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Cycloheximide Efflux in Antibiotic-Adapted Cells of the Fungus *Mucor racemosus*†

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***Mucor racemosus* cells adapted to either cycloheximide or trichodermin were approximately 40-fold more resistant to cycloheximide than nonadapted cells. Ribosomes isolated from adapted and nonadapted cells were equally sensitive to cycloheximide in an in vitro poly(U) translation assay. There was no detectable modification of cycloheximide by adapted cells. Uptake of drug by nonadapted and adapted cells was characterized by a rapid initial accumulation during the first 2 min of incubation with [^{3}H]cycloheximide, followed by a steady-state intracellular drug concentration well below that of the medium. The steady-state drug concentration was approximately 10-fold lower in adapted cells than in nonadapted cells. Treatment of cells with sodium azide or dinitrophenol abolished the difference between uptake of drug by nonadapted and adapted cells and resulted in intracellular drug levels equal to that of the medium. Direct efflux measurements showed that adapted cells loaded with cycloheximide were able to excrete the drug far more rapidly than nonadapted cells. These results suggest that both nonadapted and adapted cells possess an energy-dependent efflux mechanism for transporting cycloheximide and that resistance in adapted cells is due to increased efficiency of transport.**

Previous work in our laboratory has shown that the fungus *Mucor racemosus* will adapt phenotypically to the translation inhibitors cycloheximide and trichodermin and to the membrane-binding drug amphotericin B (12). Cells exposed to 100 μg of cycloheximide, 5 μg of trichodermin, or 0.5 μg of amphotericin B per ml were inhibited for 20 to 30 h, after which all the cells in the population recovered and grew at a rate only slightly lower than normal. Adapted cells grown in drug-free medium became fully susceptible after several generations. Cells adapted to either cycloheximide or trichodermin were cross resistant to all three drugs, while cells adapted to amphotericin B showed no cross resistance to cycloheximide or trichodermin. We have shown that the mechanism of adaptive resistance to trichodermin was the production of a trichodermin esterase which converted the drug to its less toxic alcohol derivative, trichodermol (8). The investigation reported in this paper examined the mechanism of adaptive cycloheximide resistance.

MATERIALS AND METHODS

Organisms and growth conditions. *M. racemosus* (*M. lusitanicus* ATCC 1216B) was used throughout these studies. The cells were grown either in medium consisting of 2% glucose, 0.5% Bacto-Peptone (pH 4.5; Difco Laboratories), 0.05% yeast nitrogen base without amino acids, and ammonium sulfate (Difco) or in medium containing 2% glucose and 1% yeast extract (pH 6.0; Difco). Cultures were grown at 28°C on a rotary shaker and sparged with water-saturated CO_2 at a flow rate of 1 vol/vol per min. Growth was monitored with a Klett-Summerson colorimeter equipped with a 540-nm-pore-size filter. Cells were adapted to cycloheximide and trichodermin as previously described (12).

Inhibition of in vivo protein synthesis. Log-phase cells were collected by centrifugation and suspended in fresh medium at a density of 4×10^6 cells per ml. Cycloheximide was added to 0.5-ml samples of cells to yield final concentrations of 0 to 200 μg/ml. ^{14}C-protein hydrolysate (Amersham Corp.) was added to yield a final concentration of 1 μCi/ml. Cells were incubated at 28°C for 30 min, and the reaction was stopped by the addition of 1/10 volume of 100% trichloroacetic acid. The cells were chilled on ice for 30 min and then filtered on 934-AH glass fiber filters (Whatman, Inc.). The filters were washed three times with 5 ml of 5% trichloroacetic acid and once with 5 ml of 95% ethanol and dried under an infrared lamp. Radioactivity was measured by liquid scintillation spectroscopy. Incorporation of ^{14}C-amino acids was linear with time and the amount of isotope added.

