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Sal1p, a Calcium-Dependent Carrier Protein That Suppresses an Essential Cellular Function Associated With the Aac2 Isoform of ADP/ATP Translocase in *Saccharomyces cerevisiae*

Xin Jie Chen[1]

Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9148 and Research School of Biological Sciences, The Australian National University, Canberra, ACT 2601, Australia

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ABSTRACT

Adenine nucleotide translocase (Ant) catalyzes ADP/ATP exchange between the cytosol and the mitochondrial matrix. It is also proposed to form or regulate the mitochondrial permeability transition pore, a megachannel of high conductancy on the mitochondrial membranes. Eukaryotic genomes generally contain multiple isoforms of Ant. In this study, it is shown that the Ant isoforms are functionally differentiated in *Saccharomyces cerevisiae.* Although the three yeast Ant proteins can equally support respiration (the R function), Aac2p and Aac3p, but not Aac1p, have an additional physiological function essential for cell viability (the V function). The loss of V function in *aac2* mutants leads to a lethal phenotype under both aerobic and anaerobic conditions. The lethality is suppressed by a strain-polymorphic locus, named *SAL1* (for Suppressor of *aac2* lethality). *SAL1* was identified to encode an evolutionarily conserved protein of the mitochondrial carrier family. Notably, the Sal1 protein was shown to bind calcium through two EF-hand motifs located on its amino terminus. Calcium binding is essential for the suppressor activity. Finally, Sal1p is not required for oxidative phosphorylation and its overexpression does not complement the R^- phenotype of *aac2* mutants. On the basis of these observations, it is proposed that Aac2p and Sal1p may define two parallel pathways that transport a nucleotide substrate in an operational mode distinct from ADP/ATP exchange.

THE adenine nucleotide translocase (Ant) is the most abundant protein in the mitochondrial inner membrane (Klingenberg 1985). It catalyzes the ADP/ATP exchange across the mitochondrial inner membrane as the terminal step of mitochondrial oxidative phosphorylation (Klingenberg 1989; Fiore *et al.* 1998; Nelson *et al.* 1998). Under respiring conditions, ATP produced within mitochondria is exported to cytosol through Ant to meet the energy requirement of cells. As exchange, ADP is imported into the organelle to fuel the conversion of ADP to ATP by the F_1F_0-ATP synthase. Structurally, Ant belongs to the mitochondrial carrier family (MCF) that supports a variety of transport activities across the mitochondrial inner membrane (Belenkiy *et al.* 2000; Palmieri *et al.* 2000; Kaplan 2001; Passarella *et al.* 2003). Like most MCF members, Ant is a small protein of ∼300 amino acids. It contains three tandem-repeated sequences of ∼100 amino acids made of two hydrophobic transmembrane helices joined by a large hydrophilic segment. Each tandem repeat possesses a sequence motif known as a mitochondrial energy transfer signature. Early investigations have suggested that the functional unit of Ant is a homodimer acting as a gated pore that catalyzes strictly the one-to-one exchange of ATP and ADP (Hackenberg and Klingenberg 1980). However, structural analysis by low-resolution electron crystallography of the yeast Ant isoform, Aac3p, suggests that the nucleotide substrates are most likely to pass through the membrane in the core of an Ant monomer rather than on the interface of a homodimer (Kunji and Harding 2003). Recent X-ray crystallography of an Ant monomer has revealed a deep central cavity in the carboxyatractyloside-complexed monomeric protein. It is speculated that this central cavity may undergo a transition from a "pit" to a "channel" conformation during nucleotide translocation (Pebay-Peyroula *et al.* 2003).

Ant has been shown to exist in multiple isoforms in eukaryotes. For example, three Ant isoforms have been characterized in the yeast *Saccharomyces cerevisiae* and in humans, and two isoforms have been reported in *Drosophila melanogaster*, mouse, and cow (Powell *et al.* 1989; Stepien *et al.* 1992; Ellison *et al.* 1996; Graham *et al.* 1997; Nelson *et al.* 1998; Zhang *et al.* 1999). The mammalian Ant isoforms normally share 87–93% of sequence identity and are expressed in a tissue-specific manner. The mouse, bovine, and human Ant1 isoform is predominantly expressed in skeletal and cardiac muscles. The mouse and bovine Ant2 protein, or Ant3 in humans, is ubiquitously expressed in all tissues. Humans have the

[1]*Address for correspondence:* Department of Molecular Biology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., NA5.142, Dallas, TX 75390-9148. E-mail: xinjie.chen@utsouthwestern.edu

third isoform, Ant2p, which is expressed at a low level, if at all, in brain, liver, kidney, heart, and skeletal muscle. In addition to the tissue specificity, expression of the mammalian Ant isoforms has also been shown to be differentially regulated in response to developmental stages and to specific physiological conditions (Lunardi and Attardi 1991; Lunardi *et al.* 1992; Stepien *et al.* 1992). For instance, the expression of Ant1 is markedly induced during myoblast differentiation whereas the ubiquitously expressed Ant2 (or Ant3 in humans) is increased during cell proliferation in response to thyroid hormone or growth factors. As proposed by previous investigators, the Ant isoforms may have evolved distinct biochemical properties in the ADP/ATP exchange reaction, which could match the dynamic metabolic activities during various developmental stages and in different tissues (Stepien *et al.* 1992). However, it remains unknown whether the divergence of Ant isoforms may also implicate a differentiation in the physiological function of the proteins.

Loss of Ant function is expected to affect mitochondrial energy transduction in the cell. In the mouse model, a knockout mutation of Ant1 is not lethal but it induces mitochondrial myopathy and cardiomyopathy (Graham *et al.* 1997). The loss of Ant1 inhibits oxidative phosphorylation and induces an increased H_2O_2 production and mtDNA damage (Esposito *et al.* 1999). In humans, specific mutations in Ant1 have been found to cause the neuromuscular degenerative disease autosomal dominant progressive external ophthalmoplegia (adPEO; Kaukonen *et al.* 2000). Phenotypically, the Ant1-mediated adPEO is manifested by the accumulation of multiple deletions of mtDNA in postmitotic tissues (Moraes *et al.* 1989; Zeviani *et al.* 1989; Suomalainen *et al.* 1997). It has been proposed that the mutations may disturb mtDNA maintenance mechanisms. A defect in ADP/ATP exchange could cause the depletion of matrix ADP. Low matrix ADP level subsequently affects the balance of the mitochondrial nucleotide pools and the accuracy of mtDNA replication. This would give rise to the accumulation of mutant mtDNA, thereby leading to defective energy production in the affected tissues (Suomalainen and Kaukonen 2001).

