Mutations in Yeast ARV1 Alter Intracellular Sterol Distribution and Are Complemented by Human ARV1*

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Intracellular cholesterol redistribution between membranes and its subsequent esterification are critical aspects of lipid homeostasis that prevent free sterol toxicity. To identify genes that mediate sterol trafficking, we screened for yeast mutants that were inviable in the absence of sterol esterification. Mutations in the novel gene, ARV1, render cells dependent on sterol esterification for growth, nystatin-sensitive, temperature-sensitive, and anaerobically inviable. Cells lacking Arv1p display altered intracellular sterol distribution and are defective in sterol uptake, consistent with a role for Arv1p in trafficking sterol into the plasma membrane. Human ARV1, a predicted sequence ortholog of yeast ARV1, complements the defects associated with deletion of the yeast gene. The genes are predicted to encode transmembrane proteins with potential zinc-binding motifs. We propose that ARV1 is a novel mediator of eukaryotic sterol homeostasis.

Sterols are essential structural and regulatory components of eukaryotic cellular membranes (1, 2). However, cholesterol over-accumulation is cytotoxic (3), necessitating mechanisms to maintain this metabolite at appropriate levels. A pivotal component of this homeostasis is the esterification of free sterol by acyl-coenzyme A:cholesterol O-acyltransferase (ACAT)1, 2. Indeed, the inhibition of ACAT in sterol-loaded cells induces cell death when extracellular sterol acceptors such as high density lipoproteins are absent (6, 7).

Intracellular cholesterol redistribution mediates a number of responses to elevated free sterol levels. These include elevated ACAT activity, down-regulated sterol and fatty acid biosynthesis, and reduced lipoprotein uptake via LDL receptors (8, 9). The latter two events reflect changes in transcriptional activation by sterol regulatory element-binding proteins (SREBPs) in response to sterol accumulation in regulatory pools (9), whereas ACAT activity is allosterically regulated by substrate supply (10). Sterols are maintained at a high concentration in the plasma membrane (PM) relative to the endoplasmic reticulum (ER) (1, 2), where SREBP and ACAT reside. Thus trafficking of sterol to and from the ER is a critical component of sterol homeostasis.

The process of sterol trafficking is poorly understood at the molecular level. In certain cell types, caveolin influences what has been termed “fast” movement of cholesterol to plasma membrane cholesterol-rich microdomains (caveolae) (11, 12). Mutations in the Nijmegen Pick type C (NPC1) gene result in accumulation of LDL-derived cholesterol in the lysosome (13, 14). However, not all cells express caveolin, and the movement of endogenously synthesized cholesterol to the plasma membrane in NPC1-deficient cells is normal (15).

To identify novel genes that mediate sterol trafficking in all higher cells, we utilized the genetically tractable model eukaryote, Saccharomyces cerevisiae (budding yeast). We reasoned that dependence on sterol esterification for viability would be one criterion for identifying novel sterol-trafficking genes. Yeast strains lacking the ACAT-related enzymes (encoded by the ARE1 and ARE2 genes) contain no steryl ester (16, 17), down-regulate sterol biosynthesis (16, 18), and grow normally. In a screen for genes required for viability in the absence of the ARE genes, we isolated yeast and human ARV1 (ARE2 required for viability). We demonstrate that ARV1 is required for sterol uptake and distribution in yeast.

EXPERIMENTAL PROCEDURES

Reagents, Strains, and General Techniques—Yeast extract-peptone dextrose (YPD), synthetic complete dextrose (SCD), and 5-fluoro orotic acid (5-FOA; Toronto Research Chemicals) media were prepared as described (19). Molecular biology reagents were obtained from New England Biolabs, Life Technologies, Inc., and Stratagene. [3H]Acetate (sodium salt), [9,10-3H]oleic acid, [1,2-14C]cholesterol, and [4-14C]cholesterol were from PerkinElmer Life Sciences. Other reagents were from Sigma unless noted. Nucleotide sequencing was performed by the Columbia University Cancer Center sequencing facility. Gene-specific oligonucleotides were synthesized by Genset. yeast strains used in this study were constructed by standard genetic techniques and are isogenic with strain W303–1A unless noted (16). S. cerevisiae and Escherichia coli (XL1-Blue) transformations were performed as described (20) or by electroporation (Bio-Rad). BBL gas jars and GasPak Plus (Becton Dickinson) were used for anaerobiosis according to the manufacturer’s instructions.