Ribosome isolation. Log-phase cells were collected by centrifugation and washed at 4°C with buffer A (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 100 mM potassium acetate, 2 mM magnesium acetate, 2 mM dithiothreitol [pH 7.4]). The cells were suspended in buffer A and broken in a French press at 10,000 lb/in^2. Cell debris and mitochondria were removed by successive centrifugations at 10,000 × *g* for 20 min and 30,000 × *g* for 30 min. The postmitochondrial supernatant was centrifuged at 100,000 × *g* for 90 min to sediment ribosomes. Samples of the S-100 supernatant were quick frozen in dry ice-ethanol and stored at −70°C. The ribosomal pellet was suspended in 1 ml of buffer A and layered over 4 ml of buffer B (50 mM Tris, 500 mM potassium acetate, 10 mM magnesium acetate, 10 mM dithiothreitol, 10% [wt/vol] sucrose [pH 7.4]) and centrifuged at 150,000 × *g* for 3 h. The surface of the ribosomal pellet was washed with buffer A. The ribosomal pellet was gently resuspended in buffer A to yield a final concentration of 200 A_{260} units/ml. Samples were quick frozen in dry ice-ethanol and stored at −70°C.

Inhibition of in vitro translation. The reaction mixture (50 μl) contained 20 mM Tris hydrochloride (pH 7.5) at 30°C, 50 mM KCl, 1.5 mM magnesium sulfate, 0.8 mM spermidine, 1 mM dithiothreitol, 1 mM GTP, 2 mM phosphoenolpyruvate, 15 IU of pyruvate kinase per ml, 200 μg of poly(U) per ml, 200 μg of S-100 protein per ml, 1 A_{260} unit of ribosomes, and 9 pM [^{3}H]Phe-tRNA (10^4 cpm/pM). Cycloheximide was

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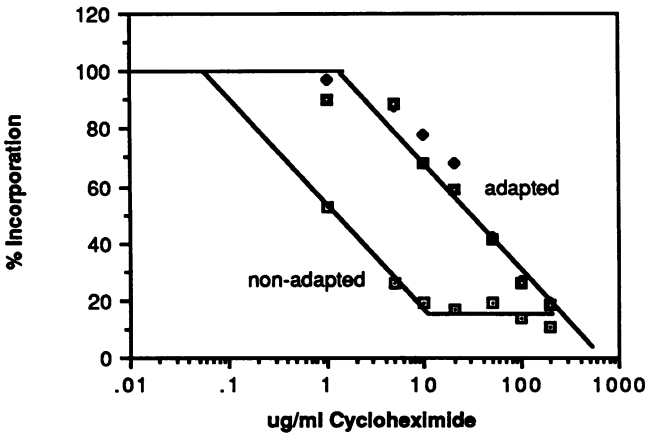

FIG. 1. Inhibition of in vivo protein synthesis by cycloheximide. Log-phase nonadapted, cycloheximide-adapted, and trichodermin-adapted cells were incubated with ^{14}C-amino acids and 0 to 200 μg of cycloheximide per ml. The percentage of acid-precipitable counts was measured at the indicated cycloheximide concentrations.

added from an aqueous stock solution to yield final concentrations of 0 to 200 μg/ml. After incubation at 30°C for 20 min, triplicate 15-μl samples were pipetted into 500 μl of 10% trichloroacetic acid. The tubes were heated at 100°C for 10 min, and the contents were filtered on nitrocellulose membranes. The filters were washed twice with 3 ml of 5% trichloroacetic acid and once with 5 ml of 95% ethanol. The filters were dried under an infrared lamp, and radioactivity was measured by liquid scintillation spectroscopy. Polymerization of poly(Phe) was linear with time and the amount of ribosomes added.

