| YPH250dl1p      | MATa ade2, his3, leu2, ly2, trpl, ura3–52, ARF1, ARF2, ARF3, arl1–1, ARL3 | This work   |
| YPH250dl3p      | MATa ade2, his3, leu2, ly2, trpl, ura3–52, ARF1, ARF2, ARF3, arl1–1, ARL3 | This work   |
| YPH250dl3p13    | MATa ade2, his3, leu2, ly2, trpl, ura3–52, ARF1, ARF2, ARF3, arl1–1, ARL3 | This work   |
| YPH252          | MATa ade2, his3, leu2, trpl, ura3–52, ARF1, ARF2, ARF3, arl1–1, ARL3 | Ref. 22     |
| YPH252dl3       | MATa ade2, his3, leu2, trpl, ura3–52, ARF1, ARF2, ARF3, arl1–1, ARL3 | This work   |
| YPH252dl3p      | MATa ade2, his3, leu2, trpl, ura3–52, ARF1, ARF2, ARF3, arl1–1, ARL3 | This work   |
| YPH252dl3p13    | MATa ade2, his3, leu2, trpl, ura3–52, ARF1, ARF2, ARF3, arl1–1, ARL3 | This work   |

* ade, adenine-requiring; arg, arginine-requiring; cyh, cyehochoxidase-resistant; cyh, cyehochoxidase-sensitive; his, histidine-requiring; trp, tryptophan-requiring; ura, uracil-requiring; leu, leucine-requiring. arl3 represents arf3;hisG;uracil; arl3 represents arf3;hisG-URA3-hisG; arl3 represents arf3;hisG-URA3-hisG; and arl1–1 represents arl1–1.
Characterization of a Yeast ARL3 Protein

pET15-b expression plasmid (Novagen), isolated on Ni²⁺-NTA resin, and further purified by SDS-PAGE. Denatured purified proteins from SDS-PAGE gel were used as antigens to raise polyclonal antibodies in rabbits essentially as described (35).

Yeast Cell Extracts and Immunoprecipitation—Whole cell extracts were prepared by homogenizing yeast with three optical density units (A₅₀₀)/ml of cells. Yeast cell concentration was assessed by absorbance at 600 nm. Cells were suspended in radioimmune precipitation buffer (50 mM Tris-HCl (pH 8.0), 0.1% SDS, 0.5% deoxycholic acid, 150 mM NaCl, and 1% Nonidet P-40) to a final A₅₀₀ of 30. Whole cell extracts were then prepared by vortexing with glass beads for 2 min at 4 °C and clarified by centrifugation. Proteins separated by SDS-PAGE were electroblotted to Immobilon-P membranes (Millipore Corp.). Incubation with antibodies was carried out in phosphate-buffered saline (pH 7.4) containing 0.1% Tween 20 and 5% dried skim milk at room temperature for 60 min. The anti-HA monoclonal antibody (HA.11, Berke- lundy Antibody Co., Richmond, CA) and horseradish peroxidase-conju- gated goat anti-mouse IgG + IgM (H + L) were each diluted 1:5000. Bound antibodies were detected with the ECL system (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Primary and secondary antibodies and lumino substrate were removed from the blot using the blot-stripping protocol (Amersham Pharmacia Biotech).

Construction of HA Epitope-tagged yARL3 and yARL(Q78L) Mutant—The ADH1 promoter (36), as a primer (gcacatatgtttcatttagtcaagg) and 5'-end primers to generate the full-length Q78L mutant sequence was amplified so that a viral bovine brain phosphoinositides (1 mg/ml), and incubated at 30 °C. Every 5 min, samples were transferred to 2 ml of ice-cold 20 mM Tris-Cl (pH 7.4), 100 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, before rapid filtration on 0.45 µm HA filters (Millipore, Bedford). The amount of nucleotide bound to the fusion protein was quantified by scintillation counting. Data were fitted to a first-order rate equation.