However, recent studies have indicated that mutations in Ant may interfere with cellular functions other than oxidative phosphorylation. This has come from the analysis of mutant alleles of the yeast Ant isoform, Aac2p. In an attempt to understand the pathogenic mechanism of adPEO caused by the evolutionarily conserved A114P mutation in Ant1p, an equivalent mutation, A128P, was introduced in the yeast Aac2p. It has been found that, while the yeast *aac2*^{A128P} allele is capable of exchanging ADP/ATP across the inner membrane as reflected by the capability of the mutant cells to grow on nonfermentable carbon sources, the mutation causes the loss of cell viability in a dominant-negative manner (Chen 2002). The Aac2p-mediated lethality is accompanied by a drastic membrane depolarization and structural swelling of mitochondria. These findings raised the possibility that the mutant Ant may form an unselective channel that causes mitochondrial dysfunction and cell death. How the unselective channel arises is unclear, but it can be speculated that it derives from the adenine nucleotide translocation channel. Alternatively, it may arise from a dysregulation of a novel channel activity associated with Ant in yeast.

The budding yeast *S. cerevisiae* has three Ant isoforms that are encoded by the *AAC1*, *AAC2*, and *AAC3* genes. *AAC2* encodes the bulk of Ant and *AAC1* is poorly expressed (Gawaz *et al.* 1990; Lawson *et al.* 1990). The *AAC3* gene is expressed exclusively under anaerobic conditions (Kolarov *et al.* 1990). In the present study, it is shown that the yeast Ant isoforms are functionally differentiated. The Aac2 and Aac3 proteins, but not Aac1, have an essential cellular function for mitotic viability of the cell. The essential function is overlapped by a novel carrier protein named Sal1p. As both Ant and Sal1p are evolutionarily conserved, the bifunctionality of Ant and the functional interaction with the proteins of the Sal1 family could have general implications for other eukaryotes.

MATERIALS AND METHODS

Media for cell growth: Complete medium for the growth of yeast cells (GYP) contains 0.5% Bacto yeast extract, 1% Bacto peptone, and 2% glucose. Glycerol medium contains 2% glycerol in place of glucose. Glucose minimal medium contains 0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, and 2% glucose. Nutrients essential for auxotrophic strains were added at 25 μg/ml for bases and 50 μg/ml for amino acids. For anaerobic conditions, GYP was supplemented with 30 μg/ml ergosterol and 0.5% Tween 80 as a source of sterol and unsaturated fatty acids.

Strain and plasmid construction: Yeast strains used in this study are listed in Table 1. *AAC2* was disrupted by replacing the first 175 codons and the flanking 325-bp sequence in the promoter region of the gene with the MX4 *kan* module. *SAL1* was disrupted by replacing the sequence from codon 134 to codon 339 with *kan*. The isogenic strains CS341 and CS415 were derived from W303-1B by disrupting *AAC2* and *SAL1*, respectively. CS523/3 was constructed by the integration of the *GAL10-AAC2* cassette into the *ura3* locus of CS415 (*sal1Δ::kan*), which was followed by the replacement of the wild-type *AAC2* gene with the *aac2Δ::LEU2* cassette. CS523/3 is therefore nonviable on glucose medium as it contains only one copy of *AAC2* controlled by the galactose-inducible and glucose-repressible *GAL10* promoter. The K48A and R96H mutants of *AAC2* and the D62R and D93R alleles of *SAL1* were generated by *in vitro* mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Isolation of *sal1-1* and *SAL1*: CS295-3C (*aac2Δ::kan sal1-1* [pSEYc58-ScAAC2]) is sensitive to 5-fluoroorotic acid (5-FOA), as it carries the *AAC2* gene on a centromeric and *URA3*-based plasmid. The strain was transformed with a *S. cerevisiae* genomic library based on the multicopy vector YEp13M4 by selecting for Leu$^+$ colonies on selective minimal medium. A total of 16,500 Leu$^+$ transformants were screened for 5-FOA resistance by two successive replica platings on minimal medium containing 5-FOA at 1 mg/ml. Plasmids were rescued in *Escherichia coli* from 22 transformants that were stable for the Leu$^+$

TABLE 1

Genotype and source of yeast strains

Strain	Relevant phenotype	Source or reference
AH22	*MAT***a**, *leu2-3, 112 his4-519 can1*	G. Fink
CH1305	*MAT***a**, *ade2 ade3 leu2 ura3 lys2 can1*	C. Holm
CS5	*MAT***a**/α, *leu2/leu2 ura3/ura3 ade2/ADE2 his4/HIS4*	Chen and Clark-Walker (1999)
CS282/1	As W303-1B, but *aac2*Δ*::LEU2*	This study
CS293-1D	*MAT*α, *ade2 ura3 leu2 trp1 his3 lys2 aac2*Δ*::LEU2 SAL1*	This study
CS294	As CS5, but *aac2*Δ*::kan/AAC2*	This study
CS295-3C	*MAT*α, *leu2 ade2 ura3 aac2*Δ*::kan sal1-1* [pSEYc58-ScAAC2]	This study
CS341	As W303-1B, but *aac2*Δ*::kan*	This study
CS354	As CS294, but [pCXJ24-ScAAC2-1]	This study
CS355	As CS294, but [pCXJ24-ScAAC2-3]	This study
CS356	As CS294, but [pCXJ24-ScAAC2]	This study
CS364/1	*MAT***a**/α, *leu2/leu2 ura3/ura3 ade2/ADE2 his3/HIS4 aac1*Δ*::kan/aac1*Δ*::kan aac3*Δ*::kan/aac3*Δ*::kan aac2*Δ*::LEU2/+*	This study
CS366	As CS294, but [pCXJ24-ScAAC2^{K48A}]	This study
CS415	As W303-1B, but *sal1*Δ*::kan*	This study
CS428	As CS294, but [pCXJ24-ScAAC2^{R96H}]	This study
CS494-2B	*MAT***a**, *his4 leu2 aac2*Δ*::kan ura3::GAL-AAC2*	This study
CS523/3	As W303-1B, but *sal1*Δ*::kan aac2*Δ*::LEU2 ura3::pURA-GAL-AAC2*	This study
M2915-6A	*MAT***a**, *ade2 leu2 ura3*	Chen and Clark-Walker (1999)
W303-1B	*MAT*α, *leu2-3, 112 his3-11- 15 ura3-1 ade2-1 trp1-1 can1-100 SAL1*	R. Rothtein

phenotype. Nucleotide sequence analysis of the 22 plasmids revealed that seven and nine of the clones correspond to the *AAC2* and *AAC3* loci, respectively. The remaining six clones harbored the YNL083w open reading frame (ORF) in the frameshifted *sal1-1* form. The full-length *SAL1* allele was amplified from W303-1B genomic DNA by PCR and resequenced using oligonucleotide primers.