Recovery and assay of cycloheximide from cells. Log-phase cycloheximide-adapted cells were collected by centrifugation and suspended in fresh medium at a final density of 5×10^4 cells per ml. [^{3}H]cycloheximide was added to 5-ml samples of cells to yield a final concentration of 1 μCi/ml. The cells were incubated at 28°C and sparged with CO_2 as noted above. After 24 h the cells had reached a density of about 1.5×10^6 and were collected by centrifugation and extracted with 1 ml of ethyl acetate at 25°C for 30 min with occasional vigorous vortexing. Approximately 5,000 cpm of the ethyl acetate extract and the medium was spotted onto silica gel plates (Polygram sil G, 250 μm; Brinkmann Instruments, Inc.). The plates were developed with ethyl acetate-isopropanol (98:2), dried, sprayed with En^{3}Hance (New England Nuclear Corp.), and exposed to X-ray film at −70°C. Control samples were prepared from cells incubated with cycloheximide for less than 5 min.

Uptake assays. Log-phase cells were collected by centrifugation and resuspended in fresh medium at 28°C to a final density of 4×10^6 cells per ml. [^{3}H]cycloheximide was added to yield a final concentration of 1.0 μCi/ml. After incubation at 28°C, 0.5-ml samples of cells were quickly filtered onto Teflon filters (type LC, 10-μm pore size; Millipore Corp.) and washed three times with 5 ml of ice-cold phosphate-buffered saline (10 mM $NaPO_4$ [pH 6.0], 150 mM KCl). The filters were dried under an infrared lamp, and radioactivity was measured by liquid scintillation. Intracellular drug concentration was estimated by calculating disintegrations per minute per microliter, assuming a 2.6-μl intracellular volume per milligram of cell protein. Intracellular volume was measured as described by Rottenberg (15). Background radioactivity was estimated by incubating cells at 0°C with [^{3}H]cycloheximide and then filtering them as described above. To investigate the effect of metabolic inhibitors, sodium azide or dinitrophenol was added to yield a final concentration of 10 or 1 mM, respectively, 10 min before the addition of [^{3}H]cycloheximide.

Efflux assays. Log-phase cells were prepared and incubated with [^{3}H]cycloheximide as described above. After 30 min, the cells were quickly filtered and washed as described above. The cells were immediately resuspended in fresh medium at 28°C to a final density of 4×10^6 cells per ml. At various times, 0.5-ml samples were removed, the cells were pelleted by centrifugation in a microfuge for 5 s, and radioactivity released into the medium was measured.

Materials. Trichodermin was the kind gift of W. O. Godtfredson, Leo Pharmaceutical Products, Ballerup, Denmark. [^{3}H]cycloheximide (70 Ci/M) was prepared by tritium exchange (Amersham) and purified by thin-layer chromatography on silica gel plates developed with ethyl acetate-isopropanol (98:2). [^{3}H]Phe-tRNA was prepared by the method of Merrick (13) with *Mucor* tRNA.

RESULTS

We determined the effect of cycloheximide on ^{14}C-amino acid incorporation in nonadapted cells and in cells that had been adapted to grow in cycloheximide (100 μg/ml). Adapted cells were 40 times more resistant to cycloheximide than nonadapted cells, since adapted cells required 40 μg/ml for incorporation to be inhibited by 50% compared with 1 μg/ml required for 50% inhibition of nonadapted cells (Fig. 1). Cells adapted to trichodermin (5 μg/ml) were equally resistant to cycloheximide.

To examine the possibility that some ribosomal modification was responsible for the resistance to cycloheximide, we measured the inhibition of in vitro translation using isolated ribosomes. Ribosomes from nonadapted cells or adapted cells were equally sensitive to cycloheximide in the in vitro poly(U) translation assay (Fig. 2). Translation assays from nonadapted, cycloheximide-adapted, and trichodermin-adapted cells required approximately 0.4 μg of cycloheximide per ml to inhibit 50% of the poly(Phe) synthesis. Similar results were obtained when the S-100 preparations from adapted cells or nonadapted cells were used.

