Characterization of an ADP-ribosylation Factor-like 1 Protein 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 are believed to participate in vesicular transport in both exocytic and endocytic pathways. Several ARF-like proteins (ARLs) have been cloned from *Drosophila*, rat, and human; however, the biological functions of ARLs are unknown. We have identified a yeast gene (ARL1) encoding a protein that is structurally related (>60% identical) to human, rat, and *Drosophila* ARL1. Biochemical analyses of purified recombinant yeast ARL1 (yARL1) protein revealed properties similar to those ARF and ARL1 proteins, including the ability to bind and hydrolyze GTP. Like other ARLs, recombinant yARL1 protein did not stimulate cholera toxin-catalyzed auto-ADP-ribosylation. yARL1 was not recognized by antibodies against mammalian ARLs or yeast ARFs. Antibodies against mammalian ARLs or yeast ARFs. An-ti-yARL1 antibodies did not cross-react with yeast ARFs, but did react with human ARLs. On subcellular fractionation, yARL1, similar to yARF1, was localized to the soluble fraction. The amino terminus of yARL1, like that of ARF, was myristoylated. Unlike *Drosophila* Arl1, yeast ARL1 was not essential for cell viability. Like rat ARL1, yARL1 might be associated in part with the Golgi complex. However, yARL1 was not required for endoplasmic reticulum-to-Golgi protein transport, and it may offer an opportunity to define an ARL function in another kind of vesicular trafficking, such as the regulated secretory pathway.

ADP-ribosylation factors (ARFs) are members of the Ras superfamily of 20-kDa guanine nucleotide-binding proteins, including both ARFs and ARF-like proteins (ARLs) (for recent reviews, see Refs. 1 and 2). Members of the ARF family have been identified in all eukaryotic cells. These include six mammalian ARFs (3–8) as well as ARFs in *Drosophila melanogaster* (9, 10), *Saccharomyces cerevisiae* (3, 11), *Giardia lamblia* (12), and *Plasmodium falciparum* (13). They share 65–96% amino acid identity and consensus sequences believed to be involved in guanine nucleotide binding and GTP hydrolysis (14). ARFs activate cholera toxin-catalyzed ADP-ribosylation (3–13). The stimulatory effects of certain phospholipids and detergents on this reaction are ARF-specific (15, 16).

ARFs, along with *ypf1*- and *rab*-related gene products (17, 18), have been implicated in vesicular transport, involving the endoplasmic reticulum, Golgi apparatus, endosomes, and nuclear membranes (19–23). It seemed likely that mammalian ARFs should have different functions and intracellular localizations. Consistent with this view, ARF1, ARF3, and ARF5 differed in their binding to the Golgi apparatus (24, 25) as well as in their dependence on accessory proteins for interaction with the Golgi apparatus and, perhaps, other cellular membranes (26, 27). In addition, recombinant human ARF6 was localized to the plasma membrane (28–30) and might, like the *Rhodopsin*-related GTPases, regulate plasma membrane architecture (29) and participate in endocytosis (30). In *vitro* assays, ARFs also have been shown to stimulate the activity of phospholipase D (31–33), raising speculation that ARFs may exert their effects by altering phospholipid metabolism (34–37). Such effects have not, however, been demonstrated in intact cells.

Recently, cDNAs encoding proteins with similarity to the ARF isoforms, designated ARF-like or ARL, have been cloned from several species (38–43), including *Drosophila* (ARL1 and ARL2), human (ARL1, ARL2, ARL3, ARL4, 2 and ARL5 2), rat (ARL1, ARL3, and ARL4), and mouse (ARL4 2). The products of these genes do not appear to activate cholera toxin and differ in GTP binding requirements (dependence on magnesium and phospholipids) from ARF isoforms. Although some of these proteins exhibit tissue- and/or differentiation-specific expression, the biological functions of ARLs remain unknown.

To determine whether ARLs are also present in *Saccharomyces*, where their functions might perhaps be more readily defined, we isolated by polymerase chain reaction a new ARL cDNA from a yeast library. This report describes the genetic, molecular, and biochemical characterization of the yeast homologue (yARL1) of mammalian and *Drosophila* ARL1. yARL1 is not required for ER-to-Golgi protein transport. Unlike *Drosophila* ARL1, yARL1 is not essential for cell viability.

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*§* The abbreviations used are: ARFs, ADP-ribosylation factors; yARF, recombinant yeast ARF (*S. cerevisiae*); ARLs, ARF-like proteins; hARL, human ARL; rARL, rat ARL2; ER, endoplasmic reticulum; PCR, polymerase chain reaction; kb, kilobase pairs; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; GTPγS, guanosine 5′-O-(3-thiotriphosphate).

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The nucleotide sequence reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number U89332. The complete sequence of this fragment had been deposited in data bases by the Yeast Genome Project (accession number Z36033, chromosome II, ORF: YBR164c).