GTP hydrolysis was determined by binding (α<sup>32</sup>P)GTP to 5.0 µm recombinant yARL3 protein, as described by Randazzo and Kahn (40) followed by its dilution (1:9) into 25 mM HEPEs (pH 7.4), 100 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, and 0.1% (v/v) Tween 20. The amount of bound GTP was measured by monitoring the production of inorganic phosphate using the Millipore timer kit.

Nucleotide Binding and Hydrolysis—Binding of GTPyS to purified recombinant yARL3 was determined by a filter trapping method (39). Unless otherwise specified, 1 µg of Histagged yARL3 fusion protein was added to 25 mM HEPEs (pH 7.5), 100 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, before rapid filtration on 0.45 µm HA filters (Millipore, Bedford). The amount of nucleotide bound to the fusion protein was quantified by scintillation counting. Data were fitted to a first-order rate equation.

Experimentally determined samples were transferred to 2 ml of ice-cold 20 mM Tris-Cl (pH 7.4), 100 mM NaCl, 10 mM MgCl₂, and 1 mM dithiothreitol before rapid filtration on 0.45 µm HA filters (Millipore, Bedford). The amount of nucleotide bound to the fusion protein was quantified by scintillation counting. Data were fitted to a first-order rate equation.

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Subcellular Fractionation by Velocity Sedimentation on Sucrose Den- sity Gradients—Cells were harvested by centrifugation from cultures (50 ml), grown in YPD to mid-exponential phase (A₅₀₀ = 1). Cells (~0.5 g) were washed by repeated suspension in ice-cold Na₂SO₄ (10 mM in double-distilled H₂O) and centrifugation, incubated with Lyticase to form spheroplasts, suspended in 0.2 ml of ice-cold lysis buffer (20 mM triethanolamine (pH 7.2), 1 mM EDTA, 0.8% s sorbitol) containing pro- tease inhibitors (aprotinin, leupeptin, and pepstatin, each 1 µg/ml; 1 mM benzamidine; and 1 mM phenylmethylsulfonyl fluoride), and disrupted on ice with 20 strokes in a Dounce homogenizer. The cell lysate was centrifuged (450 × g) twice for 10 min to remove unbroken cells and cellular debris. For gradient fractionation of cell organelles, 0.8 ml of the clarified supernatant was loaded on top of a manually generated five-step sucrose gradient containing 50, 40, 30, 20, and 10% sucrose (in lysis buffer), which was then subjected to centrifugation (~170,000 × g) for 3.5 h at 4 °C in a Beckman SW55 rotor. Twelve fractions were collected manually from the top. Proteins in samples (100 µl) of fractions were precipitated with 10% trichloroacetic acid, separated by SDS-PAGE, and analyzed by immunoblotting. Antibodies were kindly provided by Dr. Schroder-Kohne (anti-Em47p) and Dr. Dieter Gallwitz (anti-ALP).

Indirect Immunofluorescence—Cells were grown in 5 ml of minimal selective medium with 2% glucose to a density of 1–2 × 10⁷ cells/ml and prepared for indirect immunofluorescence as described (41) with the following modifications. To each culture, 0.6 ml of 37% formaldehyde was added for fixation, and the cultures were gently shaken at 30 °C for 2 h. Cells were collected by centrifugation (2500 × g for 5 min), washed once in 5 ml of 0.1 M potassium phosphate (pH 6.5) buffer, suspended in 1 ml of solution P (1.2 M sorbitol, 0.1 M potassium phosphate, pH 6.5), and incubated at 30 °C for 30 min with 5–10 µl of Lyticase (10,000 units/ml, solution P) and 1% of β-mercaptoethanol. The cells were collected by centrifugation (3000 × g for 5 min), washed with solution P, and suspended in 100–200 µl of solution P. Samples (30 µl) of cells were placed in each well of a multilwell slide that had been coated with 0.1% polylysine. Following aspiration of nonadherent excess cells, the slides were washed once with a washing buffer containing 100 mM Tris-HCl (pH 9.0) and 150 mM NaCl and then incubated for 1 h with antibody blocking buffer (100 mM Tris-HCl, pH 9.0, 150 mM NaCl, 5% nonfat milk, 0.1% Tween 20), followed by a 2-h incubation with the primary antibody in antibody blocking buffer. The slides were then washed twice with washing buffer and once with wash buffer containing 1% anti- body, were washed extensively with the washing buffer again. Mouse monoclonal anti-HA antibody 12CA5, and fluorescein isothiocyanate- conjugated secondary antibodies (Cappel) were diluted 1:1000 to 1:300 for use, respectively. Texas Red-conjugated goat anti-rabbit IgG antibody (Amersham Pharmacia Biotech) was used to detect rabbit polyclonal antibodies. Nuclei were visualized by staining with H33258.
TABLE II