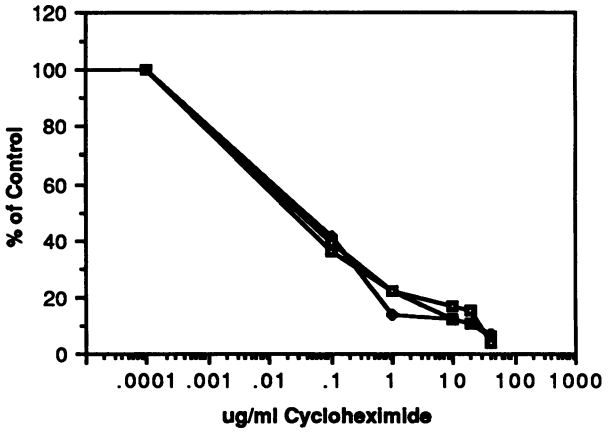

FIG. 2. Inhibition of in vitro translation by cycloheximide. Isolated ribosomes from nonadapted (■), cycloheximide-adapted (◆), and trichodermin-adapted (■) cells were incubated with 0 to 100 μg of cycloheximide per ml in a poly(U) translation mixture. The relative percentage of poly(Phe) synthesis was measured at the indicated cycloheximide concentrations.

Since several organisms are known to convert cycloheximide to derivatives of lower toxicity (10, 15, 16), we next investigated the possible modification of the drug by *M. racemosus*. Adapted cells were grown for 24 h in medium containing [^{3}H]cycloheximide. Samples of medium- and ethyl acetate-extracted cells were examined by thin-layer chromatography (Fig. 3). There was no detectable decrease in the amount of [^{3}H]cycloheximide, nor were any new radioactive compounds seen on autoradiograms.

To investigate the possibility that resistant *Mucor* cells have an altered permeability for the drug, we next measured the uptake of cycloheximide by nonadapted and adapted cells. Uptake quickly reached a plateau after approximately 2 min in both nonadapted and adapted cells (Fig. 4). Adapted cells, however, had a steady-state intracellular drug level 10- to 12-fold lower than that of nonadapted cells. The cells did not concentrate the drug against the concentration gradient; rather, the intracellular steady-state level in nonadapted cells was about one-fourth that of the extracellular level.

One possible explanation for the rapid plateau in drug uptake was that we were observing the sum of an influx-efflux system with much greater drug efflux in the adapted cells. To investigate the presence of an active influx or efflux system, we treated cells with the metabolic inhibitor sodium azide or dinitrophenol. Cells treated with 10 mM NaN_3 (Fig. 4) or 1 mM dinitrophenol (data not shown) 10 min before addition of [^{3}H]cycloheximide did not show a quick plateau but a steady increase until the intracellular drug level equalled the extracellular level. The initial rate of uptake was essentially the same as in cells without azide, indicating that drug influx is due to some energy-independent mechanism. The metabolic inhibitors abolished the 10-fold difference between uptake by nonadapted and adapted cells (Fig. 4). These results imply that the adapted cells have an enhanced

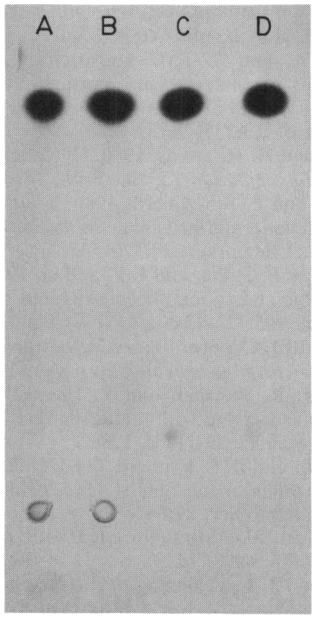

FIG. 3. Modification of cycloheximide. Cycloheximide-adapted cells were grown in the presence of 10 μg of [^{3}H]cycloheximide per ml for 24 h. Medium and cell extracts were separated by thin-layer chromatography and exposed to X-ray film. Lanes: A, medium at 0 h; B, medium at 24 h; C, cell extract at 0 h; D, cell extract at 24 h.