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MATERIALS AND METHODS

Yeast Culture—Yeast culture media were prepared as described by Sherman et al. (44). 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 (44). Sporulation, growth, and mating were carried out as described (45). Yeast cells were transformed by the lithium acetate method (46). Plasmids were constructed by standard protocols (47). Yeast strains are listed in Table I.

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, and 1 min at 72 °C, followed by extension at 72 °C for 10 min, in a solution containing 50 mM KCl, 10 mM Tris-Cl (pH 8.3), 1.5 mM MgCl2, 0.01% gelatin, 20 mM each dNTP, 0.1% Tween, 25 pmol of each amplification primer, and 2.5 units of Taq polymerase (total volume of 100 μl). Samples of reaction mixtures were subjected to electrophoresis on a 1.5% agarose gel. All PCR products were purified, subcloned, and sequenced by the dideoxy chain termination method (48). DNA sequences were analyzed, and multiple protein alignments were prepared using a geneWorks software package (IntelliGenetics, Inc./Betagen, Mountain View, CA).

Isolation of Yeast ARL1 cDNA—Yeast ARL1 was isolated initially by PCRs from a yeast agt11 cDNA library. Five PCRs were used to obtain cDNA segments and to assemble a composite sequence of the full-length coding region. Primers 1222 (5'-TTGAGAAGCCGAAACACTGTTAATG-3'), 1222B (5'-ACCGGCTCAGCAATGGAGGC3'), 1222B (5'-ACGGGCTCAGCATTGAAAT-3'), 1215 (5'-GATGCCCTGAAGGAGCAG-3') were complementary to sequences immediately upstream or downstream of the EcoRI insert site of vector agt11. Degenerate nucleotides (GTG; ARL1) and ARL1-B2 (E. coli) correspond to part of the consensus sequences WVDAGGD and TSIPLYW in human, rat, and Drosophila ARL1. The open reading frame was completed using the rapid amplification of 5'-cDNA ends procedure (49). A yeast agt11 cDNA library (CLONTECH) served as template in the one site-specific PCR used to capture 3'- and 5'-ends as described previously (10, 11).

Yeast ARL1 Gene Disruption—Yeast ARL1 DNA generated by PCR was subcloned into the pGEM-7Zf plasmid, resulting in pGyL1. The yeast URA3 gene was inserted at the single EcoRI site in the yeast ARL1 gene as follows (see Fig. 4A). The 3.8-kb DNA fragment containing the yeast URA3 gene and two hisG repeat sequences was excised from the plasmid pNKS1 (50) by digestion with BglII and BamHI; the 5'-overhangs were filled in with Klenow fragment. Plasmid pGyL1, containing the yeast ARL1 gene, was linearized at the internal EcoNI 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 pGyL1U.

Gene disruption mutants were constructed by a one-step gene replacement method (51). Briefly, the ~4.8-kb DNA fragment excised from pGyL1U by digestion with XhoI and BamHI was used to transform various yeast strains (see Table I), and all prototrophic transformants were selected. DNA blot analysis of the URA' cells confirmed that the yeast ARL1 gene contained an additional 3.8 kb, corresponding to the hisG-URA3-hisG gene. Elimination of URA3 and one hisG repeat was carried out as described previously (11). Double deletions of yeast ARL1 and yeast ARF3 were performed in the yeast arf11 mutants (arf11::hisG, ura3) and arf3 mutants (arf3::hisG, ura3) that are listed in Table I.

Expression and Purification of Recombinant Proteins—The open reading frame of yeast ARL1 was obtained by PCR using primers that incorporated unique NdeI and BamHI sites at the initiating methionine and 6 base pairs 3’ of the stop codon, respectively. For the His-tagged yARL1 fusion protein, a DNA fragment containing the yeast ARL1 coding region was generated by amplifying yeast genomic DNA with sequence-specific primers. The PCR product was purified and annealed to the expression vector pET15b (Novagen), yielding pET15bbyL1. For the nonfusion protein, PCR products were digested with NdeI and BamHI, purified, and annealed to the expression vector pET2 (52), yielding pT7yARL1. BL21(DE3) cells containing expression plasmids were grown to a density of \( \Delta_{600} = 1.0 \), at which time the inducer, isopropyl-1-thio-