| Class I ARF | yARF1 | 32 | 1 |
| Class II ARF | hARF4 | 32 | 1 |
| Class III ARF | hARF6 | 32 | 1 |
| Other ARFs | yARF1 | 34 | 24 |
| ARF-like proteins | hARL1 | 31 | 21 |
| | hARL2 | 30 | 18 |
| | hARL3 | 30 | 20 |
| | hARL4 | 33 | Footnote 2 |
| | hARL5 | 30 | Footnote 2 |
| | hARPI | 43 | 23 |
| | rARP1 | 44 | 23 |

*Percentage identity of deduced amino acid sequences to that of yARL3 was calculated using the GeneWorks program.

(2 μg/ml), which was included in mounting solution. Polyclonal anti-Kar2 antibody was kindly provided by Dr. Mark Rose. Fluorescence microscopy was performed with a Nikon Microphot SA microscope. Cells were viewed at a magnification of × 1000. Exposure times for immunofluorescence photographs were 15 or 30 s.

RESULTS AND DISCUSSION

Identification of Yeast ARL3—To date, four members of the ARF/ARL family have been characterized in S. cerevisiae: yARF1, yARF2, yARF3 (also known as yARL2), and yARL1 (3, 22, 25, 26). Evidence was provided that yARF1 and yARF2 act in ER-to-Golgi protein sorting. yARF3 is most similar to human ARF6, which has been implicated in the regulation of early endocytic transport. yARL1 may reside in the Golgi apparatus and regulate the vesicular traffic from trans-Golgi to vacuole (22).2 Sequence analysis of the S. cerevisiae genome revealed an additional gene that shares significant similarity with members of ARF/ARL family (temporarily named Lpe21 in the Yeast Genome Project). The human and rat homologues of this gene, ARP, were originally identified as products of PCR amplification using degenerate primers derived from conserved members of the ARL family (23). We propose that the yeast Lpe21 be renamed ARL3 based on its properties (see below) and following the nomenclature of Saccharomyces Genome Database guidelines. The full-length open reading frame of yARL3 (549 bases) encodes a protein of 198 amino acids. yARL3 has a calculated molecular mass of ~23 kDa. Alignment of the deduced amino acid sequence of yARL3 and related ARFs and ARLs revealed that yARL3 is more identical (43%) to mammalian ARF than to ARFs (28-33%) or ARLs (28-33%) (Table II). Like its mammalian homologues (ARP), yARL3 does not have a glycine at position 2, the site of N-myristoylation in ARF/ARL proteins, and has an insertion of eight amino acids between the GDXAGK and DVGG consensus sequences. In addition, yARL3 lacks cysteine residues near the C terminus, which are sites of isoprenylation in non-ARF members of the Ras superfamily. Alignment of the yARL3 protein with other ARL and ARF proteins is shown in Fig. 1. There are 33 amino acids that are identical in yARL3 and the mammalian ARPs, including two consensus GTP-binding sequences, WDXGGQ and NKQD, found in the ARF family proteins (Fig. 1). These are residues and sequences that are thought to be involved in binding of the guanine moiety and the magnesium ion, or in protein-protein interactions, and are very highly conserved in the ARF family. Because of its sequence similarity to ARF/ARL family, we decided to study the function of yARL3 in more detail.

