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Sac1p of *Saccharomyces cerevisiae* Is Not Involved in ATP Release to the Extracellular Fluid

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One activity ascribed to Sac1p is the transport of ATP into the lumen of the endoplasmic reticulum of *Saccharomyces cerevisiae;* therefore, the question of whether this protein plays a role in ATP efflux from yeast was addressed. Preliminary results suggested that deletion of the *SAC1* gene eliminated nigericin-stimulated ATP efflux. However, further experimentation revealed that this result was caused by a pronounced extracellular ATPase activity for *sac1*Δ cells at alkaline pH, conditions required to measure extracellular ATP in wild type cells. At acid pH, *sac1*Δ cells exhibit glucose-dependent, nigericin-stimulated ATP efflux. *sac1*Δ cells express less acid phosphatase activity in the periplasm than do wild type cells, thus increasing the stability of extracellular ATP. At alkaline pH, however, *sac1*Δ cells tend to lose structural integrity and release lactate dehydrogenase as well as an unidentified ATPase activity to the extracellular fluid. Therefore, Sac1p is not involved in ATP efflux from *S. cerevisiae.*

ATP is released from *Saccharomyces cerevisiae* both in growing cultures and in artificial conditions, and the release is regulated by the level of intracellular cAMP (1,2). Beyond the role of cAMP, little is known about the mechanism of ATP release from yeast; indeed, little is known about the mechanism of ATP release from intact mammalian cells, even though the phenomenon has long been known to exist (3). One hypothesis is that members of the ATP binding cassette family of membrane transport ATPases such as P-glycoprotein and the cystic fibrosis transmembrane conductance regulator are involved in this process (4,5,6). Recent results, however, suggest that ATP binding cassette proteins may not be involved in ATP efflux from yeast (1).

ATP is transported from the cytosol to the endoplasmic reticulum of *S. cerevisiae* by the ATP/ADP exchanger Sac1p (7). Mutation of *SAC1* causes plieotropic effects in yeast, including secretory effects and the suppression of actin mutations (8,9). The fact that Sac1p is capable of mediating ATP transport raises the question of whether it plays a role in the accumulation of extracellular ATP.

Here we describe experiments conducted to test the hypothesis that Sac1p might have a role in ATP efflux from yeast. Although the amount of extracellular ATP was decreased in *sac1*Δ cells at alkaline pH compared to the levels in wild type cells, the decrease was not directly related to the absence of Sac1p. Rather, there are two important differences between *sac1*Δ cells and wild type cells: the mutant cells express less acid phosphatase activity in the periplasm, thus affecting the stability of extracellular ATP; and the mutant cells lose structural integrity at alkaline pH, leading to the release of cytoplasmic enzymes to the extracellular fluid, including an ATPase. Because of these two differences between *sac1*Δ cells and wild type cells, the stability of extracellular ATP is greater at acid pH than at alkaline pH for the mutant cells; this is the reverse of the situation with wild type cells. It is clear, however, that cells which do not express Sac1p do exhibit glucose-dependent, nigericin-stimulated ATP efflux, which can be observed when the experiment is conducted at acid pH.

MATERIALS AND METHODS

Materials. Saccharomyces cerevisiae Strains: *sac1*Δ (CTY244) and isogenic wild type control (CTY182) are described (9) and were obtained from Vytas Bankaitis, University of Alabama, Birmingham, Alabama.

Nigericin, p-nitrophenol phosphate, and nicotinamide adenine dinucleotide (reduced form) were obtained from Sigma.

Extracellular nucleotide assays. Cells were grown to mid log phase growth (A_{650}=1) in glucose-containing media and were prepared and incubated as described (1,2); the incubation time for all experiments was 30 minutes at 25° C. The composition of the incubation buffers is indicated in the tables. ATP and ADP were measured by high performance liquid chromatography (HPLC) as described (1,2).