| Strain          | Genotypea | Source            |
|-----------------|-----------|-------------------|
| SEY2810.5       | MATa [MATa his3/3, ura3/3, leu2/2] | S. Cheng         |
| MGD 1d          | ade2, arg4, cby', his3, trp1, ura3, ARF1, ARF2, ARF3 | J. Szostek       |
| MGD 1drcp       | ade2, arg4, cby', his3, trp1, ura3, ARF1, ARF2, arf3-1 | Ref. 11          |
| MGD 1drcp1      | ade2, arg4, cby', his3, trp1, ura3, ARF1, ARF2, arf3-1, arl1 | Ref. 11          |
| MGD 1drp        | ade2, arg4, cby', his3, trp1, ura3, ARF1, ARF2, arf3, arl1 | Ref. 11          |
| MGD 1drpl1      | ade2, his3, leu2, lys2, trp1, ura3-52, ARF1, ARF2, ARF3, ARF5, ARL1 | Ref. 78          |
| YPH250          | ade2, his3, leu2, lys2, trp1, ura3-52, ARF1, ARF2, ARF3, arl1 | This work        |
| YPH250d1i       | ade2, his3, leu2, lys2, trp1, ura3-52, ARF1, ARF2, arf3, arl1 | This work        |
| YPH250d1p       | ade2, his3, leu2, lys2, trp1, ura3-52, arf1, ARF2, ARF3, arl1 | This work        |
| YPH250d1p1      | ade2, his3, leu2, lys2, trp1, ura3-52, arf1, ARF2, ARF3, arl1 | This work        |
| YPH252          | ade2, his3, leu2, lys2, trp1, ura3-52, ARF1, ARF2, ARF3, ARL1 | This work        |
| YPH252d1i       | ade2, his3, leu2, lys2, trp1, ura3-52, ARF1, ARF2, ARF3, arl1 | This work        |

The abbreviations used are: ade, adenine-requiring; arg, arginine-requiring; cby', cycloheximide-resistant; his, histidine-requiring; trp, tryptophan-requiring; ura, uracil-requiring; leu, leucine-requiring; arf1 represents arf1::hisG-URA3-hisG; arf1-1 represents arf1::hisG; arf3-1 represents arf3::hisG; arf3 represents arf3::hisG-URA3-hisG; arf11-1 represents arf11::hisG; and arf11 represents arf11::hisG-URA3-hisG.
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stop codon). The yeast HA-tagged ARL1 gene was subcloned into the pVT101U plasmid, an expression plasmid containing the ADH1 promoter (54), as a XhoI-XbaI fragment into XhoI-XbaI sites to yield pVT101yLHA1.

Expression and Myristoylation of yARL1—For expression of myristoylated ARL1 protein, competent BL21(DE3) bacteria (Novagen) were transformed with expression plasmids (pT7/yARL1, pT7/yARF1, pT7/hARL1, or pET16b/yARL1) and selected for ampicillin resistance. Yeast (S. cerevisiae) N-myristoyltransferase expression was obtained in BL21(DE3) cells using the plasmid pACYC177/Et3/dyNMT (55). To produce myristoylated proteins, strain BL21(DE3) competent bacteria were transformed with two expression plasmids and selected for both ampicillin and kanamycin resistance. [9,10^-H]Myristic acid (1 mc/ml in ethanol; Amersham Corp.) was added to a final concentration of 30 μCi/ml, and the cultures were shaken at 30 °C. After cultures reached an A_{600} of ~0.6, 10 μl of 100 mM isopropyl-β-D-thiogalactopyranoside was added. Cultures were grown for an additional 3 h, bacteria were collected by centrifugation, and the pellets were frozen at ~20 °C.

To synthesize [3H]-labeled myristoylated yARL1 in yeast, [3H]myristic acid was added to yeast culture as described by Simon and Aderem (56). Briefly, yeast cells were grown to a density of 2 × 10^6 cells/ml at 25 °C in a rotary air shaker, harvested by centrifugation, and suspended at a density of 2 × 10^6 cells/ml. Cerulein (10 mg/ml in dimethyl sulfoxide) was added to a final concentration of 2.5 μg/ml. After 10 min, [3H]myristic acid was added to a final concentration of 30 μCi/ml, and cultures were incubated for 1 h at 25 °C in a rotary shaker. Cells were collected and washed with 10 mM Na_2PO_4 in double distilled H_2O. Glass beads (300 ml) and 300 ml of lysing buffer (50 mM Tris-Cl (pH 7.5), 1% SDS, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) were added, and the mixture was agitated vigorously for 90 s at room temperature before immersion in a boiling water bath for 6 min. Immunoprecipitation, electrophoresis, and autoradiography were done essentially as described (57) using 10 μl of anti-yARL1 or anti-yARF1 serum prepared in our laboratory.

Protein Processing in the Golgi Apparatus—Yeast cells were grown at 24 °C overnight in selective minimal medium containing 200 mM (NH_4)_2SO_4 to an A_{600} of 0.5. After 1 h at 37 °C, cells were transferred to sulfate-free selective minimal medium (final A_{600} ~5) and incubated for 5 min at 37 °C before the addition of 30 μl of Pro-mix (1:1-[35S]-label of [35S]-methionine and [35S]-cysteine, 14.3 mCi/ml) per 1 ml culture. After 5 min, labeling was terminated by the addition of 1% (v/v) chase solution (0.3% (w/v) cysteine, 0.4% (w/v) methionine, and 100 mM (NH_4)_2SO_4). Samples were removed at the indicated times thereafter and added to equal volumes of ice-cold 20 mM Na_2PO_4 in double distilled H_2O. Cells were collected, and cell lysates were prepared as described above, electrophoresis, and autoradiography were done essentially as described (57) using 10 μl of anti-carboxypeptidase Y serum prepared in our laboratory.