yARL3 Is Not an Essential Gene, but yarl3 Mutants Are Cold-sensitive for Growth—To investigate the function of yARL3, we prepared strains in which the corresponding open reading frame was disrupted by URA3 marker gene (see under “Materials and Methods”). A DNA fragment containing the yARL3:hisG-URA3-hisG sequence was used to transform ura3/ura3 diploid yeast (SEY6210.5) (Table I) (29). Ura+ transformants were isolated and used to confirm the correct replacement of one of the two genomic copies of yARL3. The verified heterozygous diploids were then subjected to sporulation and tetrad dissection. On germination at 30 °C, most diploid cells gave rise to four viable spores. Ura+ spores, but not ura- spores, contained the replacement of yARL3 (data not shown) and lacked yARL3 protein as judged by immunoprecipitation (see below). As each of the strains (haploid) containing the arl3 disruption (hisG-URA3-hisG) was viable, yARL3 is not an essential gene under optimal growth conditions at 30 °C. yARL3 is a single copy gene and is located on chromosome XVI (Yeast Genome Data Bank). Total RNA from yeast (wild-type or arl3 mutant) during mid-log growth in either glucose- or galactose-containing medium was subjected to electrophoresis, transferred to GeneScreen Plus, hybridized with the yARL3 DNA probe, and after stripping, with a yeast γ-tubulin probe. The ~0.8-kb yARL3 RNA, similar to that of yARF1, was not repressed by growth in glucose and was not detected in the arl3 mutant (data not shown).

The double deletion of yARF1 and yARF2 is lethal, but each of the five known human ARF proteins can restore vegetative growth to this double deletion mutant (42, 43). Cells with double deletions of yARL3 and yARF3 or yARL3 and yARL1 were also viable (data not shown). Proper disruption of the specific genes was confirmed by PCR on genomic DNA prepared from colonies of the mutants. This result confirmed that yARL3, like yARL1 and yARF3, is not essential for cell viability and that its deletion was not being complemented by yARL1 or yARF3.

To assess whether yARL3 can affect growth phenotype, growth rates of wild-type, arl3 mutant, and overexpressed yARL3 strains were determined. We constructed a recombinant yARL3 clone with a nine-amino acid influenza virus HA epitope (44) fused to its C terminus, placed the HA-tagged allele (yARL3-HA) under control of the ADH1 promoter, and expressed it in wild-type and arl3 mutant yeast. The arl3 mutants and yeast overexpressing yARL3, had only ~10% lower specific growth rates than wild-type cells in glucose synthetic medium (data not shown).

The replacement of Gbn by Leu at position 71 in ARF1 is analogous to the transforming Ha-Ras Q61L mutation in the second highly conserved guanine nucleotide-binding region, which is essential for the function of all guanine nucleotide-binding proteins (45). In ras, this substitution reduces the intrinsic GTP hydrolytic activity significantly, rendering the protein constitutively active with GTP bound (46). An identical effect on activity was observed for ARF1(Q71L), and the effects of the ARF1(Q71L) mutant on vesicular traffic in vitro were similar to those of GTP*S on ER to Golgi and intra-Golgi transport (47). We also constructed recombinant yARL3 with Leu replacing Gln at amino acid 78 position, yARL3(Q78L).

Although overexpression of yARF1 or yARF1(Q71L) in yeast was believed to interfere with normal cellular functions and hence affect cell growth, we did not observe a similar growth
defect when wild-type yARL3 or yARL3(Q78L) was overexpressed under optimal growth conditions at 30 °C (data not shown).

