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Effect of ATP Binding Cassette/Multidrug Resistance Proteins on ATP Efflux of *Saccharomyces cerevisiae*

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Multidrug resistance (MDR) in mammalian tumors or tissues is often associated with the overexpression of the putative drug efflux pump P-glycoprotein (Pgp). One theory concerning the mechanism of Pgp activity is that efflux of ATP is coupled to drug efflux. Evidence in support of this theory has been observed in mammalian cells. Recently, the *STS1* gene, which is a multidrug resistance gene related to the mammalian Pgp's, has been characterized in *S. cerevisiae*. Also, the mouse mdr3 Pgp has been functionally expressed in yeast cells. Therefore, it was of interest to determine whether the expression of these proteins affected ATP efflux from yeast. Although both genes were shown to confer MDR, thus confirming functional expression, the endogenous glucose-dependent, drug-stimulated ATP efflux activity of yeast was not affected by expression of *STS1*, and was decreased by the expression of mouse mdr3.

One common form of multidrug resistance (MDR), the ability of cells to survive exposure to many structurally diverse chemotherapeutic drugs, is associated with the overexpression of the putative drug efflux pump P-glycoprotein (Pgp), a member of a group of proteins called the ATP Binding Cassette (ABC) superfamily that includes the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) (1,2). Abraham *et al.* (3), demonstrated both a correlation between ATP efflux and Pgp expression in Chinese hamster ovary cells and human lung tumor cells, and an ATP-specific channel associated with Pgp. In addition, the CFTR has recently been shown to facilitate the extrusion of ATP from airway epithelial cells (4). While these results provide support for the theory that ABC proteins mediate ATP efflux, there is evidence against this view (5,6,7, reviewed in 8). Because of the possibility that an activity attributed to Pgp is due to another protein present in mammalian cells that may have a regulatory relationship with Pgp, it is of interest to study mammalian Pgp activity in the heterologous system of the yeast cell. Also, it is unknown whether the expression of yeast Pgp-like genes (9-11) is correlated with ATP extrusion.

Described here are experiments using a strain of *S. cerevisiae* expressing the mouse mdr3 Pgp, described in Raymond *et al.* (12), and a strain overexpressing the yeast Pgp-like gene *STS1*, described in Bissinger and Kuchler (9), to test the hypothesis that Pgps are capable of mediating ATP efflux. Both mdr3 and *STS1* confer an MDR phenotype on the yeast cells. A drug-stimulated, glucose-dependent ATP efflux activity is present in the yeast cells: it is decreased by mouse mdr3 expression and is unaffected by *STS1* expression.

MATERIALS AND METHODS

Materials. Nigericin, valinomycin, and cycloheximide were purchased from Sigma. The anti-Pgp monoclonal antibody C219 was purchased from Signet. The 2° antibody (goat anti-mouse IGG coupled to horseradish peroxidase) was purchased from Bio-Rad. The chemiluminescent reagents for the western blots were purchased from DuPont-NEN. The two HPLC columns used were: Sax Ultrasil, purchased from Beckman; and Vydac nucleotide analysis column, purchased from Rainin.

Western blots. For figure 2, cells were first grown to post-diauxic shift in 10mL of Ura D.O. media containing either 0.1mL ethanol (-nigericin culture), or 0.1mL 10 mg/mL nigericin (+nigericin culture). 4.5mL of these cultures was then diluted into 45 mL of the same media. The 50mL cultures were then grown to exponential phase. The cultures were collected by centrifugation, and lysed with glass beads by vortexing. The suspension was spun at 700×g at 4°C to pellet unlysed cells, nuclei, and mitochodria. The supernatant was then spun at 14500×g for 30 minutes at 4°C to pellet membranes. The supernatant (cytosol fraction) was removed and the pellet (membrane fraction) was resuspended in 0.1mL resuspension buffer. Equal amounts of protein (13) of each fraction were separated by SDS-PAGE on a 7.5% gel and a Western blot (14) was performed

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Abbreviations: ABC, ATP Binding Cassette; HPLC, High Performance Liquid Chromatography; MDR, Multidrug Resistance; Pgp, P-Glycoprotein; SC, Synthetic Complete; Ura D.O., Uracil Drop Out; Leu D.O., Leucine Drop Out.

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using C219 as 1° Ab and goat anti-mouse as 2° Ab. Immune complexes were visualized using the chemiluminescent reagents for horseradish peroxidase activity.