Synthetic Lethality Screen—An adenine auxotrophy-based colony color sectoring assay was used to identify are mutants (21). are1Δ are2Δ ade2Δ ade3Δ strains of opposite mating type were generated. To facilit-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF290878.

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1 The abbreviations used are: ACAT, acyl-coenzyme A:cholesterol O-acyltransferase; LDL, low density lipoprotein; SREBP(s), sterol regulatory element-binding proteins; PL, plasma membrane; ER, endoplasmic reticulum; YPD, Yeast extract-peptone dextrose; SCD, synthetic complete dextrose; 5-FOA, 5-fluoro orotic acid; bp, base pair; PCR, polymerase chain reaction; AHD, Arv1 homology domain.

2 O-acyltransferase; LDL, low density lipoprotein; SREBP(s), sterol regulatory element-binding proteins; PL, plasma membrane; ER, endoplasmic reticulum; YPD, Yeast extract-peptone dextrose; SCD, synthetic complete dextrose; 5-FOA, 5-fluoro orotic acid; bp, base pair; PCR, polymerase chain reaction; AHD, Arv1 homology domain.
Sterol Trafficking in Yeast

Irate complementation analysis, the are2::LEU2 allele of one parent was converted to are2::LEU2::TRP1 (22). Both strains were transformed with plasmid pAT001 (2870-bp PmlI-XhoI fragment of ARE2 at the SacII/NruI sites of plasmid pCH122 (21), which conferred sterol esterification in are1Δ are2Δ strains (data not shown). Parent strains were grown in scid-Uracil media and exposed to a UV transilluminator (10–15% survival). Mutants requiring the ARE2 gene were placed in complementation groups by constructing diplogs and scoring plasmid retention by colony sectoring or growth on 5-FOA-containing media. Seven are1 alleles were recovered, whereas other complementation groups were represented by four or less alleles.

Identification of ARV1—Standard techniques (16) were used to isolate and characterize the complementing gene on plasmid pAT005–4, which rescued all alleles of are1-1 identified in the screen. Deletion analysis of pAT005–4 indicated that open reading frame YLR242C was essential for are1-1 complementing activity. To confirm that the are1-1 mutation resided in YLR242C, a strain bearing a LEU2-marked wild-type allele of YLR242C was crossed with an are1-1 strain. All haploid progeny from this cross were either temperature-sensitive or Leu+, indicating linkage of YLR242C and are1-1.

Construction of are1 Deletion Allele—Deletion of ARV1 (are1Δ) was accomplished using a PCR-generated allele and one-step gene replacement (23). Oligomers were as follows: 5′ KOARV1′ (AAGGCGATGAAA-GCCGATCAAAAAAGTATATGGCTGTAACACG) catcgcaatctcgggtgtragggggtgtagTATV1 (TAAGATATCTACTGATGATTTTTTTATTGGTGGGTAATT), and the upprase sequence corresponds to Kluveromyces lactis URA3. The following three independent ARV1/are1Δ diploids were confirmed using oligomers: 5′ARV1′ (CCTGATAGAAC-GCATCAAAGAG), 3′ARV1′ (GTTGATGATTAAATCCCAAGTGGC), and 3′KIURA3 (gAgCAATgAACCCTAAATCgaAATC). Diploid sporulation and tetrad manipulation of three heterozygous diploids was performed as described (24). PCR analysis of genomic DNA from haploid progeny showed 2:2 segregation of the deletion alleles.