Growth of wild-type cells, those overexpressing yARL3(Q78L), arl3 mutant cells, and those overexpressing yARL3 was also determined at different temperatures. Wild-type and arl3 mutant yeast cells were transformed with vectors harboring no insert (pVT101U) or with yARL3(Q78L) or yARL3 expression plasmids, respectively. At 37 and 30 °C, all of these cells grew nearly as well as the wild-type strain (Fig. 2). At 15 °C, however, growth of the arl3 mutant was severely impaired. Expression of yARL3 complemented the growth defect of arl3 mutant, indicating that the growth defect of the null mutant was caused by disruption of yARL3. Moreover, overexpression of yARL3(Q78L) in wild-type yeast can cause growth defective at 15 °C. It is conceivable that overexpressed yARL3(Q78L), similar to ARF1(Q71L) (44), might interfere with yARL3-mediated vesicular transport at 15 °C.

Specific Immunoreactivity of Antibody against yARL3—Recombinant proteins were purified and antibodies were prepared as described under "Materials and Methods." Purified His-tagged fusions with yARL3, arl3 mutant cells, and those overexpressing yARL3 was also determined at different temperatures. Wild-type and arl3 mutant yeast cells were transformed with vectors harboring no insert (pVT101U) or with yARL3(Q78L) or yARL3 expression plasmids, respectively. At 37 and 30 °C, all of these cells grew nearly as well as the wild-type strain (Fig. 2). At 15 °C, however, growth of the arl3 mutant was severely impaired. Expression of yARL3 complemented the growth defect of arl3 mutant, indicating that the growth defect of the null mutant was caused by disruption of yARL3. Moreover, overexpression of yARL3(Q78L) in wild-type yeast can cause growth defective at 15 °C. It is conceivable that overexpressed yARL3(Q78L), similar to ARF1(Q71L) (44), might interfere with yARL3-mediated vesicular transport at 15 °C.

Specific Immunoreactivity of Antibody against yARL3—Recombinant proteins were purified and antibodies were prepared as described under “Materials and Methods.” Purified His-tagged fusions with yARL3, yARL1, yARF1, yARF2, and yARF3 (~30–40 ng) were subjected to SDS-PAGE under reducing conditions, transferred to polyvinylidene difluoride membranes, and visualized by silver staining. At a dilution of 1:5000, the polyclonal antibody specific for yARL3 did not cross-react with yARF1, yARF2, yARF3, or yARL1 (data not shown). In addition, polyclonal antibodies against yARF1, yARF2, yARF3, and yARL1 failed to react with yARL3 on Western blot analysis (data not shown). Immunoblotting with the yARL3 antiserum allowed detection of 1–2 ng of yARL3, whereas no signal was detected with recombinant yARF1, yARF2, yARF3, and yARL1 (up to 100 ng) (data not shown). Thus, yARL3 was immunologically distinguishable from yARF and yARL1.

Expression of Endogenous yARL3—To confirm the presence
of yARL3 protein in yeast, proteins from lysates of wild-type, arl3 mutant, and wild-type cells overexpressing yARL3 were separated by SDS-PAGE and stained with Coomassie Blue (Fig. 3A). Although overexpressed yARL3 was detected by the antibody against yARL3, endogenous yARL3 was not detected (Fig. 3A).

As a more sensitive means to identify endogenous yARL3 protein, we prepared lysates from cells that had been incubated with Pro-mix L-35S label (blend of [35S]methionine and [35S]cysteine) for 1 h. Immunoprecipitation with yARL3 antibody permitted detection of [35S]-labeled endogenous yARL3 protein from wild-type and those overexpressing yARL3(Q78L), but not arl3 mutant cells (Fig. 3B). These results demonstrated the existence of yARL3 in yeast, although in much lesser abundance (less than 0.005%) than yARF1 and yARF2, which represent approximately 0.03–0.1% of total yeast protein (25).