Extracellular nucleotide sample preparation. Cells were grown to log phase, washed with 20 mM Tris-Citrate pH 5.5, resuspended in the appropriate incubation buffer at 0°C and at a cell density of 5.2 $\times 10^8$ cells/ml (A_{650}=1 represented 1.6 $\times 10^7$ cells/ml), and moved to room temperature (23-28°C) at time T=0. The samples were incubated at room temperature for 30 minutes, after which each sample was filtered through a 0.2 μm syringe-tip filter. The filtrate was then stored at −70°C until HPLC analysis was performed as described in Pogolotti & Santi, 1982 (15). 0.1 mL of each sample was subjected to HPLC analysis and the data in Table 2 represents the amount of ATP associated with 5.2 $\times 10^7$ cells.

Intracellular nucleotide sample preparation. Cell suspensions were prepared and incubated as described for the extracellular ATP samples. Intracellular nucleotides were then isolated as described (15). Briefly, at the end of each incubation, the cells were pelleted, resuspended in 0.5 ml 0.6 M trichloroacetic acid, and incubated at 0°C for at least 10 min. The precipitate was removed by centrifugation, and the supernatant was extracted with freon/TOA. The samples were stored at −70°C until HPLC analysis was performed.

RESULTS

Multidrug Resistance

The results presented below suggest that the mdr3 gene product, when expressed in yeast, interacts with valinomycin and nigericin. Valinomycin and nigericin have been shown to affect the growth of yeast by interfering with mitochondrial function (16). Valinomycin has been shown to moderate the MDR phenotype of animal cells (18), and Kuchler and Thorner (18) have shown that human MDR1 confers valinomycin resistance to yeast cells. It can be seen from Figure 1A that mdr3 expression results in valinomycin and nigericin resistance as assayed on solid media (see Table 1 for a description of strains). In liquid culture, both cell types are able to grow if the culture is seeded at a sufficient density (data not shown). When mdr3 control and mdr3 cells are grown in liquid culture in the presence of nigericin, mdr3 cells express an elevated level of Pgp (Fig.