Lipid Analyses—Cells grown for 18 h to mid-log phase in media containing [3H]acetate and/or [1-14C]oleic acid (final concentrations were 2 µCi/ml and 0.02 µCi/µl, respectively) were washed twice in 0.5% tergitol and once in water and frozen at −70 °C prior to being lyophilized. Lyophilized cell pellets (between 2 and 6 mg) were resuspended in 50 µl of lytic case solution (1700 units/ml in 10% glycerol, 0.02% sodium azide) for 15 min at 37 °C to remove the cell wall. Treated cell suspensions were incubated at −70 °C for 1 h and 37 °C for 15 min and vortexed with 200 µl of isopropanol. Lipids were extracted with 5 ml of isopropanol/hexane (1:2) and 5 ml of KCl (3 M) to precipitate the organic layer. The cell suspension was re-extracted with 5 ml of isopropanol/hexane. For measuring the total sterol pools, 3 ml of methanol/KOH (60% (v/v)) were added to the lyzed cell/isopropanol suspension, incubated at 70 °C for 2 h, and extracted twice with hexane (25). The organic phases were dried down and analyzed by TLC (26). Lipid mass was visualized on TLC plates following exposure to iodine vapor or charring at 180 °C following brief immersion in 10% cupric sulfate solution (27).

Subcellular Fractionation—Membrane preparations from cells grown for 18 h were subjected to discontinuous gradient ultracentrifugation as described (28) except that renograin-76 was replaced with reno-grafin-76 (Bracco Diagnostics; a generous gift of Dr. Z. Haskal). 250 µl of each fraction were resuspended in 5 ml H2O/isopropanol (4:1), extracted twice with hexane, and analyzed by TLC as before. 15 µl of each fraction was used for SDS polyacrylamide gel electrophoresis and immunoblot analysis of marker proteins (29).

Analysis of Sterol and Oleate Uptake—The pep2-1 mutation (30) was back-crossed six times into the W303 genetic background and was followed by measuring net [4-14C]cholesterol accumulation in haploid progeny. Lysed cell suspensions prepared as before from cells grown for 18 h in media containing 1% tyloxapol:ethanol (1:1), 0.01 µCi/ml [4-14C]cholesterol, and 0.25 µCi/ml [9,10-3H]oleic acid were added directly to scintillation fluid followed by counting. PCR amplification from genomic templates and HphI digestion of a 221-bp fragment were also used to identify pep2-1. The pep2-1 mutation (G to A transition at nucleotide 2663; see Ref. 31) creates a novel HphI restriction site.

Identification of ARV1—A cDNA was obtained from the IMAGE consortium corresponding to hARV1 (gbAA461156). Both strands of the 1465-bp insert were sequenced. An ~1500-bp EcoRI fragment of the image clone containing the putative hARV1 open reading frame (GenBank accession number AF290878) was ligated into pRS416-VP (29) at EcoRI downstream of the GAL11/10 promoter to produce pARV1. Expression of hARV1 in transformants was induced by growth on 3% galactose and confirmed by Northern analysis. A human hepatoma (HepG2) cDNA yeast expression library (32) was transformed into an are1Δ mutant, and viable colonies were selected at 37 °C. Resucing plasmids were recovered in E. coli and analyzed by retransformation of yeast, nucleotide sequencing, and PCR.

RESULTS AND DISCUSSION

Identification of ARV1—We screened for mutants of S. cerevisiae (yeast) that were inviable in the absence of the genes encoding the ACAT-related enzymes (Are1p and Are2p) (16, 17). Mutants were identified by their inability to grow in the absence of a plasmid encoding Are2p in an are1Δ are2Δ deletion background. We named these mutants arv1, for ARV1 required for viability. Four complementation groups were obtained. We chose ARV1 for further study because it was the most frequently mutated gene identified in the screen (seven independent arv1 alleles, arv1-1 to arv1-7).