Cholera Toxin A-catalyzed ADP-ribosylation Assay—5 μg of purified His-tagged yARL1 or yARF1 fusion protein were tested for their ability to stimulate cholera toxin-catalyzed auto-ADP-ribosylation in reaction mixtures (total volume of 100 μl) containing 50 mM potassium phosphate (pH 6.5), 0.5 mM MgCl_2, 20 mM theophylline, 0.1 mM GTP, 10 μM [3H]GTP (2 mCi/ml), and 1 μg of activated cholera toxin A. After incubation at 30 °C for 1 h, reactions were terminated by the addition of 1.0 ml of ice-cold 7.5% trichloroacetic acid. After precipitation overnight at 4 °C and centrifugation, proteins were dissolved in 60 mM Tris (pH 6.8), 10% glycerol, 5% 2-mercaptoethanol, 3% SDS, and 0.006% bromphenol blue (10 min, 65 °C); separated by SDS-PAGE on 12% gels; and transferred to nitrocellulose membranes, which were exposed to x-ray film for 24 h.

Nucleotide Binding and Hydrolysis—Binding of GTP-S to purified recombinant yARL1 was determined by a filter trapping method (58). Unless otherwise specified, 1 μg of His-tagged yARL1 fusion protein was incubated at 30 °C in 20 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 0.5 mM MgCl_2, 20 mM bovine serum albumin, and 10 μM [35S]GTP-S (>1000 Ci/mmol; Amersham Corp.) without or with 3 mM sonified 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 MgCl_2, and 1 mM dithiothreitol. The conversion of GTP to GDP was determined by thin-layer chromatography as described (59). A blank without protein was used to determine background, which was subtracted from each experimental value.

Subcellular Fractionation by Velocity Sedimentation on Sucrose Density Gradients—Cells were harvested by centrifugation from cultures (50 ml) grown in YMD medium to midexponential phase (A_{600} = 1). Cells (~0.5 g) were washed by repeated suspension in ice-cold H_2O and centrifugation, incubated with lyticase to form spheroplasts, suspended in 0.2 ml of ice-cold lysis buffer (10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 125 μM succrose) containing protease inhibitors (1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride), and disrupted on ice with 20 strokes in a Dounce homogenizer. The cell lysate was centrifuged (1500 × g) for 5 min to remove unbroken cells and large 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 (0.8 ml each of 60, 50, 40, 30, and 20% sucrose in lysis buffer) and then subjected to centrifugation at 37,000 rpm (~170,000 × g) for 3 h at 4 °C in a Beckman SW 55 rotor. Ten fractions were collected manually from the bottom. Samples (12 μl) of fractions were boiled for 5 min in SDS-PAGE sample buffer containing 5% SDS, subjected to SDS-PAGE, and analyzed by immunoblotting.

Indirect Immunofluorescence—Cells were grown in 5 ml of minimal selective medium (final A_{600} ~1) to a cell density of 1–2 × 10^7 cells/ml and prepared for indirect immunofluorescence as described (60) with the following modifications. To each culture was added 0.6 ml of 37% formaldehyde for fixation, and the cultures were gently shaken at 30 °C for 60 min. Cells were collected by centrifugation (2500 × g, 5 min), washed once in 5 ml of solution P (1.2 mM sorbitol and 0.1 mM potassium phosphate (pH 6.5)), suspended in 1 ml of solution P, transferred to a microscope slide, and incubated with a buffer containing 100 mM Tris-HCl (pH 9.0) and 150 mM NaCl and with a buffer containing 100 mM Tris-HCl (pH 9.0) and 150 mM NaCl and 50 mM MgCl_2. Affinity-purified rabbit anti-yARL1, mouse monoclonal anti-HA (12CA5), and fluorescein isothiocyanate-conjugated secondary antibodies (Cappel) were diluted 1:1000 for use. 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 and 30 s.

RESULTS AND DISCUSSION

Analyses of Protein Sequences of Yeast ARL1—The yeast cDNA homologue of human and rat ARL1 (yARL1) was identified as a product of PCR amplification using degenerate probes derived from conserved sequences in members of the ARL family (see “Materials and Methods”). The full-length open reading frame of yARL1 (549 bases) encodes a protein of 183 amino acids. Alignment of the deduced amino acid sequence of yARL1 and other related ARFs and ARLs revealed that yARL1 is more identical (61%) to mammalian ARL1 than to ARFs (48–56%) or other ARLs (35–45%) (Table II). The entire S. cerevisiae genome sequence data base was searched for mammalian ARL1 sequences; yARL1 was the only ARL homologue identified. Like other ARL proteins, yARL1 has a glycine at position 2, the site of N-myristoylation in ARF proteins. In addition, yARL1 lacks cysteine residues near the carboxyl terminus, which are sites of isoprenylation in non-ARF members of the Ras superfamily. Alignment of the yARL protein with other ARF and ARL proteins is shown in Fig. 1. A number of amino acids are conserved in all four ARL1 proteins,
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TABLE II