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TABLE 1

Extracellular ATP of Wild Type and *sacl*Δ Cells[a]

	Wild type (pmoles/30 min./ 5×10^7 cells)	*sacl*Δ (pmoles/30 min./ 5×10^7 cells)
pH 8.8[b]		
+Nigericin, +glucose	186	45
−Nigericin, +glucose	47	31
pH 5.5[c]		
+Nigericin, +glucose	114	671
−Nigericin, +glucose	N.D.[d]	292
+Nigericin, −glucose	N.D.	81
−Nigericin, −glucose	N.D.	102

[a] Cells were prepared and incubated as described in Materials and Methods, +nigericin indicates the presence of 25 mg/ml nigericin, +glucose indicates the presence of 2 % (w/v) glucose.
[b] The incubation buffer is 20 mM Tris, pH 8.8.
[c] The incubation buffer is 20 mM Tris-citrate, pH 5.5.
[d] N.D. = None Detected.

Acid phosphatase assay. Periplasmic acid phosphatase activity was measured as described (10). Briefly, cells were grown to mid log phase (A_{650}=1). 8×10^7 cells of each type were then pelleted and resuspended in 0.1 ml 20 mM Tris-Citrate pH 5.5. 10 μl of the cell suspension was then added to 1.0 ml 100 mM sodium acetate, pH 4.0 containing 2 mM p-nitrophenol phosphate. At the times indicated in Figure 4, 2.0 ml of 0.5 N NaOH was added. The p-nitrophenol released by acid phosphatase activity was then measured by reading the absorbance of the solution at 405 nm.

Lactate dehydrogenase assay. Lactate dehydrogenase was detected by measuring the rate at which NADH was converted to NAD^+. Cells were prepared and incubated as for the experiments measuring extracellular nucleotide. Following the filtration step, the LDH activity of the filtrate was measured as follows: to 845 μl of filtrate in a cuvette, 5 μl of 100 mM pyruvate was added. The cuvette was placed in a Beckman spectrophotometer and 150 μl of 1 mM NADH (in 50 mM Tris pH 7.5) was added and rapidly mixed. The A_{340} of the solution was immediately monitored for the next 5 minutes at 3 second intervals. A decrease in absorbance was due to oxidation of NADH to NAD^+.

RESULTS

In order to measure extracellular ATP in yeast, an experiment was first conducted at alkaline pH in order to inactivate the periplasmic acid phospatases as described (1). Table 1, lines 1 & 2, show that extracellular ATP is present with both wild type control and *sac1*Δ cells. The efflux of ATP for both cell types is strictly dependent upon glucose, because no extracellular ATP was detected when glucose was omitted (data not shown). Furthermore, ATP efflux from wild type cells is stimulated by nigericin, as has been reported (1,2). In contrast, nigericin has little or no effect on the *sac1*Δ cells. This result suggests that Sac1p may indeed play a role in nigericin-stimulated ATP efflux from yeast.

The experiment just described was conducted at alkaline pH because previous results showed that this was the only way to measure ATP efflux from yeast expressing periplasmic acid phosphatase (1,2). However, since acid pH is more physiologically relevant to yeast, an important control is to conduct the same experiment at lower pH. In Table 1, lines 3-6, are shown the measurement of extracellular ATP at pH 5.5. As expected, extracellular ATP is not detected for the wild type control cells unless both nigericin and glucose are present (line 3), because in the absence of nigericin the smaller amount of ATP released is hydrolyzed by periplasmic acid phosphatase. Surprisingly, however, with *sac1*Δ cells there is a large amount of ATP in the extracellular fluid. The release of ATP is glucose-dependent and nigericin-stimulated. Therefore, Sac1p does not appear to have a role in ATP efflux; rather, the *sac1*Δ phenotype may result in a different composition of extracellular ATPase enzymes that is affected by pH.

To test this idea, the stability of extracellular ATP was tested at both acid and alkaline pH. Table 2 shows that, when ATP is added to the cell suspensions at a concentration of 5.75 nmoles/5×10^7 cells, the wild type control cells display the same pattern of extracellular ATP stability as has been reported (1,2). Specifically, ATP is stable at alkaline pH and hydrolyzed at acid pH, most likely to adenosine, explaining the lack of accumulation of ADP. The *sac1*Δ cells, however, are completely different. At alkaline pH, ATP is very rapidly hydrolyzed to ADP, whereas at acid pH, ATP is nearly as stable as for the wild type cells at alkaline pH. Table 2 reveals two differences between sac1Δ and wild type cells: first, extracellular ATP is more stable at acid pH for the mutant cells; second, incubation in alkaline buffer causes the mutant cell suspension to exhibit a pronounced extracellular ATPase activity that results in the accumulation of ADP.

