ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 2000, p. 1743–1744 Vol. 44, No. 6
0066-4804/00/$04.00+0

Cyclic AMP and Fluconazole Resistance in *Saccharomyces cerevisiae*

D. P. KONTOYIANNIS[1]* AND STEFFEN RUPP[1,2]

Department of Internal Medicine Specialties, Section of Infectious Diseases, The University of Texas M. D. Anderson Cancer Center, Houston, Texas,[1] and Fraunhofer IGB, Stuttgart, Germany[2]

Received 30 November 1999/Returned for modification 2 February 2000/Accepted 11 March 2000

Cyclic AMP (cAMP) is involved in the stress response in *Saccharomyces cerevisiae*. We show that cAMP is required for resistance to fluconazole in *S. cerevisiae*. In addition, activation of *Ras2*, a regulator of cAMP generation, results in some protection from fluconazole toxicity in a fashion independent of the efflux transporter Pdr5p.

Saccharomyces cerevisiae, a genetically tractable fungus related to *Candida albicans*, is an attractive organism for the study of fluconazole resistance (4). Despite advances in understanding the mechanisms of fluconazole resistance in *S. cerevisiae*, however, the components of the response pathways are not fully known.

In *S. cerevisiae*, the *RAS1* and *RAS2* genes are activators of adenylate cyclase, which catalyzes the formation of cyclic AMP (cAMP), a central signaling molecule (10). Ras1p and Ras2p are highly homologous to the mammalian *ras* proteins (10). Activation of mammalian *ras* has been shown to affect the sensitivity of tumors to various antineoplastic agents via activation of the *MDR-1* (multidrug resistance) gene, which is the mammalian homologue of *PDR5* (1, 3). Since an important mechanism of fluconazole resistance in *S. cerevisiae* is mediated by *PDR5* (pleiotropic drug resistance 5), an efflux transporter (2, 4), we examined whether cAMP and activation of the Ras pathway in *S. cerevisiae* similarly affect the resistance to fluconazole.

cAMP is required for resistance to fluconazole. Standard procedures to prepare the media and to manipulate *S. cerevisiae* were used (5). To test whether cAMP has a function in resistance to fluconazole we used a strain in which internal cAMP levels can be changed by the addition of cAMP to the medium (strain SR 959 [*MAT***a**/α *ras1*/*ras1 ras2*/*ras2 pde2*/*pde2*] [7]). Thus, we can mimic functions of Ras connected to changing cAMP levels in the cell by changing external cAMP concentrations (7). Despite its very low intracellular cAMP level, the SR959 mutant does not require external cAMP for growth on synthetic complete (SC) medium (7). We compared the sensitivity of strain SR959 to fluconazole with that of the isogenic wild type (SR607 [7]) in the presence or absence of cAMP. As shown in Table 1, SR959 was very sensitive to fluconazole (100 μg in a paper disk by applying 20 μl of a 5-mg/ml solution in sterile water) in the absence of cAMP on SC medium (zone of inhibition [ZI], 51 ± 1 mm). Addition of 0.1 mM cAMP to the medium restored resistance to fluconazole to wild-type levels (ZI, 38 ± 1 mm [Table 1]). Higher concentrations of cAMP (1 mM) did not increase the resistance to fluconazole appreciably (ZI, 36 ± 1 mm). Similarly, the sensitivity of SR607 (wild type) to fluconazole was not affected significantly by the addition of 1 mM cAMP (ZI, 36 ± 1 and 34 ± 1 mm respectively [Table 1]). The plates were grown at 30°C for 24 h. Experiments were done in triplicate at different time points. These results could be interpreted as evidence that cAMP is required for activation of response mechanisms to protect the cell from fluconazole. The lack of a significantly increased resistance in the wild type by increased amounts of exogenous cAMP can be explained by the known resistance of wild-type strains to external cAMP and by the high internal levels of cAMP in wild-type cells (11).

Expression of *RAS2*^{Val19} results in resistance to fluconazole in a *PDR5*-independent fashion. *RAS2*^{Val19} is a well-characterized dominant active allele of *RAS2* (10). We transformed the *Saccharomyces* strain 10560-14C (*MAT***a** *ura3-52 leu2*::*hisG his3*::*hisG* [supplied by the Fink laboratory]) and DK 13-5D (*MAT***a** *pdr5*::Tn*3*::*LEU2*::*lacZ ura3-52*::*leu2*::*hisG* [6]) with either a single-copy plasmid containing *URA3* as a selection marker and the allele *RAS2*^{Val19} (S. Rupp, unpublished data) or with a *URA3* plasmid containing no insert (pRS316 [8]). We selected the corresponding Ura$^+$ transformants (10560-14C/*URA3*, *pdr5*/*URA3*, 10560-14C/*URA3-RAS2*^{Val19}, and *pdr5*/*URA3-RAS2*^{Val19}) in SC-uracil plates. To compare the fluconazole sensitivities of 10560-14C and DK13-5D with those of the respective isogenic strains expressing *RAS2*^{Val19}, the resulting strains were inoculated in 5 ml of liquid SC-uracil medium. Cultures were grown to saturation at 30°C, diluted (1:1,000) in 10 ml of SC-uracil medium, and grown at 30°C into late log phase. Yeast growth was examined by spreading approximately 10^5 cells of each culture on SC-uracil plates containing 100 μg of fluconazole in a paper disk. Growth on plates at 30°C was evaluated for 24 h. Experiments were performed in triplicate at different time points. As shown in Table 1, the expression of *RAS2*^{Val19} resulted in a small increase in fluconazole resistance in both the 10560-14C/*RAS2*^{Val19} (ZI, 38 ± 1 mm in 10560-14C; ZI, 33 ± 2 mm in 10560-14C/*RAS2*^{Val19}) and in DK13-5D/*Ras2*^{Val19} strains respectively (ZI, 52 ± 1 mm in DK13-5D; ZI, 44 ± 2 mm in DK13-5D/*Ras2*^{Val19}). The observed small protective effect in strains expressing *Ras2*^{Val19} was not specific to fluconazole, because protection from cycloheximide (5 μg in a paper disk) was also seen in these strains on SC-uracil plates. On the other hand, toxicity from 5-fluorocytosine (50 μg in a paper disk) was no different in the strains expressing *Ras2*^{Val19} (data not shown). Cycloheximide but not 5-fluorouracil is a substrate of Pdr5p (2).