Comparison of deduced amino acid sequences of yeast ARL1 and those of ARFs and other ARLs

| Identity to yARL1a | Ref. | % |
|-------------------|------|---|
| Class I ARF       |      |   |
| hARF1 b           | 56   | 5 |
| hARF2             | 54   | 4 |
| hARF3             | 55   | 5 |
| dARF1             | 55   | 9 |
| Class II ARF      |      |   |
| hARF4             | 51   | 8 |
| hARF5             | 52   | 7 |
| hARF6             | 55   | 10 |
| Class III ARF     |      |   |
| hARF6             | 52   | 7 |
| dARF3             | 51   | 10 |
| Other ARFs        |      |   |
| yARF1             | 55   | 3 |
| yARF2             | 52   | 60 |
| yARF3             | 48   | 11 |

ARF-like proteins

hARL1 61 43
vARL1 61 40
dARL1 60 38
hARL2 45 39
hARL3 42 41
dARL4 40 7
hARL5 35 9

a Percentage identity of deduced amino acid sequences to that of yARL1 was calculated using the GeneWorks program.

b The prefixes h, v, d, y, and r represent human, bovine, Drosophila, yeast, and rat, respectively.

c F.-J.S. Lee, C.-F. Huang, W.-L. Yu, L.-M. Buu, C.-Y. Lin, J. Moss, and M. Vaughan, unpublished data.

d Including Phe5, Ile23, Gln39, Leu62, Leu69, the 7th–Ser74, Cys81, Ala88, Asp99, Lys164, and two consensus GTP-binding sequences for GTP binding and hydrolysis are underlined and boxed.

% Percentage identity of deduced amino acid sequences to that of yARL1.

**FIG. 1.** Alignment of deduced amino acid sequences of ARLs and ARFs from yeast and other eukaryotes. Sources of sequences are as follows: yARF1, Ref. 3; yARF2, Ref. 60; yARF3, Ref. 11; yARL1, this study; Drosophila ARL1 (dARL1), Ref. 38; rat ARL1 (yARL1), Ref. 40; hARL1, Ref. 41; hARL2, Ref. 39; hARL3, Ref. 41; hARL4, Footnote 2; and hARL5, Footnote 2. Amino acids identical in at least 6 of the 11 sequences compose the consensus sequence shown at the top. Consensus sequences for GTP binding and hydrolysis are underlined. Amino acids identical in four ARL1 sequences or identical in yARL1 and in two of the other ARL1 sequences are boxed.

the function of yARL1, the gene and flanking regions were isolated from genomic DNA using PCR, and a construct was created in which its open reading frame was disrupted by the URA3 marker gene (Fig. 4A). A DNA fragment containing the ARL1::hisG-URA3-hisG sequence was used to transform yeast yARL1 was immunologically distinguishable from yeast ARFs, and the anti-hARL1 antibody proved to be a sensitive and specific probe for yARL1 protein. Immunoblotting with this antiserum allowed detection of yARL1 at nanogram levels (Fig. 2A). At a dilution of 1:5000, the polyclonal antibody specific for yARL1 did not cross-react with yARF1, yARF2, yARF3, or yARF1 (Fig. 2B). It reacted, however, with human ARFs (hARL5 > hARL1 > hARL3). In addition, polyclonal antibodies against yARF1, yARF2, and yARF3, and yARF1 failed to react with yARL1 on Western analysis (data not shown). Thus, yARL1 was immunologically distinguishable from yeast ARFs, and the anti-hARL1 antibody proved to be a sensitive and specific probe for yARL1 protein. Immunoblotting with this antisera allowed detection of yARL1 at nanogram levels (~1–2 ng), whereas no signal was detected with recombinant yARF1 and yARF2 (up to 100 ng) or yARF3 (up to ~40 ng). DNA and RNA Blot Analyses—Yeast ARL1 is a single copy gene and is located on chromosome II (Yeast Genome Data Bank). Total RNA from mid-log growth yeast cultures in either glucose- or galactose-containing medium was subjected to electrophoresis, transferred to GeneScreen Plus, hybridized with the yARL1 cDNA probe, and, after stripping, with a yeast β-tubulin probe. The ~0.75-kb yARL1 RNA, similar to that of yARF1, was not repressed by growth in glucose (Fig. 3).

Yeast ARL1 Is Not an Essential Gene—To learn more about
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ARF1 and ARF2 is lethal, but each of the five known human ARF proteins can restore vegetative growth to this double deletion mutant (61–63). Cells with double deletions of yeast ARL1 and ARF3 or ARL1 and ARF1 were also viable (data not shown). Proper disruption of the specific genes was confirmed by PCR on genomic DNA prepared from colonies of the mutant yeast (38). Proper disruption of the specific genes was confirmed by PCR on genomic DNA prepared from colonies of the mutant yeast. The specific ARF gene, and its deletion, is not essential for cell viability, and its deletion is lethal, but each of the five known human ARF proteins can restore vegetative growth to this double deletion mutant (61–63). Cells with double deletions of yeast ARL1 and ARF3 or ARL1 and ARF1 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 yeast ARL1, unlike Drosophila Arl1 (38), is not essential for cell viability, and its deletion was not being complemented by yeast ARF1 or ARF3.