Function of yARL3 in Vesicular Transport—To evaluate the role of yARL3 in vesicular transport, we examined both endocytic and exocytotic pathways. First, we measured the glycosylation and proteolytic processing of CPY and vacuole ALP, enzymes that are transported from the ER to Golgi and finally to vacuole by distinct sorting machineries (48). Pulse-chase labeling with [35S]-labeled cysteine and methionine of wild-type, arl3, and arf1 mutant cells at the permissive temperature (37 °C) or nonpermissive temperature (15 °C) was followed by immunoprecipitation of CPY or ALP. The core-glycosylated P1 form of the CPY proenzyme in the ER is converted to the P2 form by further glycosylation in the Golgi apparatus and finally is proteolytically processed in the vacuole to the mature form. ALP is a type II membrane protein that is delivered to the Golgi to the vacuole is reported to differ from that of CPY (48). Upon arrival at the vacuole, precursor ALP is cleaved at a site near the C terminus to yield a mature membrane-spanning form of the hydrolase. At the permissive temperature, similar to the wild-type cells, the arl3 mutant converted CPY and ALP from the ER to Golgi and vacuole forms (Fig. 4, A and C). The arf1 mutant, however, accumulated core-glycosylated CPY in the P1 form and pro-ALP form as expected. At the nonpermissive temperature, processing of alkaline phosphatase and carboxypeptidase Y in arl3 mutant was slower than it was at 37 °C (Fig. 4, B and D). Thus, yARL3 may have a biological function different from that of yARF1/yARF2, with involvement in a distinct ER to Golgi or Golgi to vacuole protein transport pathway.

Effect of the arl3 Mutant on Fluid-phase Endocytosis—Previous work demonstrated that some ARF proteins participate in endosome fusion reactions, as well as in traffic through the secretory pathway (47, 49). In addition, the mammalian homologue of yARL3, ARP, was detected on the plasma membrane and thus might be involved in some plasma membrane-related events (e.g. endocytosis). To determine whether yARL3 might function in an endocytic pathway, we investigated the effect of yARL3 on the uptake of the fluid-phase marker, Lucifer Yellow (LY). LY is a small fluorescent organic anion that is often used as a marker for fluid-phase endocytosis (38). The uptake of LY is time- and energy-dependent (50) and requires certain proteins that are important for endocytosis (Ref. 51 and references therein). Wild-type and arl3 mutant cells were incubated with LY at either the permissive temperature (30 °C) or the nonpermissive temperature (15 °C) for various times. Cells were washed, mounted, and viewed under phase-contrast and fluorescence optics. At the permissive temperature, arl3 mutant cells appeared defective in accumulation of LY after incubation for 30 min but not after incubation for 90 min (data not shown). Throughout this experiment, vacuolar morphology of both wild-type and arl3 mutants appeared normal. At the nonpermissive temperature, fluid-phase endocytosis of LY was found to be impaired in arl3 mutant compared with wild-type cells after incubation for 2 h (Fig. 5, left column). After incubation with LY for 4 h at the nonpermissive temperature, wild-type cells exhibited unambiguous vacuolar staining, whereas most of the arl3 cells had less clearly stained vacuoles. Moreover, arl3 mutants were found to contain vacuoles of aberrant sizes after incubation at the nonpermissive temperature for 4 h (Fig. 5H).
Prior study has shown that overexpression of mutant ARF1 protein inhibited fluid-phase endocytosis (47). A GTPase-activating protein of yeast ARF1, Gcs1, was also found to participate in endocytosis at the nonpermissive temperature (52). More recently, ARF was shown to have a fundamental role in regulating membrane dynamics, peroxisome biogenesis and was required for maintenance of yeast Golgi and endosome structure and function (53, 54). Hence, the action of yARL3 may be similar to that of ARF in regulating membrane dynamics and organelle biogenesis.

**Subcellular Localization of yARL3**—As the cellular localization of yARL3 could provide clues to its function, a cell lysate was subjected to sucrose density centrifugation. Samples of fractions were subjected to SDS-PAGE, and proteins were transferred to polyvinylidene difluoride filters; yARL3, yARF1, a Golgi marker (Emp47p), and a vacuole marker (ALP) were identified with specific antibodies and detected using the ECL system with exposure to Hyper-film-MP. Lane W contains yeast total lysate; lane M indicates positions of protein standards (from top: 105, 98, 53, 33, and 24 kDa). Gradient fractions are numbered from the top.