Expression of *SAL1*$^{1-220}$ and calcium-binding assay: The first 220 codons of *SAL1* and its mutant variants were amplified in a PCR by using two oligonucleotide primers and the *SAL1*, *sal1*^{D62R}, and *sal1*^{D93R} alleles as templates. The PCR products, encompassing a His_6 tag immediately after the ATG initiation codon and a stop codon immediately downstream of L220, were cloned into the *E. coli* expression vector pKK261. Expression of the constructs from the *E. coli* DH5αF′ strain was induced by isopropyl-β-d-thiogalactopyranoside at 1 mm. After cell lysis, the polypeptides were purified by the Talon metal affinity columns (CLONTECH, Palo Alto, CA) under nondenaturing conditions. For the Ca^{2+}-binding assay, Sal1$^{1-220}$, Sal1$^{1-220(D62R)}$, and Sal1$^{1-220(D93R)}$ were resolved on a 15% SDS-PAGE and transferred to a nylon membrane. The membrane was then incubated in the buffer (10 mm imidazole-HCl, pH 6.8, 5 mm $MgCl_2$, 60 mm KCl, $^{45}Ca^{2+}$ at 1.87 mCi/ml) for 10 min. After rinsing three times with 40% ethanol, the membrane was air dried and exposed to X-ray film at −80° overnight.

Mitochondrial membrane potential measurement: Mitochondrial membrane potential was estimated by using flow cytometry. Cells were loaded with the mitochondrial-membrane-potential-sensitive fluorescent probe $DiOC_6$ (3,3′-dihexyloxacarbocyanine iodine; Molecular Probes, Eugene, OR) at 0.1 μm (Bouillaud *et al.* 1994) and intracellular accumulation of the dye was measured with a Becton Dickinson FACscan flow cytometer.

RESULTS

The yeast *AAC2* and *AAC3*, but not *AAC1*, have a novel cellular function essential for cell viability: The yeast Aac2p shares 90.6% of identical sequences with Aac3p. Although Aac1p is distantly related to Aac2p with an identity score of 78.2%, early investigations have shown that these two proteins do not have a drastic difference in their nucleotide transport capacity (Gawaz *et al.* 1990). When placed under the control of the *AAC2* promoter and introduced in a strain lacking the endogenous *AAC2* (but *SAL1*, see below; Figure 1A), the three proteins contribute equally to respiratory growth on medium containing a nonfermentable carbon source such as glycerol (Figure 1B).

However, disruption of *AAC2* was found to be a lethal event in most laboratory strains. Meiotic progeny receiving a disrupted allele of *AAC2* from the diploid CS294, heterozygous for *AAC2/aac2*Δ*::kan*, were segregated into nonviable microcolonies on medium containing glucose as a carbon source (Figure 1C). Microscopic inspection revealed that the microcolonies contain ∼2000–4000 cells, indicating that these cells can undergo 11–12 cell divisions before ceasing to grow. Under these conditions the *AAC3* gene is repressed whereas *AAC1* is poorly expressed.

As shown in Figure 1C, overexpression of *AAC3* from the *AAC2* promoter was found to be able to rescue the nonviable *aac2* spores. Surprisingly, the overproduced Aac1p failed to do so despite its ability to replace *AAC2* for supporting respiratory growth (Figure 1B). The simplest interpretation for this observation is that *AAC2* and *AAC3*, but not *AAC1*, have a novel function essential for a cell's mitotic viability. The failure in complementation by the catalytically competent Aac1 isoform suggests that the novel function is distinct from ADP/ATP exchange. The moderately diverged Aac1p can support respiratory growth only by catalyzing ADP/ATP exchange

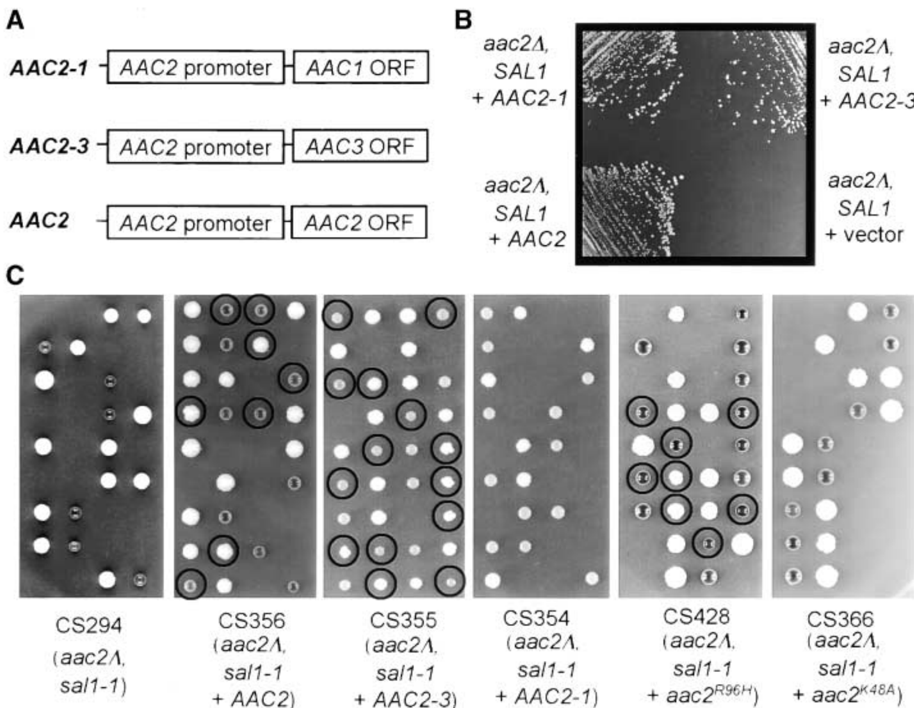

FIGURE 1.—Functional differentiation and bifunctionality of the *S. cerevisiae* Ant isoforms. (A) Schematic of the *AAC2-1*, *AAC2-3*, and *AAC2* constructs. The yeast *AAC1* and *AAC3* ORFs were amplified by PCR and fused to the *AAC2* promoter for overexpression. (B) Respiratory growth of CS341 (*aac2*Δ*::kan SAL1*) transformed with the *AAC2-1*, *AAC2-3*, and *AAC2* constructs and with the empty centromeric vector pCXJ24 (LEU2; CHEN 1996). Leu^+ transformants were streaked onto complete medium containing 2% glycerol as a sole carbon source. The plate was incubated at 30° for 4 days before being photographed. (C) Tetrad dissection of CS294 (*leu2/leu2 sal1-1/sal1-1 aac2*Δ*::kan/AAC2*) and its transformants carrying the *AAC2* (CS356), $aac2^{K48A}$ (CS366), and $aac2^{R96H}$ (CS428) alleles and the *AAC2-3* (CS355) and *AAC2-1* (CS354) fusions based on pCXJ24. The Leu^+ transformants were sporulated and the asci were dissected onto complete glucose medium. The plates were incubated at 30° for 3–5 days before being photographed. Meiotic progenies carrying the *aac2*Δ*::kan* allele are segregated into either nonviable microcolonies or large colonies as a result of rescue by the plasmids (outlined). In all the transformants, 40–60% of the viable colonies were Leu^+, which marks the retention of the plasmids.

across the mitochondrial inner membrane but does not provide the novel cellular function shared by Aac2p and Aac3p. These observations raise the possibility that Aac2p and Aac3p are bifunctional molecules. In addition to their role in respiratory growth, these two proteins also support the cell's mitotic viability on fermentable carbon sources. These two distinct roles of Ant have been designated the R (for respiration) and the V (for viability) functions, respectively.