To assess whether yeast arl1 can affect growth phenotype, the growth rates of wild-type, arl1 mutant, and overexpressed yARL1 strains were determined. We constructed a recombinant yARL1 clone (yARL1-HA) with a 9-amino acid influenza virus HA epitope (64) fused to its C terminus, placed the HA-tagged allele (yARL1-HA) under the control of the ADH1 promoter, and expressed it in wild-type and arl1 mutant yeast. The arl1 mutants and yeast overexpressing yARL1 had 15–20 and ~40% lower specific growth rates, respectively, than wild-type cells in glucose synthetic medium (data not shown). It is conceivable that overexpressed yARL1, similar to yARF1, may interfere with ARF (or ARL)-mediated vesicular transport (65).

Expression of yARL1 was confirmed by using antibody against yARL1 (Fig. 5B). However, Western analyses with either anti-yARL1 or anti-HA antibodies detected two proteins of ~21 and ~22 kDa when yARL1 was overexpressed in wild-type or arl1 mutant strains (Fig. 5, B and C). Overexpression of yARF3 in the same system did not yield a doublet pattern. We currently do not have a good explanation for this observation. It is necessary to investigate further possible post-translational modifications of yARL1. Expression of yARL1 protein, like that of yARF1, was not carbon

**Fig. 2.** Specific immunoreactivity of antibody against yARL1. 20–40 ng of purified recombinant His-tagged ARFs and ARLs were subjected to SDS-PAGE on a 15% gel. yARF1 (temporarily named Lpe21p in the Yeast Genome Project) is the yeast homologue of mammalian ARF1 (77). A, silver-stained gel; B, proteins transferred to nitrocellulose and reacted with anti-hARL1 antibody, followed by detection using the ECL system. Positions of protein standards are indicated on the left.

**Fig. 3.** Northern blot of yeast total RNA. Total RNA (~20 μg) from mid-log yeast cultures grown in YPD (lane a) or YPGal (lane b) medium was separated on a formaldehyde-containing 1.2% agarose gel, transferred to GeneScreen Plus, and hybridized with random-primed [32P]-labeled yeast ARL1. Labeled probes (~6 × 10⁶ cpm/mg) were purified on MicroSpin G-50 columns (Pharmacia Biotech Inc.). The blot was also hybridized with a random-primed [32P]-labeled yeast β-tubulin probe as an internal control. Positions of RNA standards (stained with ethidium bromide) are indicated on the left.

**Fig. 4.** Genetic disruption of yeast ARL1 gene. A, the 3.8-kb histG-URA3-histG fragment was inserted into the EcoNI site of the yARL1 DNA clone pGyL1 plasmid, resulting in pGyL1U (the open box represents yARL1, and the line represents pGEM). The solid bar above the construct indicates hybridization probe A used for blot analysis. B, shown are the results from DNA blot analysis of disrupted yeast ARL1. DNA was isolated from spore colonies from the arl1-disrupted diploid cell SEY6210.5 (URA₃, lanes 1 and 2; Ura₃, lanes 3 and 4), a heterozygous disrupted diploid cell SEY6210.5 (lanes 5 and 6), and a wild-type SEY6210.5 (lanes 7 and 8). Isolated DNA was digested with EcoRI (E) or BamHI/EcoRI (B/E), separated by electrophoresis on a 1% (w/v) agarose gel using Tris borate buffer, transferred to GeneScreen Plus, and hybridized with a [32P]-labeled 584-bp fragment of yARL1.

**Fig. 5.** Detection of yARL1 by Western blot analysis. Samples of yeast proteins (~30–40 μg) containing HA-tagged yARL1 protein synthesized in the yeast arl1 mutant (lane 1) or wild-type yARL1 (lane 2) and similar protein samples from the yeast arl1/arf3 double mutant (lane 3), the yeast arl1 mutant (lane 4), and wild-type yARL1 (lane 5) were subjected to SDS-PAGE. Proteins were stained with Coomassie Blue (A). After transfer of proteins to polyvinylidene difluoride filters, yARL1 (B) and HA-tagged yARL1 (C) were identified with specific antibodies against yARL1 and HA (antibody 12CA5), respectively, using the ECL system and a 5-min exposure of Hyperfilm-MP. Positions of protein standards are indicated on the left.
Characterization of a Yeast ARL1 Protein

source-dependent (data not shown).