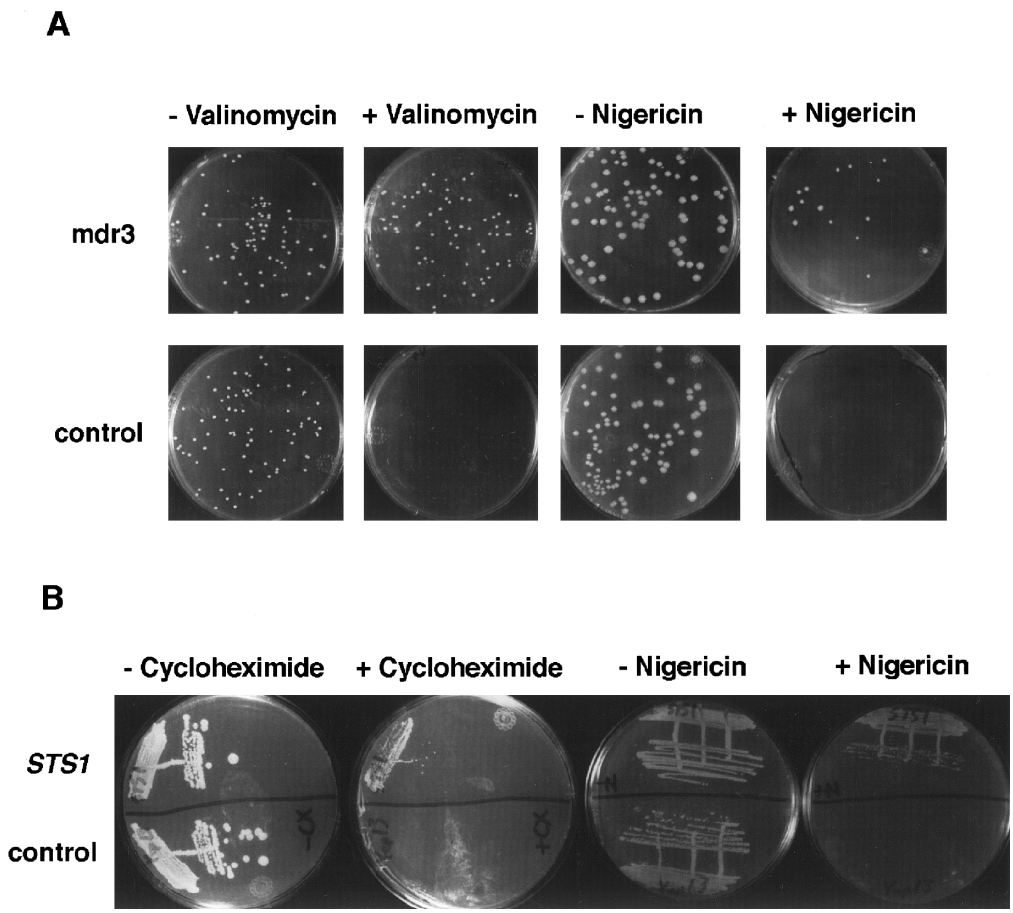

FIG. 1. Multidrug resistance due to mdr3 and *STS1* expression. All cells are grown and plated on media indicated in Table 1. A.) An equal number of cells was spread onto each plate. +valinomycin plates contain 63.4μg/mL valinomycin. +nigericin plates contain 49.7μg/mL nigericin. B.) A loopful of liquid culture was streaked onto the plates. +cycloheximide plates contain 1 μg/mL cycloheximide. +nigericin plates contain 25 μg/mL nigericin.

TABLE 1

Saccharomyces cerevisiae Strains[a]

Name in text	Description	Growth media[b]
mdr3 control cells	JPY201 cells with pVT plasmid	Ura D.O.
mdr3 cells	JPY201 cells with pVT-mdr3 plasmid	Ura D.O.
STS1 control cells	YKKA-7 cells with Yep13 plasmid	Leu D.O.
STS1 cells	YKKA-7 cells with Yep13-STS1 plasmid	Leu D.O.

[a] mdr3 control and mdr3 cells were obtained from Martine Raymond and Phillipe Gros (11). YKKA-7 cells and the Yep13-STS1 plasmid were obtained from Karl Kuchler (8). The Yep13 plasmid was obtained from Nancy Kleckner.

[b] Media is synthetic drop out as described in Sherman *et al.* (18).

2). This is additional evidence of an interaction between nigericin and the mdr3 gene product expressed in yeast. These data extend the results of Raymond *et al.* (12, 20), concerning the functional expression of mdr3 in yeast and suggest that valinomycin and nigericin are substrates for the mdr3 Pgp in yeast *in vivo.*

Figure 1B shows that *STS1* expression results in cycloheximide resistance, in support of previous results (9). Also shown in Figure 1B is that *STS1* expression results in resistance to nigericin. These results confirm the functional overexpression of both mdr3 and *STS1* in yeast; furthermore, the results demonstrate that both the mdr3 and *STS1* gene products interact with nigericin.

ATP Efflux

Having established the functional expression of mdr3 and *STS1* in yeast and demonstrated the interaction of toxins with the mdr3 and *STS1* gene products, we tested the hypothesis that drug transport via Pgps is associated with ATP efflux by measuring the extracellular ATP levels in the presence and absence of valinomycin, cycloheximide, and nigericin. This was done at pH 8 to prevent ATP hydrolysis by acid phosphatase in the extracellular compartment (21).

Table 2 shows that ATP is present in the extracellular fluid of all cells and that the amount of extracellular ATP is increased greatly by the presence of glucose. With the control cells, nigericin causes a 3-fold increase in ATP release whether it is present only for the duration of the experiment (lines 1 and 2; lines 11 and 12) or used in the growth medium to select nigericin resistant cells (lines 3 and 4). In the latter case, additional nigericin does not have to be added during the efflux measuement, probably because it remains associated with the cells. With the cells expressing mdr3 (lines 6, 7, 8, and 9) and *STS1* (lines 14 and 15), nigericin also causes a 2-fold increase in ATP release; however, the amount of extracellular ATP with the mdr3 cells is approximately half of that with the control cells. Importantly, addition of valinomycin has no effect on ATP release from the mdr3 cells (lines 5 and 10) nor does the addition of cycloheximide affect ATP release from the STS1 cells (lines 13 and 16) in spite of the evidence that these compounds interact with the ABC proteins.

Intracellular nucleotide levels do not vary considerably between either mdr3 control and mdr3 cells, or between *STS1* control and *STS1* cells, nor are they affected by exposure to nigericin (Table 1, Intracellular ATP columns). The only real variation in intracellular ATP levels is observed in cells incubated in the absence of glucose, in which case, the intracellular ADP concentration rises (data not shown). This rise is not reflected in the extracellular medium when nigericin is included in the incubation (data not shown). Therefore, the effect of nigericin is not to lyse or permeabilize the cells in a general way.