To support the bifunctionality notion of Aac2p, a genetic dissection for the R and V functions of the protein was attempted. To do this, point mutations that are respiratory deficient (R^-) were tested to see whether they still retain the V function. As exemplified in Figure 1C, the respiratory-deficient $aac2^{R96H}$ allele (KOLAROV *et al.* 1990; LAWSON *et al.* 1990), known as *op1* (KOVAC *et al.* 1967), is able to rescue *aac2* lethality (Figure 1C). Together with the R^+V^- phenotype associated with the naturally occurring Aac1p variant, the R^-V^+ nature of $aac2^{R96H}$ provides compelling evidence that the two functions of Ant can be genetically bisected.

The absence of Aac2p, which is the most abundant protein in the inner membrane, could also alter the protein/lipid ratio and the biophysical properties of the membrane and subsequently cause the loss of cell viability. To exclude this possibility, several *aac2* alleles were examined for their ability to support cell viability. These alleles have previously been shown to be correctly expressed and targeted into mitochondria (MULLER *et al.* 1996). As exemplified in Figure 1C, one of these alleles, $aac2^{K48A}$, is unable to support cell growth on glucose medium, suggesting that the death of *aac2* mutants is not caused by the physical absence of Aac2p in the membrane.

Identification of the *SAL1* locus that suppresses the lethal phenotype of the *aac2* mutant: *AAC2* has been successfully disrupted in haploid strains of W303 background by several laboratories (LAWSON *et al.* 1990; DRGON *et al.* 1991). However, attempts to disrupt *AAC2* in the present study have never given rise to a correct replacement of the wild-type *AAC2* by the *aac2*Δ*::LEU2* cassette in the haploid strains M2915-6A, AH22, and CH1305. The failure in disrupting *AAC2* in the non-W303 strains raised the possibility that a suppressor gene that allows for the survival of *aac2*Δ cells may be present in W303. To test this hypothesis, CS282/1 (W303-1B/*aac2*Δ*::LEU2*) was crossed to M2915-6A, AH22, and CH1305, which have different origins and are unrelated to W303. The resulting diploids, CS291, CS292, and CS293, were sporulated and 21, 32, and 61 asci were dissected, respectively, on complete glucose medium. Indeed, in the three crosses, 58, 50, and 50% of the Leu^+ spores were found to be segregated into viable colonies. All the viable Leu^+ segregants are respiratory deficient. The rest of the Leu^+ spores were segregated into nonviable microcolonies of 2000–4000 cells as described in Figure 1C. These observations strongly suggested that there is a single locus in W303 that suppresses the inviability but not the respiratory growth of *aac2* cells. This suppressor locus, *SAL1*, and its nonsup-

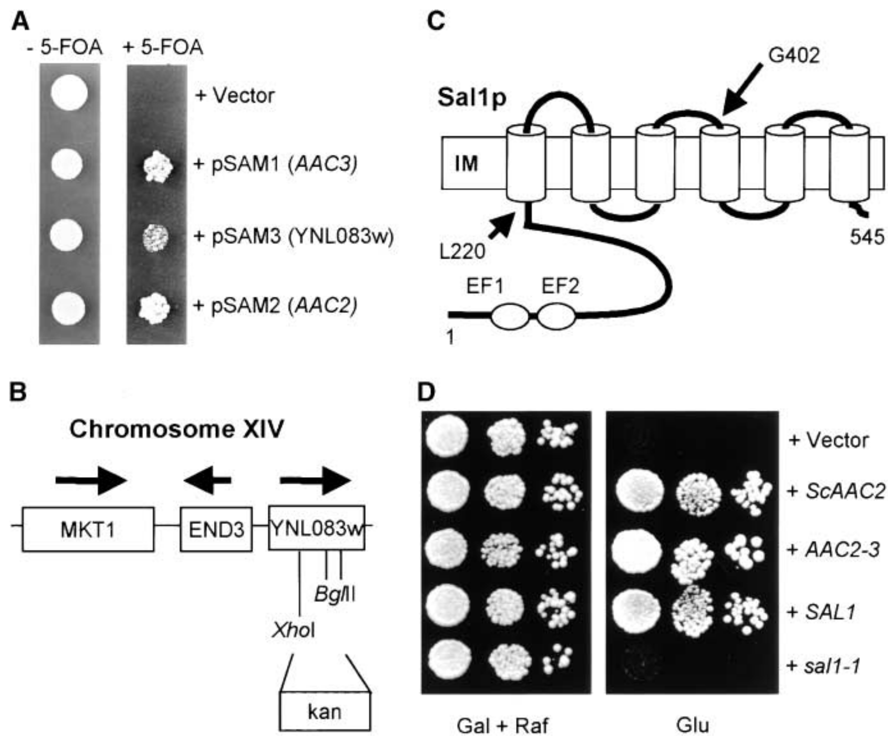

FIGURE 2.—Isolation and characterization of *SAL1*. (A) Growth phenotype of CS295-3C transformants in the presence or absence of 5-FOA. Yeast transformants were diluted in water, spotted onto minimal glucose medium supplemented with adenine (20 μg/ml), leucine (40 μg/ml), uracil (10 μg/ml), and 5-FOA (1 mg/ml). The plates were incubated at 30° for 5 days before photography. CS295-3C (*aac2*Δ*::kan* [pSEYc58-ScAAC2]) carries the unique copy of *AAC2* on a *URA3*-based plasmid and is sensitive to plasmid elimination by 5-FOA. Transformants of the genomic clones pSAM1, pSAM3, and pSAM2, carrying *AAC3*, *sal1-1*, and *AAC2*, respectively, are 5-FOA^R because the *LEU2*-based genomic clones can rescue cell death after the elimination of pSEYc58-ScAAC2. (B) Physical map of the insert DNA from the *S. cerevisiae* chromosome XIV in the genomic clone pSAM3. Also shown is the location of the *Bgl*II and *Xho*I sites used for creating internal deletion and the knockout allele of YNL083w (SAL1). (C) Predicted structural organization of Sal1p. Sal1p displays six transmembrane domains on its carboxyl terminus across the mitochondrial inner membrane (IM) and two well-conserved EF-hand motifs (EF1 and EF2) with putative Ca^{2+}-binding capacity. G402 indicates the position where the frameshift occurs in the *sal1-1* allele. The leucine 220 (L220) residue marks the boundary between the hydrophilic N-terminal sequence and the first transmembrane domain. (D) Growth phenotype of yeast transformants showing the difference between *SAL1* and *sal1-1* in suppressing *aac2* lethality when expressed from a centromeric vector. CS494-2B (*leu2 aac2*Δ*::kan ura3::GAL-AAC2*) contains a chromosomal copy of *AAC2* under the control of the *GAL10* promoter and is viable on medium with galactose and raffinose as carbon sources. Glucose repression leads to growth arrest as a result of *AAC2* depletion. The cell lethality is rescued by the introduction of a full-length *SAL1* allele on the centromeric plasmid pCXJ24 (*LEU2*). The truncated *sal1-1* allele is unable to suppress the lethality under the same conditions. The *AAC2-3* fusion on monocopy (see Figure 1A) can also suppress *aac2*Δ as can the wild-type *AAC2* allele.