The greater stability of extracellular ATP for the mutant cells at acid pH is consistent with the hypothesis that *sac1*Δ cells express less acid phosphatase in the periplasm than do the wild type cells. This idea was tested by measuring acid phosphatase activity directly with p-nitrophenyl phosphate as substrate at pH 4.0 (10). The rate of p-nitrophenyl phosphate hydrolysis for the *sac1*Δ cells is 9.0 A_{405} units/min/8×10^6 cells, while that for the wild type cells is 15.5 A_{405} units/min/8×10^6 cells, or 72% higher than that for the *sac1*Δ cells.

The appearance of an ATPase activity, leading to the accumulation of ADP, when *sac1*Δ cells are subjected to alkaline conditions is consistent with the idea that some of the cells are lysing under these conditions, thus releasing cytoplasmic ATPases to the extracellular fluid. This is a reasonable speculation due to the fact that acid pH is a more physiological medium for yeast, and in light of the fact that the *sac1*Δ mutation is known to exhibit a complex phenotype, and therefore may exhibit an unforeseen tendency to lyse under alka-

TABLE 2

Extracellular ATPase Activity of Wild Type and *sacI*Δ Cells[a]

	Wild type		*sacl*Δ	
	ATP (nmoles/5 × 10^7 cells)	ADP (nmoles/5 × 10^7 cells)	ATP (nmoles/5 × 10^7 cells)	ADP (nmoles/5 × 10^7 cells)
pH 8.8[b]	5.50 +/− 0.01	0.63 +/− 0.50	0.94 +/− 0.35	4.50 +/− 0.09
pH 5.5[c]	3.93 +/− 0.00	0.35 +/− 0.03	5.10 +/− 0.01	0.14 +/− 0.04

[a] Cells were prepared and incubated as described in Materials and Methods. All incubations initially contained exogenous ATP (5.75 nmoles/5 × 10^7 cells). All incubations were 30 minutes in duration. Data represent means +/− variance, n=2.
[b] The incubation buffer is 20 mM Tris, pH 8.8.
[c] The incubation buffer is 20 mM Tris-citrate, pH 5.5.

line conditions. If the *sac1*Δ cells are lysing under alkaline conditions, it should be possible to detect other cytoplasmic enzymes, along with the unidentified ATPase, in the cell free filtrate. Lactate dehydrogenase activity of the filtrates of cells incubated under both acid and alkaline conditions was measured in order to test this hypothesis. Both wild type and mutant cells incubated at acid pH showed lactate dehydrogenase activities 50 % above background, and wild type cells at alkaline pH gave a value of 100 % above background. Mutant cells incubated at alkaline pH gave a value of 1000 % above background. This result confirms that the reason for the appearance of a novel extracellular ATPase when *sac1*Δ cells are incubated at alkaline pH is that at least some of the cells are lysing, thus releasing intracellular contents, including cytoplasmic ATPases.

DISCUSSION

The results presented here indicate that Sac1p is not involved in ATP efflux from yeast. At alkaline pH, conditions required to measure extracellular ATP for wild type cells, it appeared as though *sac1*Δ cells did not exhibit nigericin-stimulated ATP efflux; however, further analysis showed that glucose-dependent, nigericin-stimulated ATP efflux occurs for the mutant cells at acid pH (Table 1). Extracellular ATP is detectable at acid pH for the mutant cells because these cells exhibit less extracellular ATPase activity (Table 2) due to the expression of less acid phosphatase activity in the periplasm. There is no apparent nigericin-stimulation of ATP efflux from the mutant cells at alkaline pH (Table 1) because under these conditions, the extracellular ATPase activity of the mutant cells is very high (Table 2), suggesting that cell lysis, and a consequent release of cytoplasmic ATPases, is responsible for the appearance of the extracellular ATPase activity at alkaline pH. This idea is confirmed by the detection of lactate dehydrogenase, a cytoplasmic enzyme, in the filtrate of *sac1*Δ cells incubated at alkaline pH. Sac1p clearly is not responsible for nigericin-stimulated ATP efflux from yeast, but the deletion of *SAC1* does result in a phenotype that has interesting consequences regarding extracellular ATP.