Even in the presence of a functional ARE2 gene, arv1-1 strains grow slowly compared with controls and are inviable at 37 °C (Fig. 1A), suggesting that sterol esterification cannot compensate for a critical function of Arv1p, which becomes essential at elevated temperature. The ARV1 gene was isolated by complementation of the temperature-sensitive phenotype of an are1Δ strain using a yeast genomic library (33) and shown to be allelic with arv1 by conventional genetic methods. Sequencing of PCR-generated DNA from are1Δ genomic templates identified a stop codon halfway through the predicted ARV1 open reading frame (see Fig. 3B). A complete gene deletion (arv1Δ) was constructed and found to confer growth characteristics similar to arv1-1. Further, in a genetic cross between are1Δ and are2Δ strains, triple mutants were not recovered, indicating dependence on sterol esterification for viability of strains lacking ARV1. The deletion allele was thus used in subsequent studies.

Intracellular Sterol Accumulation in arv1Δ Mutant Cells—We hypothesize that mutations in sterol-trafficking genes could alter membrane sterol levels. Interestingly, arv1Δ cells are sensitive to nystatin (Fig. 1A), a polyene antibiotic that disrupts membranes in part by interaction with sterols (34). Because nystatin partitioning into membranes is sensitive to both sterol organization and levels (34) we analyzed sterol levels in these cells as reflected by incorporation of [3H]acetate during exponential growth. arv1Δ mutants showed an ~50% decrease in free sterols and an ~75% decrease in steryl ester levels (Fig. 1B) consistent with the dependence of arv1 mutants on sterol esterification for viability. Iodine staining or charring of sterol mass following extraction and TLC analysis further reflected these differences (not shown). These data suggest a role for Arv1p in determining cellular sterol levels.

Altered Sterol Distribution in arv1Δ Mutant Cells—To identify which membranes in arv1Δ mutants might be accumulating sterol, subcellular fractionation and lipid analyses were performed on membrane preparations from arv1Δ and wild-type strains. Sterols and phospholipids were assayed by labeling cells for 18 h to steady state with [3H]acetate and [14C]oleate, respectively. Colorimetric assays of sterols and phospholipids indicated that metabolic labeling over this period was reflective of mass (not shown). The enrichment of free sterol relative to phospholipids distinguishes the PM from other membranes in the cell. The highest sterol to phospholipid ratios were seen in the PM fractions of both mutant and wild-type cells, as defined by the PM-specific antigen, Pma1p. However, relative to wild-type cells, arv1Δ mutants showed significantly elevated sterol levels in ER and vacuolar membrane fractions and decreased overall levels in the plasma membrane fractions (Fig. 1C). This altered distribution was confirmed by densitometric analysis of charred TLC plates of fractions from
unlabeled cells (not shown). These data suggest a role for Arv1p in sterol distribution.

**ARV1 Is Essential for Growth during Anaerobiosis and Sterol Uptake**—In the absence of sterol esterification, the elevated level and altered distribution of sterol in *arv1* mutants could result in sterol toxicity. Therefore, reduction of intracellular sterol levels should alleviate the requirement for *ARE2*. To limit sterol levels in *arv1Δ* cells, we utilized anaerobic growth conditions where cells require exogenous sterols and fatty acids (35). *arv1Δ* and wild-type yeast were incubated anaerobically with oleate and a range of ergosterol concentrations. Surprisingly, *arv1Δ* cells were inviable anaerobically (Fig. 2A).

One explanation for anaerobic inviability of *arv1* mutants is that Arv1p mediates sterol uptake. To address this, we constructed *arv1Δ* strains that carry *upc2–1*, a gain of function mutation that confers aerobic sterol uptake (30). *UPC2* encodes a predicted zinc finger transcription factor of the Zn(2)-Cys(6) binuclear cluster domain class (30). *arv1Δ upc2–1* double mutants accumulated 50% less exogenous [14C]cholesterol than *upc2–1* cells (Fig. 2B) but similar levels of exogenous [3H]oleate (data not shown), consistent with a role for *ARV1* in sterol uptake. Interestingly, sterol uptake in double mutants was significantly elevated compared with wild-type and *arv1Δ* controls (Fig. 2B), suggesting *upc2–1* mediated *ARV1*-independent sterol transport mechanisms. Moreover, the *upc2–1* mutation suppressed *arv1Δ* anaerobic inviability (Fig. 2A). Taken together, these data support the idea that Arv1p plays a critical role in sterol uptake.