pressing version in non-W303 strains were temporarily designated *sal1-1*. *SAL1* was found to suppress the inviability of *aac2* cells in a dominant manner, because a diploid strain homozygous for *aac2*Δ/*aac2*Δ but heterozygous for *SAL1/sal1* is viable on complete glucose medium (data not shown).

Isolation of *SAL1*: The *SAL1* gene was identified in a screen for genes whose overexpression suppresses *aac2* lethality in a *sal1* background. A *S. cerevisiae* genomic library based on a *LEU2*-bearing multicopy vector was introduced into CS295-3C (*aac2*Δ*::kan* [pSEYc58-ScAAC2]), which carries the only wild-type copy of *AAC2* on the *URA3*-based plasmid pSEYc58-ScAAC2. Leu$^+$ colonies resistant to the elimination of pSEYc58-ScAAC2 by 5-FOA were screened. 5-FOA^R cells were assumed to contain genomic clones acting as a suppressor of *aac2*Δ. Among the identified suppressor clones were pSAM2, pSAM1, and pSAM3 that contain, respectively, *AAC2*, *AAC3*, and a 7.1-kb genomic fragment from chromosome XIV (Figure 2A). The latter harbors the *MKT1*, *END3* genes and the open reading frame YNL083w (Figure 2B). This clone was therefore assumed to contain a suppressor gene, although the suppressor phenotype appears to be significantly weaker than what was observed with the plasmids containing *AAC2* and *AAC3* (Figure 2A). Subcloning of the insert DNA in pSAM3 has shown that YNL083w is responsible for the suppressor phenotype, as the deletion of a 219-bp *Bgl*II internal fragment within YNL083w abolishes the suppressor activity of pSAM3 (not illustrated).

Sequencing of YNL083w on the genomic clone pSAM3 revealed an open reading frame of 494 amino acids. It was subsequently found that YNL083w is allelic to the *SAL1* locus in W303-derived strains. This was demonstrated as follows. The YNL083w locus of the W303 derivative, CS293-1D (*aac2*Δ*::LEU2 SAL1*), was first marked by the integration of a *URA3*-based plasmid preserving a functional copy of YNL083w. The resulting strain was crossed to M2915-6A (*AAC2 sal1-1*) and the diploid was sporulated and dissected for tetrad analysis. From 18 tetrads analyzed, 14 Leu$^+$ but respiratory-deficient meiotic segregants were scored. These segregants are the survivors of the *aac2*Δ spores resulting from the suppression by *SAL1*. More importantly, all the suppressed *aac2*Δ*::LEU2* segregants were Ura$^+$, which marks the chromosomal locus defined by YNL083w. The remaining 22 Leu$^+$ spores were segregated into nonviable microcolonies, which were all deduced to be Ura$^-$. These

data strongly suggest that the suppressor *SAL1* locus in the W303-derived strains is allelic to YNL083w.

The allelism between *SAL1* and YNL083w would have to suggest that the suppressor gene is functionally active in W303 but inactive in non-W303 strains such as M2915-6A. To find the molecular basis for the strain-polymorphic phenomenon, YNL083w was amplified by PCR from M2915-6A and W303-1B and subjected to sequence analysis. Indeed, sequence difference was found between the two strains. YNL083w from M2915-6A encodes a polypeptide of 494 amino acids whereas that from W303-1B is capable of encoding a protein of 545 residues. Instead of having the sequence stretch 5′-GGGT GGGC-3′ at codon 402 in W303-1B, the sequence 5′-GGG GGGGGG-3′ was found in M2915-5A. The insertion of an extra base in the latter results in a frameshift that gives rise to the truncated protein of 494 residues. Similar sequence polymorphism at YNL083w has previously been reported by other investigators (Belenkiy *et al.* 2000). From these data, it was concluded that the suppression of *aac2* lethality in W303-derived strains is mediated by the expression of the full-length *SAL1* locus. The truncated version of the gene in non-W303 strains, *sal1-1*, is functionally inactive.

The YNL083w suppressor clone initially identified from the genomic library has an identical sequence to that from M2915-6A. The genomic library had thus been constructed from a non-W303 strain. However, if the 494-residue Sal1-1 protein is functionally inactive, how can we explain the recovery of the truncated *sal1-1* allele in the suppressor screening? One possibility that could reconcile the discrepancy is that the *sal1-1* is a multicopy suppressor as the genomic library has been constructed with a 2-μm-based vector. This was found to be the case. As shown in Figure 2C, the truncation at the codon 402 would cause a major structural change to the proteins in the last three transmembrane domains (see below). Expression of the truncated *sal1-1* allele from a monocopy vector failed to suppress *aac2*Δ (Figure 2D), in contrast to a moderate suppressor phenotype as shown in Figure 2A when the gene was expressed from a multicopy plasmid. As expected, the full-length *SAL1* allele from W303-1B suppressed *aac2*Δ on monocopy as efficiently as do the native *AAC2* and *AAC3* under the control of the *AAC2* promoter.

Sal1p is an evolutionarily conserved bipartite mitochondrial carrier protein: The data described above demonstrated that *SAL1* is a strain-polymorphic gene that is synthetically lethal with the disruption of *AAC2*. Sal1p displays characteristic features of the MCF proteins (Figure 2C). Six transmembrane helices can be predicted from its carboxyl terminal sequence that is composed of three mitochondrial carrier protein signatures. This part of the protein shows 25–30% sequence identity to various MCF proteins, including Aac2p. Unlike the majority of MCF members, which have a size of ∼300 amino acids (Nelson *et al.* 1998; Belenkiy *et al.* 2000), Sal1p has an extension of ∼220 residues on its amino terminus. The hydrophilic N-terminal extension harbors two sequences matching the calcium-binding site known as the elongation factor (EF)-hand motif.