These results show that expression of *STS1*p and mdr3 Pgp in yeast cells does not cause an increase in ATP efflux; in the cells expressing mdr3 Pgp, in fact, there is a decrease in ATP release compared to the control cells.

DISCUSSION

The results presented here, that the mouse mdr3 (Fig. 1A and 2) and the yeast *STS1* (Fig. 1B) genes can confer drug resistance to yeast cells, support evidence in the literature on this point. Raymond and associates have shown that expression of mdr3 Pgp in yeast can complement a null ste6 mutation (12) and confer resistance to the antifungal agent FK520 (20), and Kuchler and Thorner (18) reported resistance to valinomycin of

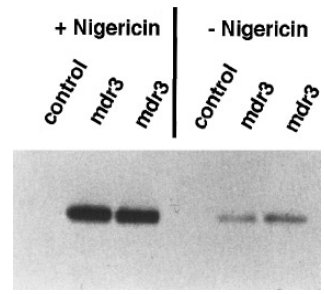

FIG. 2. Growth in nigericin results in the expression of an elevated level of mdr3 gene product. Experiments were performed as described in Materials and Methods. All lanes represent 10μg of membrane fraction protein. Lane 1 contains protein from mdr3 control cells grown in the presence of nigericin. Lanes 2 and 3 contain protein from duplicate cultures of mdr3 cells grown in the presence of nigericin. Lane 4 contains protein from mdr3 control cells grown in the absence of nigericin. Lanes 5 and 6 contain protein from duplicate cultures of mdr3 cells grown in the absence of nigericin.

TABLE 2

ATP Efflux[a]

		Extracellular ATP (pmoles/30 min./5 × 10^7 cells)		Intracellular ATP (nmoles/5 × 10^7 cells)	
		+Glucose[b]	−Glucose	+Glucose	−Glucose
1	mdr3 control cells	179.3 ± 18.1	22.0 ± 3.7	10.3 ± 1.3	5.8 ± 0.6
2	mdr3 control cells + nigericin[c]	532.3 ± 5.6	35.0 ± 2.9	9.9 ± 0.3	6.5 ± 0.1
3	nig. grown[e] mdr3 control cells	581.1 ± 21.1	31.4 ± 0.9	10.1 ± 0.3	6.9 ± 0.1
4	nig. grown mdr3 control cells + nigericin	603.0 ± 17.9	33.5 ± 1.6	10.4 ± 0.4	7.1 ± 0.1
5	mdr3 control cells + valinomycin[d]	191.9 ± 7.3	12.5 ± 3.3	8.6 ± 1.4	5.7 ± 1.1
6	mdr3 cells	112.5 ± 7.0	27.98 ± 5.2	11.4 ± 1.2	5.1 ± 0.2
7	mdr3 cells + nigericin	252.8 ± 13.9	30.4 ± 3.1	9.6 ± 0.2	6.1 ± 0.2
8	nig. grown mdr3 cells	249.4 ± 3.0	32.8 ± 2.2	9.7 ± 0.0	4.4 ± 0.1
9	nig. grown mdr3 cells + nigericin	268.8 ± 6.9	30.5 ± 1.6	9.4 ± 0.1	4.5 ± 0.0
10	mdr3 cells + valinomycin	111.5 ± 4.8	25.0 ± 12.0	11.6 ± 2.1	4.3 n = 1
11	STS1 control cells	55.5 ± 26.2	2.4 ± 3.4	8.9 ± 0.9	1.5 ± 1.0
12	STS1 control cells + nigericin	160.8 ± 6.4	N.D.	8.2 ± 0.7	3.2 ± 1.7
13	STS1 control cells + cycloheximide[f]	34.2 ± 0.1	N.D.	6.5 ± 0.1	2.7 ± 1.0
14	STS1 cells	92.9 ± 25.5	7.5 ± 5.1	6.8 ± 1.1	2.9 ± 2.4
15	STS1 cells + nigericin	182.1 ± 3.5	6.3 ± 6.3	9.0 ± 1.8	5.9 ± 1.4
16	STS1 cells + cycloheximide	79.8 ± 2.2	5.6 ± 5.6	6.4 n = 1	5.6 ± 1.3