Function of yARL1 Differs from That of yARF—To evaluate the role of yARL1 in protein transport from the ER to the Golgi apparatus or within the Golgi apparatus, we measured the glycosylation and proteolytic processing of carboxypeptidase Y, an enzyme that traverses this secretory pathway en route to the vacuole. Pulse-chase labeling with [3H]-labeled cysteine and methionine of wild-type, arfl, and arfl mutant cells was followed by immunoprecipitation of carboxypeptidase Y. The core-glycosylated P1 form of the carboxypeptidase Y 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. Similar to the wild-type cells, the arfl mutant converted carboxypeptidase Y from the ER to Golgi apparatus and vacuole forms (Fig. 6). The arfl mutant, however, accumulated core-glycosylated carboxypeptidase Y in the P1 form, as expected (57). Therefore, yARL1 has a biological function clearly different from that of yARF1 and is not required for ER-to-Golgi protein transport.

Amino Terminus of yARL1 Is Myristoylated—ARF proteins are myristoylated in vivo, and the cellular functions of ARF involve its reversible association with specific intracellular membranes in myristoylation-dependent and guanine nucleotide-regulated reactions (66, 67). Binding of the activating nucleotide (GTP) results in a conformational change in the protein with increased affinity for phospholipids and membranes (68, 69). The inactive GDP-bound form of ARF is soluble (69, 70). Although ARL3 has been reported not to be de-regulated reactions (66, 67). Binding of the activating nucleotide clearly different from that of yARF1 and is not required for ER-to-Golgi protein transport.

Materials and Methods.

Yeast cells expressing HA-tagged yARL1 were subjected to SDS-PAGE on a 15% gel. Lane a, gel fixed and stained with Coomassie Blue; lane b, gel fixed in 10% acetic acid and 45% methanol for 30 min, incubated in Amplify (Amer sham Corp.) for 20 min, dried, and exposed to Hyperfilm-MP for 41 h at −80 °C. Arrows indicate the positions of yeast N-myristoyltransferase (upper arrows) and myristoylated recombinant proteins (lower arrows). A, recombinant proteins (yARF1, hARL1, yARL1, and His-tagged yARL1) were synthesized in E. coli expressing yeast N-myristoyltransferase and grown in medium containing [3H]myristic acid. Samples of bacterial or yeast proteins (∼20 µg) were subjected to SDS-PAGE on a 15% gel. Lane a, gel fixed and stained with Coomassie Blue; lane b, gel fixed in 10% acetic acid and 45% methanol for 30 min, incubated in Amplify (Amer sham Corp.) for 20 min, dried, and exposed to Hyperfilm-MP for 41 h at −80 °C. Arrows indicate the positions of yeast N-myristoyltransferase (upper arrows) and myristoylated recombinant proteins (lower arrows). B, shown is the immunoprecipitate of myristoylated yARL1 in yeast. Cells were grown in [3H]myristic acid medium. Cell lysates were immunoprecipitated with anti-yARL1 or anti-yARF1 antibodies as indicated. Lanes 1 and 4, cell lysate from wild-type yeast expressing HA-tagged yARL1; lanes 2 and 5, from the arfl mutant; lanes 3 and 6, from wild-type yeast. Positions of protein standards are indicated on the left.

Subcellular fractionation to localize yARL1. The lysate of spheroplasts of wild-type yeast was fractionated by sucrose gradient centrifugation. Samples of fractions were subjected to SDS-PAGE, and proteins were transferred to polyvinylidene difluoride filters; yARL1, yARF1, and a Golgi marker (Emp47p) were identified with specific antibodies and detected using the ECL system by exposure to Hyperfilm-MP. Positions of protein standards are indicated on the left. Gradient fractions are numbered from the bottom. Lane W indicates the yeast total lysate.

membranes upon cell lysis, we sought to investigate the cellular localization of yARL1 by indirect immunofluorescence. Using affinity-purified anti-yARL1 antibodies, a punctate staining was observed (Fig. 9). This is reminiscent of what has been seen by others with antibodies directed against Golgiand transport vesicle-associated proteins, such as Ypt1p (71, 72), Kex2p (73), Sed5p (74), and Ypt31p (75). As shown in Fig. 9, a control experiment with antibody 12C5 directed against the HA epitope of HA-Pmr1, which is assumed to localize primarily to the Golgi compartment (76), resulted in a picture similar to that obtained with anti-yARL1 antibodies. In both cases, neither perinuclear staining typical for ER localization nor staining of the vacuole was evident. From the combined results of subcellular fractionation and indirect immunofluorescence, we

![Image of subcellular fractionation to localize yARL1](image-url)
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Biochemical Properties of Recombinant yARL1 Protein—To determine whether the yeast ARL1 gene product has ARF activity, a recombinant protein was constructed, expressed, and purified from E. coli. The His-tagged yARL1 fusion protein did not stimulate auto-ADP-ribosylation of cholera toxin A1 protein in the presence of 100 μM GTPγS, a nonhydrolyzable GTP analogue, and detergent (SDS) (data not shown). It is known that myristoylation is not absolutely required for the activation of cholera toxin A by rat ARF1.