A protein database search revealed that Sal1p belongs to a subfamily of proteins called Ca^{2+}-binding mitochondrial carriers (del Arco and Satrustegui 1998). Members of this protein family have a similar molecular architecture: the presence of EF-hand Ca^{2+}-binding motifs in their N-terminal domains and the characteristic features of the mitochondrial carrier family proteins in their C-terminal domains. Many eukaryotic species have multiple genes that exhibit significant homology with Sal1p. As exemplified in Figure 3, Sal1p shares 28–29% sequence identity with the uncharacterized proteins XP_027668, NP_766273, and NP_199918 from humans, mouse, and *Arabidopsis thaliana*, respectively. The most conserved sequences appear to occur in the putative transmembrane and the Ca^{2+}-binding domains. Mashima and co-workers have recently reported on the rat MCSC protein, which is likely a homolog of the human protein encoded by XP_027668 (Mashima *et al.* 2003).

Sal1p is a Ca^{2+}-binding protein and Ca^{2+} binding is essential for its physiological function: Sal1p contains two putative EF-hand Ca^{2+}-binding motifs at positions 62–74 and 93–105 (Figure 4A). To demonstrate that Sal1p is a Ca^{2+}-binding protein, the 220-residue segment on its N terminus was expressed in *E. coli* and purified in a His_6-tagged form. *In vitro* Ca^{2+}-binding assay demonstrated that the 220-residue polypeptide binds free Ca^{2+} (Figure 4B, wild type). The Ca^{2+}-binding activity was found to be dependent on the EF-hands. When the highly conserved acidic D62 and D92 residues were individually replaced with lysine by *in vitro* site-specific mutagenesis, the resulting mutant alleles could no longer bind Ca^{2+}. It appears that the presence of both EF-hands is essential for the Ca^{2+}-binding activity of the protein.

To know whether Ca^{2+} binding is required for Sal1 function, the $sal1^{D62R}$ and $sal1^{D93R}$ alleles defective in the ion binding were tested for their biological function *in vivo*. As shown in Figure 4C, the loss of Ca^{2+} binding abrogates the ability of the protein to suppress *aac2*Δ. These observations strongly suggest that Sal1p is a Ca^{2+}-dependent mitochondrial carrier protein and that the ion binding is essential for its physiological function.

Overexpression of *SAL1* does not support the respiratory growth of *aac2* mutants: *SAL1* was disrupted by replacing the internal 627-bp *Xho*I-*Bgl*II fragment with the *kan* module (Figure 2B). Strains carrying a disrupted *SAL1* allele did not show any notable respiratory defect on the nonfermentable glycerol medium at both 30° and 37°. Therefore, it is unlikely that *SAL1* plays any critical role in mitochondrial respiration in the presence of a functional *AAC2*.

As described above, overexpressed *AAC3* can replace *AAC2* for both respiratory growth and the life-supporting function, whereas *AAC1* can complement only the

FIGURE 3.—Amino acid sequence similarities between Sal1p (ScSal1) and its homologs from human (XP_027668), mouse (NP_766273), and *A. thaliana* (At, NP_199918). Identical residues are on a black background and conserved ones are on a white background. The conserved EF-hand motifs are indicated. Predicted transmembrane domains are underlined by asterisks.

respiratory defect but not the mitotic cell death of *aac2* mutants. To know whether *SAL1* has a dual role like *AAC3*, the gene was cloned into several multicopy vectors and introduced into CS341 (*aac2*Δ *SAL1*). The results showed that the transformants carrying an overexpressed *SAL1* could not grow on complete medium with glycerol as a carbon source. Under the same conditions, the poorly expressed *AAC1* gene controlled by its native promoter can significantly complement the respiratory deficiency in CS341 (Figure 5). These observations would support the view that the primary role of *SAL1* is not the ADP/ATP exchange across the mitochondrial inner membrane. Rather, it has a more specific function for supporting cell viability.

Irreversible loss of cell viability induced by a combined inactivation of *AAC2* and *SAL1*: To understand the novel cellular function defined by *AAC2* and *SAL1*, a haploid strain was constructed in which the *SAL1* allele was disrupted and the *AAC2* gene was placed under the control of the galactose-inducible *GAL10* promoter. The resulting strain CS523/3 could divide for approximately seven to eight generations in the repressible glucose or noninducible raffinose medium before ceasing cell growth as a result of Aac2p depletion. Electron

Figure 4.—Ca^{2+} binding is essential for the physiological function of Sal1p. (A) Amino acid sequence of the two putative EF-hand motifs. The residues that contribute to the formation of the octahedral Ca^{2+} coordination cage are labeled *x, y, z*, $-x$, $-y$, and $-z$. D62 and D93 at the *x* position are in boldface type. (B) Ca^{2+}-binding activity of the amino-terminal half of Sal1p and its mutant variants. Affinity-column-purified His_6-tagged $Sal1^{1-220}$ (lane 1), $Sal1^{1-220(D62R)}$ (lane 2), and $Sal1^{1-220(D93R)}$ (lane 3) were resolved in a 15% SDS-PAGE and the proteins (26 kD) were visualized by Coomassie Brilliant Blue staining (left). The His_6-tagged Mgm101p (Zuo *et al.* 2002) was included as a negative control (lane 4). The proteins were transferred to a nylon membrane and incubated with $^{45}Ca^{2+}$ (right). Autoradiography detected the binding of $^{45}Ca^{2+}$ to $Sal1^{1-220}$ but not to $Sal1^{1-220(D62R)}$ or $Sal1^{1-220(D93R)}$. (C) Growth phenotype of yeast transformants showing that the $sal1^{D62R}$ and $sal1^{D93R}$ alleles failed to rescue *aac2* lethality. Transformants of CS295-3C (*aac2*Δ*::kan* [pSEYc58-ScAAC2]) carrying the *SAL1*, $sal1^{D62R}$, and $sal1^{D93R}$ alleles were tested for their ability to rescue cell growth after the elimination of the endogenous plasmid pSEYc58-ScAAC2 by 5-FOA as described in Figure 2A.

Figure 5.—Overexpression of *SAL1* is unable to complement the R-deficient phenotype of the *aac2* mutant. *SAL1*, *AAC1*, and *AAC2* with their native promoters were cloned into the 2-μ-based multicopy vector pCXJ17 (X. J. Chen, unpublished results) and introduced into CS341 (*aac2*Δ *SAL1*). The transformants were tested for respiratory growth on complete medium containing glycerol as a carbon source. The plate was incubated at 30° for 7 days before being photographed. Note that the overexpressed *AAC1*, but not *SAL1*, complements the R^- phenotype of the *aac2* mutant.

microscopic examination of the cells inactivated in both *AAC2* and *SAL1* did not detect any major morphological changes to mitochondria (data not shown). However, mitochondria appeared to suffer irreversible damage. After a prolonged incubation in glucose medium before being returned to the inducible galactose medium, the vast majority of the Aac2p-depleted cells formed nonviable microcolonies (Figure 6A).