In conclusion, our data suggest that cAMP is required for resistance to fluconazole. This is in agreement with our finding that the activation of the *Ras* pathway exerts some protective effect against fluconazole toxicity in *Saccharomyces*. The exact mechanism of protection is not known. However, cAMP and

* Corresponding author. Mailing address: Department of Internal Medicine Specialties, Section of Infectious Diseases, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Box 47, Houston, TX 77030. Phone: (713) 792-6237. Fax: (713) 794-4351. E-mail: dkontoyi@notes.mdacc.tmc.edu.

TABLE 1. Effects of cAMP and activated *Ras* on fluconazole resistance in *S. cerevisiae*[a]

Strain	Mean ZI ± SD (mm) with cAMP at the following concn:		
	0 mM	0.1 mM	1 mM
SR959[b]	51 ± 1	38 ± 1	36 ± 1
SR607[b]	36 ± 1	36 ± 1	34 ± 1
10560-14C/*URA3*[c]	38 ± 1		
10560-14C/*URA3 Ras2*^{Val19}[c]	33 ± 2		
DK13-5D/*URA3*[c]	52 ± 1		
DK13-5D/*URA3 Ras2*^{Val19}[c]	44 ± 2		

[a] Resistance tested using 100 μg of fluconazole by applying 20 μl of a 5-mg/ml fluconazole solution in sterile water to a paper disk. All experiments were performed in triplicate.

[b] Approximately 10^5 cells were spread on SC plates, and growth was examined after 24 h at 30°C.

[c] Approximately 10^5 cells were spread on SC-uracil plates, and growth was examined after 24 h at 30°C.

the *Ras* pathway may regulate either directly or indirectly the major facilitator superfamily network of transporters and thus the efficiency of drug efflux in *Saccharomyces*. This hypothesis is supported by the finding that *CDC25*, an upstream regulator of *RAS2*, affects the activity of the major facilitator superfamily glucose transporters (9). Elucidation of the role of cAMP in azole resistance could shed light on the mechanisms of resistance of the inherently resistant pathogenic fungi.

Part of this work was performed at the Whitehead Institute for Biomedical Research (Fink laboratory) where D.P.K. was a fellow in the Clinical Investigator Training Program (supported by Pfizer, Inc.) at the Harvard-Massachusetts Institute of Technology Division of Health Sciences and Technology, Cambridge, Mass., and a fellow in Infectious Diseases at Massachusetts General Hospital, Harvard Medical School, Boston, Mass. This work was also supported by the Cancer Center (Core) Grant (CA16672) from The University of Texas M. D. Anderson Cancer Center.

We thank members of the Fink laboratory for helpful advice.

REFERENCES

1. **Chin, K. V., K. Ueda, I. Pastan, and M. M. Gottesman.** 1992. Modulation of activity of the promoter of the human *MDR1* gene by ras and p53. Science **255:**459–462.
2. **Decottignies, A., and A. Goffeau.** 1997. Complete inventory of the yeast ABC proteins. Nat. Genet. **15:**137–145.
3. **Grunicke, H., J. Hofmann, I. Utz, and F. Überall.** 1994. Role of protein kinases in antitumor drug resistance. Ann. Hematol. **69:**S1–S6.
4. **Joseph-Horne, T., and D. W. Hollomon.** 1997. Molecular mechanisms of azole resistance in fungi. FEMS Microbiol. Lett. **149:**141–149.
5. **Kaiser, C., S. Michaelis, and A. Mitchell.** 1994. Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Plainview, N.Y.
6. **Kontoyiannis, D. P.** 1999. Genetic analysis of azole resistance by transposon mutagenesis in *Saccharomyces cerevisiae*. Antimicrob. Agents Chemother. **43:**2731–2735.
7. **Rupp, S., E. Summers, H.-J. Lo, et al.** 1999. MAP kinase and cAMP filamentation signaling pathways converge on the usually large promoter of the yeast *FLO11* gene. EMBO J. **18:**1257–1269.
8. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:**19–27.
9. **Sillje, H. H., E. G. ter Schure, A. J. Verkleij, et al.** 1996. The Cdc25 protein of *Saccharomyces cerevisiae* is required for normal glucose transport. Microbiol. **142:**1765–1773.
10. **Toda, T., I. Uno, T. Ishikawa, et al.** 1985. In yeast, *Ras* proteins are controlling elements of adenylate cyclase. Cell **40:**27–36.
11. **Wilson, R. B., G. Renault, M. Jacquet, et al.** 1993. The *pde2* gene of *Saccharomyces cerevisiae* is allelic to *rca1* and encodes a phosphodiesterase which protects the cell from extracellular cAMP. FEBS Lett. **325:**191–195.