Lysate of spheroplasts from wild-type cells overexpressing yARL3 was fractionated by sucrose gradient centrifugation. Samples of fractions were subjected to SDS-PAGE, and proteins were transferred to polyvinylidene difluoride filters; yARL3, yARF1, a Golgi marker (Emp47p), and a vacuole marker (ALP) were identified with specific antibodies and detected using the ECL system with exposure to Hyper-film-MP. Lane W contains yeast total lysate; lane M indicates positions of protein standards (from top: 105, 98, 53, 33, and 24 kDa). Gradient fractions are numbered from the top.

Because yARL3 and yARF1 appeared to dissociate from membranes upon cell lysis, we investigated the intracellular localization of yARL3 by indirect immunofluorescence. Fixed, permeabilized wild-type cells were incubated with anti-yARL3 antibody and then with fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies. Nuclei were labeled with the DNA-binding dye H33258. No specific signal was observed (data not shown), presumably due to the low abundance of yARL3 in wild-type yeast. When yARL3-HA was overexpressed in wild-type cells, most of the immunoreactive yARL3 appeared concentrated in a continuous circum-nuclear distribution typical of ER (Fig. 7). This is reminiscent of what has been seen by others with antibodies directed against perinuclear staining typical for ER proteins, such as Kar2p (55), Sce62p (56), or Sec63p (57). In parallel experiments, we observed similar staining patterns with both antibody 12CA5 directed against the HA-epitope of yARL3-HA, and anti-Kar2p (55). In both cases, neither punctate staining typical for Golgi localization nor staining of the vacuole was evident. Because yARL3 was overexpressed using a multicopy-plasmid, large variations from cell to cell in levels of HA-yARL3 expression were seen and more cytosolic yARL3 was detected as diffuse than as reticular staining. From the combined results of subcellular fractionation and indirect im-

![Fig. 4](image-url) **Immunoprecipitation of labeled CPY and ALP.** Wild-type (ARF1/ARL3), arl3 mutant, and arf1 mutant cells were grown and radiolabeled with 35S-labeled methionine and cysteine at the permissive (37 °C) or nonpermissive (15 °C) temperature. Immunoprecipitates were prepared as described under “Materials and Methods.” P1 CPY is the core-glycosylated form found in the ER, P2 is the outer chain-glycosylated Golgi form, and the mature form (M) results from proteolytic processing in the vacuole. pro-ALP is proenzyme form, and mALP is the mature form in the vacuole. Chase time (min) is indicated. A, CPY at the permissive temperature; B, CPY at the nonpermissive temperature; C, ALP at the permissive temperature; D, ALP at the nonpermissive temperature.

![Fig. 5](image-url) **Accumulation of LY in vacuole.** The LY assays were performed on the wild-type and arl3 mutant as described under “Materials and Methods.” Wild-type (A, B, E, and F) or arl3 mutant (C, D, G, and H) was grown at 15 °C and transferred to fresh YPD before addition of LY for 2 h (A–D) or 4 h (E–H) and then viewed by phase-contrast (right) and fluorescence (left) microscopy.

![Fig. 6](image-url) **Subcellular fractionation to localize yARL3 protein.** Lysate of spheroplasts from wild-type cells overexpressing yARL3 was fractionated by sucrose gradient centrifugation. Samples of fractions were subjected to Western blot analysis (Fig. 6). Most of the yARL3, apparently a soluble cytoplasmic form, was at the top of the gradient. Although yARF1 is known to function in Golgi transport, >90% of it was also in the soluble fraction as found previously (22). Because yARL3 and yARF1 appeared to dissociate from membranes upon cell lysis, we investigated the intracellular localization of yARL3 by indirect immunofluorescence. Fixed, permeabilized wild-type cells were incubated with anti-yARL3 antibody and then with fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies. Nuclei were labeled with the DNA-binding dye H33258. No specific signal was observed (data not shown), presumably due to the low abundance of yARL3 in wild-type yeast. When yARL3-HA was overexpressed in wild-type cells, most of the immunoreactive yARL3 appeared concentrated in a continuous circum-nuclear distribution typical of ER (Fig. 7). This is reminiscent of what has been seen by others with antibodies directed against perinuclear staining typical for ER proteins, such as Kar2p (55), Sce62p (56), or Sec63p (57). In parallel experiments, we observed similar staining patterns with both antibody 12CA5 directed against the HA-epitope of yARL3-HA, and anti-Kar2p (55). In both cases, neither punctate staining typical for Golgi localization nor staining of the vacuole was evident. Because yARL3 was overexpressed using a multicopy-plasmid, large variations from cell to cell in levels of HA-yARL3 expression were seen and more cytosolic yARL3 was detected as diffuse than as reticular staining. From the combined results of subcellular fractionation and indirect im-
munofluorescence, we conclude that yARL3 is probably associated in part with ER membranes, which differs from the recently reported plasma membrane localization of mammalian ARP (23).