The irreversible loss of cell viability induced by a combined inactivation of *AAC2* and *SAL1* might result from oxidative damage to mitochondria. Impairment in the Ant function could lead to the depletion of matrix ADP as a result of a defect in ADP import. A low matrix ADP could subsequently cause the inhibition of F_1F_0-ATP synthase, the hyperpolarization of the inner membrane, and the overproduction of reactive oxygen species (Esposito *et al.* 1999). To know whether mitochondrial hyperpolarization/oxidative damage underlies the irreversible loss of cell viability in the yeast *aac2 sal1* double mutants, the mammalian mitochondrial uncoupling protein 1 (UCP1; Bouillaud *et al.* 1994) was expressed in yeast to see whether an uncoupling of the mitochondrial inner membrane could alleviate membrane hyperpolarization and rescue cells from the irreversible death. The results have shown that UCP1 expression was unable to rescue the nonviable meiotic segregants derived from CS294 (*AAC2/aac2*Δ*::kan sal1-1/sal1-1*; data not shown). In fact, when the mitochondrial membrane potential (Δψ) in the *aac2* and *sal1* double mutant was measured by flow cytometry, it was found that these cells have a Δψ lower than that of wild type but comparable with that from the *aac2*Δ single mutant (Figure 6B). Single disruption of *SAL1* does not have a significant decrease in Δψ.

A possible oxidative damage to mitochondria was also addressed in *aac2 sal1* mutants in the following experiments. First, the diploid strain CS294 (*AAC2/aac2*Δ*::kan*, *sal1-1/sal1-1*) was directly dissected on glucose medium supplemented with *N*-tert-butyl-α-phenylnitrone. It was found that the inclusion of the free radical scavenger failed to prevent the *aac2 sal1-1* segregants from forming nonviable microcolonies (data not shown). Second, the *aac2 sal1-1* segregants were examined for their ability to form viable colonies under anaerobic conditions. To do this, the diploid strain CS364 (*aac1*Δ*::kan/aac1*Δ*::kan*

*aac3*Δ*::kan/aac3*Δ*::kan aac2*Δ*::LEU2/+ sal1-1/sal1-1*) was constructed, in which both the *AAC1* gene and the anaerobically inducible *AAC3* are disrupted. Following sporulation, the meiotic segregants were incubated under anaerobic conditions. To exclude the possibility that a failure in colony formation by the Δ*aac2* segregants is caused by a defect in importing the cytosolic ATP into mitochondria, the plasmid pCXJ22-AAC2-1 was introduced into CS364 before sporulation. pCXJ22-AAC2-1 can support ADP/ATP exchange by the overexpression of *AAC1* from the *AAC2* promoter. As shown in Figure 6C, the two *aac2*Δ*::LEU2* spores from each tetrad of CS364 are all segregated into nonviable microcolonies even in the presence of an active Aac1p. As a control, the expression of *AAC3* could rescue the *aac2*Δ*::LEU2* spores from lethality under the same conditions. Taken together, these data clearly indicate that anaerobiosis could not suppress the loss of cell viability in the *aac2 sal1* double mutant. It is unlikely that the irreversible loss of cell viability induced by the simultaneous inactivation of *AAC2* and *SAL1* is related to cellular damage by reactive oxygen species.