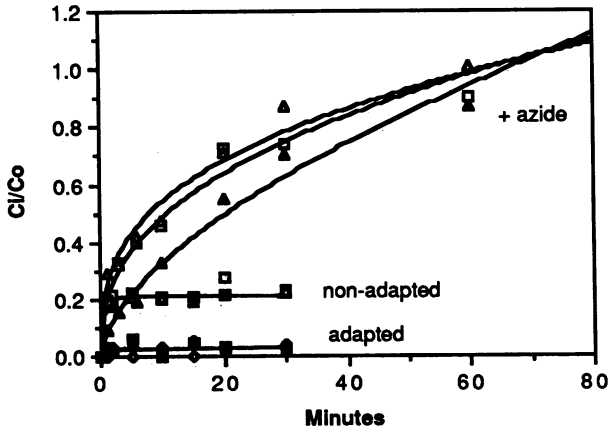

FIG. 4. Time course of cycloheximide uptake. Log-phase nonadapted (□ and ■), cycloheximide-adapted (◇, ◆, and △), and trichodermin-adapted (■ and ▲) cells were incubated with 10 μg of [^{3}H]cycloheximide per ml. Azide-treated cells were incubated with 10 mM sodium azide for 10 min before the addition of 10 μg of [^{3}H]cycloheximide per ml. The ratio of intracellular (Ci) to extracellular (Co) concentration of cycloheximide was measured at the indicated time points.

efflux system that results in a markedly lower intracellular drug concentration. To examine directly the difference in efflux, we loaded cells with [^{3}H]cycloheximide and measured radioactivity released when the cells were quickly placed in drug-free medium. Adapted cells were able to excrete the drug far more rapidly than nonadapted cells (Fig. 5). Adapted cells were able to clear the drug in 6 min, while nonadapted cells had 68% of the drug left at 6 min and 15% left at 45 min.

DISCUSSION

Numerous cycloheximide-resistant mutants have been described in higher and lower eucaryotes during the past 20 years. In nearly every case, the resistance has been mediated by a modification of the ribosomes so that the binding affinity of the drug is reduced (1–3, 7, 19). Phenotypic

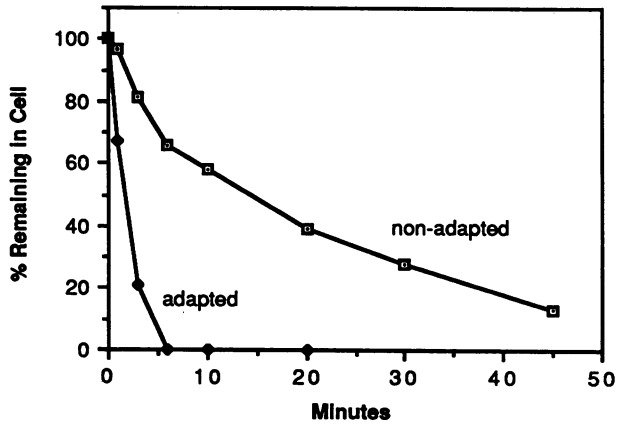

FIG. 5. Efflux of cycloheximide. Nonadapted and cycloheximide-adapted cells were incubated with 10 μg of [^{3}H]cycloheximide per ml for 30 min, washed, and placed in drug-free medium at 28°C. The percentage of drug remaining in the cells was measured at the indicated time points.

adaptation to cycloheximide has been noted in the fungus genera *Cunninghamella* (10), *Candida* (18), *Achyla* (9), *Cladosporium* (9), and *Sclerotium* (16); in plant cells (4, 17); and in the protozoan genus *Tetrahymena* (20). In the case of *Candida* (18) and *Tetrahymena* (20) spp., resistance was apparently due to a reversible ribosomal modification such as phosphorylation. Resistance in *Cunninghamella* and *Sclerotium* spp. was apparently due to drug modification. Cycloheximide was acetylated by *Cunninghamella* spp. (10) and converted to isocycloheximide by *Sclerotium* spp. (16). Both cycloheximide derivatives have reduced ability to inhibit translation. The mechanism of phenotypic resistance is unknown in the other cases.