Biochemical Properties of Recombinant yARL3 Protein—To determine whether the yARL3 gene product has ARF activity, recombinant yARL3 synthesized in and purified from E. coli was assayed as described under “Materials and Methods.” The His-tagged yARL3 fusion protein did not stimulate auto-ADP-ribosylation of cholera toxin A1 protein, in the presence of 100 μM GDP, and was maximal in 60 min at 30 °C. With and without dimyristoylphosphatidylcholine/cholate, recombinant yARL3 bound, respectively, 3.2 ± 0.3 and 1.5 ± 0.2 pmol of GDP/S/μg protein. Therefore, GDP/S binding to yARL3, like that to ARF, was modified by the added phospholipid/detergent (data not shown) (17). In contrast, binding of GDP/S to hARL2 and hARL3 was affected very little by added lipid or detergent (18, 20). Purified mammalian ARF1 and ARF3 lack detectable of GTPase activity (below 0.0015 min⁻¹) (40). Maximal rates of GTP hydrolysis by hARL2 and hARL3 were 0.0074 and 0.005 min⁻¹, respectively (18, 20). Recombinant yARL3 had a rate of ~0.01 min⁻¹, considerably less than that of the mammalian homologue ARP, which was 0.093 min⁻¹ (23).

At least four small GTPases (ARF1, ARF2, SAR1, and YPT1) are known to be involved in ER-to-Golgi vesicular transport in yeast. Sar1 is an essential gene, the product of which is required for vesicle budding from the ER (58, 59). The Ypt1 gene product is involved in either ER-to-Golgi or early Golgi transport and is required for cell viability (60, 61). Like yARL1 and yARF3, yARL3, at its normal level of expression, clearly cannot replace yARF1 and yARF2, as their double deletion is lethal. Recently, mammalian and yeast ARL1 were reported to be localized to Golgi membranes (22, 62). Although several ARL proteins have been identified, little is known about their cellular function. We report here the identification and characterization of yeast ARL3 with structural similarity to mammalian ARP. yARL3 was not essential for cell viability, but disruption of the yARL3 gene resulted in cold-sensitive cell growth. With the temperature-sensitive arl3 mutant, we can isolate suppressors that complement the cold-sensitive growth phenotype of the arl3 null mutant. Although ER-to-Golgi vesicular traffic did not appear affected in the arl3 mutant at permissive temperature (37 °C), retardation in the maturation of vacuolar proteins (ALP and CPY) and an impairment in fluid phase endocytosis at the nonpermissive temperature (15 °C) were observed. ARF is widely believed to play a critical role in recruiting coatomer (COPI) to Golgi membranes to initiate vesicle budding. Newer observations indicate, however, that organelle morphology is significantly more affected than transport in the arf mutants, suggesting a fundamental role for ARF in regulating membrane dynamics (53, 54). The newly identified yARL3, which is, in part, associated with ER-nuclear envelope, may participate in another kind of vesicular transport. Although the involvement of yARL1 and yARL3 in the vesicular transport pathway has been demonstrated, their specific cellular functions and molecular mechanisms of action remain subjects for investigation.

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