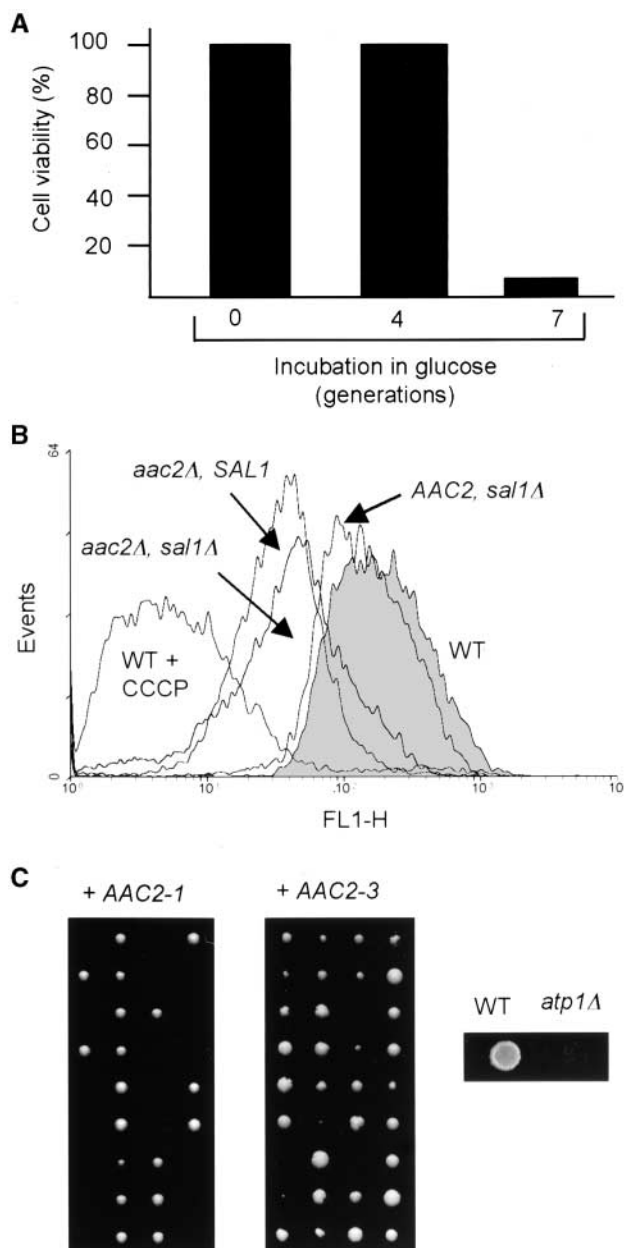

Figure 6.—Irreversible cell death, mitochondrial membrane potential (Δψ), and anaerobic growth of *aac2* mutants. (A) Irreversible cellular damage caused by inactivation of *AAC2* and *SAL1*. CS523/3 (*sal1*Δ*::kan aac2*Δ*::LEU2 GAL10-AAC2*) was grown in complete galactose medium for 24 hr. Cells were washed with sterile water and diluted into complete glucose medium to a cell density of 2.8×10^5 cells/ml. Cells were allowed to grow at 30° for four and seven generations. Aliquots were examined for colony-forming ability on complete galactose medium that induces the expression of *GAL10-AAC2*. A prolonged incubation under the *AAC2*-depleting conditions in glucose medium caused low cell viability as indicated by the formation of nonviable microcolonies when returned to galactose medium. (B) Mitochondrial membrane potential of yeast cells measured as the intracellular accumulation of the fluorescent dye $DiOC_6$ by flow cytometry. The number of cells (*y*-axis) relative to fluorescence intensity (*x*-axis) is represented. The *x*-axis is in a logarithmic scale and a shift of the curve toward the left indicates a decreased membrane potential. W303-1B (WT), CS341 (*aac2*Δ *SAL1*), CS415 (*AAC2 sal1*Δ), and CS523/3 (*aac2*Δ *sal1*Δ *GAL10-AAC2*) were grown in the complete galactose plus raffinose medium to late exponential phase, washed with water, and transferred to the noninducible medium containing 2% raffinose. After incubation for six to seven generations at 30°, cells were loaded with $DiOC_6$ in the presence or absence of the mitochondrial uncoupler CCCP (carbonyl cyanide *m*-chloro-phenylhydrazone) at 50 μm before being analyzed in a FACscan flow cytometer. (C) Anaerobiosis cannot rescue *aac2*-lethality. Transformants of CS364/1 (*aac1*Δ*::kan/aac1*Δ*::kan aac3*Δ*::kan/aac3*Δ*::kan aac2*Δ*::LEU2/AAC2*) carrying the overexpressed *AAC1* (*AAC2-1*) and *AAC3* (*AAC2-3*) were sporulated and dissected on complete glucose medium supplemented with ergosterol and Tween. The meiotic segregants were incubated under anaerobic conditions for 7 days before being photographed. The wild-type M2915-6A and its isogenic *atp1* mutant were included as positive and negative controls for the anaerobic conditions (Chen and Clark-Walker 1999).

DISCUSSION

Ant has been intensively studied for its role in promoting ADP/ATP exchange across the mitochondrial inner membrane. This study reported that the yeast Aac2 and Aac3, but not the Aac1 isoform of Ant, have a cellular function essential for maintaining the cell's mitotic viability on fermentable carbon sources. The role of Aac2p for maintaining mitochondrial R and mitotic V can be genetically bisected by the analysis of mutant alleles that affect only one of the two functions. Furthermore, it has been shown that the strain-polymorphic locus, *SAL1*, can complement the V but not the R function of the *aac2* mutant. These data suggest that Aac2p is a bifunctional molecule. In addition to its role in catalyzing ADP/ATP

exchange required for respiratory growth, it carries out an extra function during cell proliferation.

The functional differentiation and bifunctionality of Ant could have general implications. Many eukaryotic genomes encode multiple isoforms of Ant. Higher eukaryotes can have Ant isoforms that are diverged from each other as much as in yeast. For example, the two Ant homologs in *D. melanogaster* share only 78% of identical sequences (Zhang *et al.* 1999), an identity score very similar to that for the functionally differentiated Aac1p and Aac2p in yeast. In this respect, it is noteworthy that the Ant1, but not Ant2, isoform from mouse can dominantly induce apoptosis when they are overexpressed. The Ant1p-induced apoptosis is apparently independent of the ADP/ATP exchange activity as revealed by mutagenic analysis (Bauer *et al.* 1999). It is also interesting to note that the two Ant isoforms from rat have been shown to be differentially distributed inside mitochondria. The peripheral inner membrane contains both Ant1p and Ant2p whereas the crystal membrane apparently contains exclusively Ant2p (Vyssokikh *et al.* 2001). It remains to be seen whether there is a specialization of the mammalian Ant isoforms at the biochemical level.

A significant contribution from this study is the identification of the evolutionarily conserved *SAL1* gene that functionally overlaps with the V function of *AAC2*. *SAL1* is a novel yeast gene uncharacterized so far. The full-length *SAL1* allele encodes a putative protein of 545 amino acids with a calculated molecular weight of 61 kD. Sal1p is evolutionarily conserved and belongs to a novel subclass of proteins in the mitochondrial carrier family. Members of this protein subclass are predicted to have a bipartite molecular architecture with the presence of EF-hand Ca^{2+}-binding motifs in their N-terminal domains and six transmembrane helices in their C-terminal domains. Among these members are the human citrin and aralar1 proteins that catalyze aspartate/glutamate exchange across the mitochondrial inner membrane (Kobayashi *et al.* 1999; Palmieri *et al.* 2001). As was found for citrin and aralar1, the yeast Sal1p does bind Ca^{2+} *in vitro* and Ca^{2+} binding is apparently essential for its physiological function *in vivo*. Thus, although no significant sequence homology can be found between Sal1p and citrin, these bipartite molecules are likely to operate in a similar manner, which is to promote a Ca^{2+}-regulated transport across the mitochondrial inner membrane.

The nature of the Sal1p-based transport activity and the Aac2p-associated V function remains to be determined. The failure in complementing the V^- phenotype of *aac2* mutants by Aac1p, which is otherwise active in catalyzing ADP/ATP exchange, and the apparent lack of a Sal1-based respiration-supporting activity would suggest that Sal1p and Aac2p have a transport activity distinct from ADP/ATP exchange. With the currently available data, the simplest explanation would be that the two proteins transport adenine nucleotide(s) in a mode different from the one-to-one ADP/ATP exchange. In fact, in addition to ADP/ATP exchange, it has been proposed for a long time that mitochondria possess a transport mechanism that allows a net accumulation of adenine nucleotide during organelle proliferation (Aprille 1993). The maintenance of the adenine nucleotide level in the mitochondrial matrix is essential for mitochondrial biogenesis because the function of many mitochondrial proteins (such as the chaperones) is nucleotide dependent. The pioneering work by Aprille and colleagues has led to the proposition that two distinct pathways allow the net increase or decrease of adenine nucleotides in the mitochondrial matrix. Both pathways have been shown to exchange ATP-Mg^{2+} or ADP (but not AMP) on one side of the inner membrane and HPO_4^{2-} on the other side. The first pathway is Ca^{2+} dependent and carboxyatractyloside insensitive, whereas the second pathway appears to be Ca^{2+} independent and sensitive to carboxyatractyloside inhibition. The latter pathway has been suggested to represent a novel function of Ant or to involve a novel carrier protein sensitive to the drug. Under this context, it would be interesting to know whether the novel functions associated with Sal1p and Aac2p parallel the pathways required for the net import of adenine nucleotide into mitochondria.

The discovery of a novel cellular function defined by proteins of the Ant and Sal1 families could have further implications. Ant has long been suspected of being involved in the formation or regulation of the mitochondrial permeability transition pore, a high-conductance megachannel of low selectivity on the mitochondrial inner membranes with broad pathophysiological implications (Haworth and Hunter 1979; Hunter and Haworth 1979; Zoratti and Szabo 1995; Bernardi 1999; Kokoszka *et al.* 2004). It has also been shown that gain-of-function mutations in Ant1 cause human diseases such as adPEO (Kaukonen *et al.* 2000). In light of the present study, it would be very interesting to know whether the novel function of Ant contributes to the pathophysiology of these conditions.

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