M. racemosus is quite resistant to a number of growth inhibitors and exhibits an acquired phenotypic resistance to cycloheximide. In contrast to *Candida* (18) and *Tetrahymena* (20) spp., ribosomes from *Mucor* cells adapted to cycloheximide were fully sensitive to the drug (Fig. 2). We were unable to detect drug modification similar to that noted in *Cunninghamella* (10) and *Sclerotium* (16) spp. (Fig. 3). Adapted cells, however, showing a striking difference in drug uptake (Fig. 4).

Based on the effect of metabolic inhibitors and direct efflux measurement, the differential uptake in nonadapted versus adapted cells is the result of an enhanced energy-dependent efflux system in the adapted cells. The cytochrome oxidase inhibitor sodium azide completely abolished the difference between uptake by nonadapted and adapted cells and resulted in uptake equal to the extracellular drug concentration (Fig. 4). Even nonadapted cells of *M. racemosus* apparently have a constitutive efflux system that is able to transport cycloheximide out of the cell. Nonadapted cells were able to maintain intracellular levels of cycloheximide at one-fourth to one-fifth of the extracellular concentration (Fig. 4). Adapted cells had a much greater rate of efflux, so that the intracellular concentration was only 1/30 to 1/40 that of the medium. In the fungus *Aspergillus nidulans*, a similar efflux system has been reported for mutants resistant to the sterol synthesis inhibitor fenarimol. Wild-type as well as resistant mutants exhibited an energy-dependent efflux system with a much greater efflux efficiency in the mutant (6). It is interesting that the fenarimol-resistant mutants are also cross resistant to cycloheximide (5). However, the mechanism of this cross resistance has not been studied.

The elevated efflux rate in adapted cells may be due to a new inducible efflux system or simply an increased activity of the existing system. The specificity of the efflux mechanism for cycloheximide is unknown. Perhaps the constitutive efflux system is capable of pumping various xenobiotics and hence renders even nonadapted cells resistant to a number of inhibitors (e.g., anisomycin [500 μg/ml] and pactamycin [100 μg/ml]; unpublished observations).

How are the cells able either to produce a new efflux system with its ancillary proteins or to overproduce the constitutive system when translation is inhibited by cycloheximide? Although protein synthesis is largely inhibited by cycloheximide, about 3 to 5% remains. During the 20- to 30-h adaptation period, the organism may be concentrating its remaining protein-synthetic ability toward the production of the efflux system proteins in a shock response similar to that seen in *Neurospora* sp. exposed to cycloheximide (14). It is interesting that in preliminary experiments we have noted a new 112-kilodalton membrane protein in cycloheximide-adapted cells. This protein may play some role in efflux in a manner similar to that of the membrane *p*-glycoprotein thought responsible for increased drug efflux and resistance in cancer cells (11). We are currently investigating the role of this new protein.

The intriguing question of regulation of the adaptive response remains. Exposure of *Mucor* cells to either cycloheximide or trichodermin results in acquired resistance to both drugs by simultaneous induction of two mechanisms (efflux of cycloheximide and degradation of trichodermin). When cells are grown in the presence of cycloheximide for 30 generations, they retain their elevated trichodermin esterase levels and trichodermin resistance, even though there is no direct selective pressure to do so (unpublished observations). Apparently there is some common signal responsible for the induction and maintenance of these different mechanisms of drug resistance. We are currently studying the molecular biology of the induction of these resistance mechanisms.

ACKNOWLEDGMENTS

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