To assess the binding of GTPγS, a slowly hydrolyzed analogue of GTP, to yARL1, the nitrocellulose filter trapping method was used. Binding had achieved steady state in 60 min at 30 °C (Fig. 10, inset). GTPγS binding to yARL1, like that to ARF, Drosophila ARL1, and rat A1, was influenced by added phospholipids and was concentration-dependent (Fig. 10) (38, 43). In contrast, binding of GTPγS to hARL2 and hARL3 was affected very little by added lipid or detergent (39, 41). Maximal rates of GTP hydrolysis by hARL2 and hARL3 were 0.0074 and 0.005 min⁻¹, respectively (39, 41). Recombinant yARL1 had a rate of −0.02 min⁻¹, similar in magnitude to that of Drosophila ARL1 (0.05 min⁻¹) (38).

ARL1 was first identified in D. melanogaster as an essential gene encoding a structural relative of the ARF family that is 55% identical to mammalian ARF1 (38). PCRs using highly degenerate primers led to the identification of cDNA fragments encoding at least five human ARL proteins and several rat and mouse homologues (38–43). The ARL proteins are at least 40% identical to the ARF proteins, but little is known about their cellular function. We report here the identification and characterization of yeast ARL1 with the functional characteristics of an ARL1 protein that is expressed widely in Drosophila, human, and rat tissues. yARL1, at its normal level of expression, clearly cannot replace yARF1 and yARF2, as their double deletion is lethal (61). The specific antibody described should prove useful in defining the cellular role(s) of ARL1 and in distinguishing among the many structurally related members of the ARF family. There are still many gaps in our understanding of the roles of ARL proteins in membrane budding and fusion processes. The newly identified yARL1, which is apparently associated with the Golgi apparatus, is not essential for ER-to-Golgi protein transport. Thus, it offers an opportunity to define its function in another kind of membrane vesicular transport, such as the regulated secretory pathway.

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REFERENCES
1. Moss, J., and Vaughan, M. (1995) J. Biol. Chem. 270, 12327–12330
2. Boman, A. L., and Kahn, R. A. (1995) Trends Biochem. Sci. 20, 147–150
3. Sewell, J. L., and Kahn, R. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4620–4624
4. Price, S. R., Nightingale, M., Tsai, S.-C., Williamson, K. C., Adamik, R., Chen, H.-C., Moss, J., and Vaughan, M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5498–5501
5. Bobak, D. A., Nightingale, M., Murtagh, J. J., Price, S. R., Moss, J., and Vaughan, M. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 6101–6105
6. Kahn, R. A., Goddard, C., and Newkirk, M. (1988) J. Biol. Chem. 263, 8292–8297
7. Tsuchiya, M., Price, S. R., Tsai, S.-C., Moss, J., and Vaughan, M. (1991) J. Biol. Chem. 266, 2772–2777
8. Monaco, M., Murtagh, J. J., Newman, K. B., Tsai, S.-C., Moss, J., and Vaughan, M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2266–2270
9. Murtagh, J. J., Jr., Lee, F.-J. S., Deak, J., Hall, L. M., Monaco, L., Lee, C.-M., Stevens, L. A., Moss, J., and Vaughan, M. (1989) Biochemistry 22, 6911–6918
10. Lee, F.-J. S., Stevens, L. A., Hall, L. M., Murtagh, J. J., Jr., Kao, Y. L., Moss, J., and Vaughan, M. (1994) J. Biol. Chem. 269, 21555–21560
11. Lee, F.-J. S., Stevens, L. A., Kao, Y. L., Moss, J., and Vaughan, M. (1994) J. Biol. Chem. 269, 20953–20957
12. Murtagh, J. J., Jr., Mowatt, M. R., Lee, C.-M., Lee, F.-J. S., Mishima, K., Nash, T. E., Moss, J., and Vaughan, M. (1992) J. Biol. Chem. 267, 8654–8662
13. Lee, F.-J. S., Patton, W. A., Lin, C.-Y., Moss, J., Vaughan, M., Goldman, N. D., and Syin, C. (1997) Mol. Biochem. Parasitol. 87, 217–223
14. Price, S. R., Barber, A., and Moss, J. (1990) in ADP-Ribosylating Toxins and G Proteins: Insights into Signal Transduction (Moss, J., and Vaughan, M., eds) pp. 397–424 American Society for Microbiology, Washington, D.C.
15. Bobak, D. A., Bliiziotis, M. M., Noda, M., Tsai, S.-C., Adamik, R., and Moss, J. (1990) Biochemistry 29, 855–861
16. Price, S. R., Welsh, C. F., Haun, R. S., Stanley, S. J., Moss, J., and Vaughan, M. (1992) J. Biol. Chem. 267, 17766–17772
17. Salminen, A., and Novick, P. (1987) Cell 49, 527–533
18. Bourne, H. R. (1988) Cell 53, 669–671
19. Serafini, T., Orci, L., Amherdt, M., Brunner, M., Kahn, R. A., and Rothman, J. E. (1991) Cell 67, 239–253
20. Zeuzem, S., Fricke, P., Zimmerman, P., Haase, W., Kahn, R. A., and Schulz, I.