*Arv1p, a Putative Zinc-binding Transmembrane Protein*—The yeast *ARV1* gene is predicted to encode a novel 321-amino...
acid transmembrane protein with a potential zinc-binding motif (Fig. 3, A and B). The spacing of the NH2-terminal cysteine residues of Arv1p resembles that seen in zinc ribbons (Fig. 3A), which have been described in general transcription factors where they bind DNA or mediate protein-protein interaction (36). Interestingly, intragenic complementation between two arv1 alleles (arv1–2 and arv1–4) suggests that Arv1p functions as a multimer. Zinc-binding motifs serve a number of functions in membrane-associated proteins including regulation of small molecule transport (37), recruitment of signaling molecules to PM microdomains (38), and charged lipid binding (39). Interestingly, endosomes rich in negatively-charged lysobisphosphatidic acid accumulate cholesterol when luminal zinc concentrations are elevated (40), consistent with a role for zinc-binding proteins in cholesterol homeostasis.

ARV1 Is Conserved in Multicellular Eukaryotes—A search of the data base of expressed sequence tags revealed Caenorhabditis elegans, Arabidopsis thaliana, and ubiquitously expressed human sequences with similarity to Arv1p (Fig. 3A). A full-length human cDNA (hARV1) was sequenced and predicts a 271-amino acid protein (Fig. 3B). The identity of the human, worm, plant, and yeast sequences is found in a 61-amino acid NH2-terminus containing the putative zinc-binding motif and a conserved block of 33 residues, which together comprise a novel domain (Arv1 homology domain (AHD), ProDom PD038388 (41); see Fig. 3, A and B).

The conservation of the AHD and 6 putative transmembrane domains between yeast and human ARV1 led us to examine whether these genes are functionally related. Expression of the hARV1 coding sequence from the inducible yeast GAL1/10 promoter rescued arv1Δ growth defects in a galactose-dependent manner (Fig. 3C). Further, hARV1-containing plasmids were obtained in a selection for rescue of arv1Δ inviability at 37°C using a human hepatoma (HepG2) cell-derived cDNA yeast expression library (32). These observations confirm that hARV1 and yeast ARV1 share a conserved function.

The phenotypes of arv1 mutants (synthetic lethality with are1Δ are2Δ, altered sterol distribution and levels, nystatin sensitivity, anaerobic inviability, and reduced sterol uptake) are consistent with a role for Arv1p in sterol homeostasis. The sterol esterification requirement of arv1Δ cells likely arises from their elevated sterol levels, which would presumably become cytotoxic in the absence of ARE2. However, ARE2 does not suppress all the phenotypes associated with deletion of ARV1, indicating roles for Arv1p other than simply determining cellular sterol levels. Indeed, the altered sterol distribution in arv1Δ mutants suggests that Arv1p is required for appropriate sterol trafficking. Nystatin sensitivity of arv1Δ mutants might be a consequence of altered sterol trafficking to the plasma membrane. This may reflect or cause altered composition of glycosphingolipid- and ergosterol-enriched plasma membrane microdomains (“rafts”; see Ref. 42). Changes in plasma membrane structure may also account for the anaerobic inviability of arv1Δ cells and their reduced capacity to absorb extracellular sterol. A unifying hypothesis for these observations is that in arv1 mutant cells, the plasma membrane is an inefficient acceptor of sterols. Because human ARV1 can functionally substitute for yeast ARV1, it is likely that Arv1p represents a conserved compo-

nent of eukaryotic sterol homeostasis. Understanding the biochemical function of these novel proteins will undoubtedly illuminate mechanisms underlying intracellular sterol trafficking in all cells.

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