It is clear from the data in Table 2 and the results of hydrolysis of p-nitrophenyl phosphate described in the results that the sac1Δ cells express less periplasmic acid phosphatase. This is an unexpected, but not particularly surprising result. Mutations in *SAC1* are known to cause pleiotropic effects including secretory effects (8,9). It has been shown that the rate of intracellular transport of proteins through the secretory pathway is slowed in *sac1*Δ cells (7). Therefore, it is not surprising that the *SAC1* deletion affects acid phosphatase secretion.

It is clear from the detection of lactate dehydrogenase activity that some of the mutant cells in the alkaline incubation suspension lyse, releasing cytoplasmic components including the unidentified ATPase and lactate dehydrogenase. This result is also unexpected but not particularly surprising. Because the deletion of *SAC1* causes such a broad range of effects including secretory effects, suppression of actin mutations, and suppression of mutations in a phospholipid exchanger (8,9), it is not unreasonable to expect the cells to be compromised in structural integrity. This may be caused by the secretory defects, which may impair secretion of cell wall components, leading to a cell wall that is breached at alkaline pH. Alternatively, the structure of the plasma membrane may be weakened and susceptible to alkaline pH due to the loss of Sac1p-mediated phospholipid exchange. The loss of structural integrity could also be caused by the missing interaction between Sac1p and actin. This may result in a weakened structural network in the inner surface of the plasma membrane. A similar phenomenon has been observed with the expression in yeast of the virion-associated protein of human immunodeficiency virus type 1, Vpr (11). Expression of a fragment of Vpr resulted in osmotic sensitivity and a tendency of the cells to be very large. The expressed region of Vpr has homology to Sac1p, and it was suggested that Vpr interfered with the normal function of Sac1p, leading to the observed phenotype.

The deletion of *SAC1* has many effects on *S. cerevisiae,* including secretory defects leading to less periplasmic acid phosphatase, and structural defects that lead to a loss of structural integrity at alkaline pH. However, it is apparent that the deletion of *SAC1* does not eliminate ATP efflux activity. Moreover, the results presented here demonstrate that great care should be taken in interpreting results involving transmembrane transport in the *sac1*Δ strain. One must be certain that the membrane in question is fully intact before assigning to Sac1p a transmembrane transport activity.

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REFERENCES

1. Boyum, R., and Guidotti, G. (1997a) *Biochem. Biophys. Res. Comm.* **230,** 22–26.
2. Boyum, R., and Guidotti, G. (1997b) *Microbiology,* in press.
3. Dubyak, G. R., and El-Moatassim, C. (1993) *Amer. J. Physiol.* **265,** C577–C606.
4. Abraham, E. H., Prat, A. G., Gerweck, L., Seneveratne, T., Arceci, R. J., Kramer, R., Guidotti, G., and Cantiello, H. F. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 312–316.
5. Schwiebert, E. M., Egan, M. E., Hwang, T. H., Fulmer, S. B., Allen, S. S., Cutting, G. R., and Guggino, W. B. (1995) *Cell* **81,** 1063–1073.
6. Guidotti, G. (1996) *Chem. Biol.* **3,** 703–706.
7. Mayinger, P., Bankaitis, V. A., and Meyer, D. I. (1995) *J. Cell Biol.* **131,** 1377–1386.
8. Cleves, A. E., Novick, P. J., and Bankaitis, V. A. (1989) *J. Cell Biol.* **109,** 2939–2950.
9. Whitters, E. A., Cleves, A. E., McGee, T. P., Skinner, H. B., and Bankaitis, V. A. (1993) *J. Cell Biol.* **122,** 79–94.
10. Schweingruber, M. E., Fluri, R., Maundrell, K., Schweingruber, A., and Dumermuth, E. (1986) *J. Biol. Chem.* **261,** 15877–15882.
11. Macreadie, I. G., Castelli, L. A., Hewish, D. R., Kirkpatrick, A., Ward, A. C., and Azad, A. A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 2770–2774.