[a] Data were collected as described in Materials and Methods and represent means ± standard deviations, n = 2–5 except where indicated.
[b] +Glucose indicates the presence of 2% (w/v) glucose.
[c] +nigericin indicates the presence of 25 μg/mL nigericin.
[d] +valinomycin indicates the presence of 25 μg/mL valinomycin.
[e] nig. grown refers to the use of cells grown in 50 μg/mL nigericin.
[f] +cycloheximide indicates the presence of 500 μg/mL cycloheximide.
N.D. = None Detected.

yeast expressing the human MDR1 Pgp. Bissinger and Kuchler (9) showed that expression of *STS1*p made yeast cells resistant to sporidesmin and cycloheximide. This report, however, is the first demonstration of the interaction of P-glycoproteins with nigericin. The conclusion is that *STS1*p and mdr3 Pgp are expressed and function to confer resistance to structurally unrelated compounds.

The results of experiments on the relationship between the presence of ABC proteins and ATP efflux are surprising (Table 2). In the first place, ATP is released from all yeast cells at a steady rate when glucose is present in the medium; the rate is equivalent to 0.1% of the cellular ATP per min. The second surprise is that nigericin causes a 2- and 3-fold increase in efflux in both control cells and those expressing the ABC proteins; whereas, neither valinomycin nor cycloheximide stimulates ATP efflux in either control cells or those expressing the ABC proteins even though these substances interact with the proteins. These results suggest that ATP efflux and drug-ABC protein interactions are not coupled. Thirdly, the presence of *STS1*p and mdr3 Pgp in the cells either has no effect or actually decreases, respectively, the amount of ATP efflux from the cells. We conclude that the presence of functional ABC proteins is not sufficient to stimulate ATP release from yeast cells, in contrast to the situation in mammalian cells where a direct relationship exists between the rate of ATP efflux and the amount of Pgp (3).

One explanation for this discrepancy is that ABC proteins are not directly involved in ATP movement, but rather interact or couple with an ATP transporter that is present in mammalian cells but not in yeast. Another possibility is that the ABC proteins can promote ATP movement directly, but this activity in inhibited by a factor in yeast cells.

The mechanism of ATP release from yeast cells and its stimulation by nigericin remain interesting questions.

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REFERENCES

1. Endicott, J. A., and Ling, V. (1989) *Annu. Rev. Biochem.* **58,** 137–171.

2. Gottesman, M. M., and Pastan, I. (1993) *Annu. Rev. Biochem.* **62,** 385–427.
3. 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.
4. 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.
5. Reddy, M. M., Quinton, P. M., Haws, C., Wine, J. J., Grygorczyk, R., Tabcharani, J. A., Hanrahan, J. W., Gunderson, K. L., and Kopito, R. R. (1996) *Science* **271,** 1876–1879.
6. Grygorczyk, R., Tabcharani, J. A., and Hanrahan, J. W. (1996) *J. of Membrane Biol.* **151,** 139–148.
7. Li, C., Ramjeesingh, M., and Bear, C. E. (1996) *J. of Biol. Chem.* **271,** 11623–11626.
8. Guidotti, G. (1996) *Cem. & Biol.* **3,** 703–706.
9. Bissinger, P. H., and Kuchler, K. (1994) *J. Biol. Chem.* **269,** 4180–4186.
10. Balzi, E., Wang, M., Leterme, S., Dyck, L. V., and Goffeau, A. (1994) *J. Biol. Chem.* **269,** 2206–2214.
11. Servos, J., Hasse, E., and Brendel, M. (1993) *Mol. Gen. Genetics.* **236,** 214–218.
12. Raymond, M., Gros, P., Whiteway, M., and Thomas, D. Y. (1992) *Science* **256,** 232–234.
13. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
14. Towbin, H. T., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76,** 4350–4354.
15. Pogolotti, A. L., and Santi, D. V. (1982) *Anal. Biochem.* **126,** 335–345.
16. Kovac, L., Bohmerova, E., and Butko, P. (1982) *Biochim. Biophys. Acta* **721,** 341–348.
17. Daoud, S. S., and Juliano, R. L. (1989) *Cancer Res.* **49,** 2661–2667.
18. Kuchler, K., and Thorner, J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 2302–2306.
19. Sherman, F., Fink, G. R., and Hicks, J. A. (1986) Laboratory Manual for Yeast Genetics (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
20. Raymond, M., Ruetz, S., Thomas, D. Y., and Gros, P. (1994) *Mol. and Cell. Biol.* **14,** 277–286.
21. Nosaka, K. (1990) *Biochim. Biophys. Acta.* **1037,** 147–154.