Saccharomyces cerevisiae Spheroplasts Are Sensitive to the Action of Diphtheria Toxin

SEISHI MURAKAMI, JAMES W. BODLEY, AND DENNIS M. LIVINGSTON*

Department of Biochemistry, University of Minnesota, Minneapolis, Minnesota 55455

Received 8 September 1981/Accepted 18 December 1981

Diphtheria toxin kills spheroplasts of *Saccharomyces cerevisiae* but not the intact yeast cells. After 2 h of exposure to ca. 10^{-7} M toxin, less than 1% of spheroplasts were able to regenerate into intact cells. The same high levels of toxin inhibited the rate of protein synthesis by more than 90% within 1 h, whereas RNA and DNA synthesis were not inhibited until 4 h of exposure. Both killing and protein synthesis inhibition were dependent on toxin concentration. The nature of the toxin-cell interaction was also studied by using fragments of intact toxin and mutant toxin proteins. Neither toxin fragment A nor CRM45 nor CRM197 affected spheroplasts, but CRM197 and ATP prevented the inhibitory action of intact toxin. These results suggest that toxin acts on *S. cerevisiae* spheroplasts in much the same manner as it acts on sensitive mammalian cells.

Protein synthesis elongation factor 2 (EF-2) from *Saccharomyces cerevisiae* has recently been shown to contain an unusual posttranslationally derived amino acid, diphthamide, which is the site of ADP ribosylation by diphtheria toxin in vitro (22, 23). *S. cerevisiae* should be an ideal organism in which to study the biosynthesis and importance of this unusual amino acid because of the possibility of easily deriving mutations in the biosynthetic process. Unfortunately, *S. cerevisiae* cells are not inhibited by toxin in vivo and are therefore seemingly not useful for physiological studies. We have investigated the cause of this refractoriness and have found that the cell wall of *S. cerevisiae* prevents penetration of the toxin to the cell membrane. In this report we show that regenerable *S. cerevisiae* spheroplasts are sensitive to toxin inhibition and thus provide a basis for characterization of toxin action in this genetically malleable organism.

MATERIALS AND METHODS

Diphtheria toxin. Diphtheria toxin was obtained from Connaught Laboratory Ltd. (Willowdale, Ontario, Canada). The mutant forms of toxin, CRM45 and CRM197 (18, 19), were generously provided by A. M. Pappenheimer, Jr. Fragment A was prepared from intact toxin after treatment with trypsin, using previously described methods (3, 5-7). The ADP-ribosylating activity by toxin of EF-2 was measured as previously reported (21).

***S. cerevisiae* growth, spheroplast formation, and spheroplast manipulation.** *S. cerevisiae* D13-1A (*MATa his3 trp1 gal2*) was used in these experiments. Cell growth, spheroplast formation, and spheroplast regeneration have been previously described (1). The liquid generation medium used for these studies included

0.17% yeast nitrogen base (Difco Laboratories, Detroit, Mich.) without amino acids and ammonium sulfate, 0.15% ammonium sulfate, 2% glucose, 1 M sorbitol, and other nutrients as specified.

Measurement of protein synthesis. (i) **Continuous labeling.** Spheroplasts were prepared and suspended in regeneration medium (1) containing 40 μ g of tryptophan per ml and 5 μ Ci (20 μ g/ml) of [3 H]histidine (New England Nuclear Corp., Boston, Mass.). Diphtheria toxin was added either directly afterwards or at the times indicated in the figures. Spheroplasts were incubated at 25°C, and samples (100 or 150 μ l) were removed at various times for determination of incorporation of [3 H]histidine into protein. After chilling to 0°C, bovine serum albumin was added to 0.2 mg/ml, and trichloroacetic acid was added to 10% (wt/vol). The precipitate was collected, and radioactivity was determined as previously described (10).

(ii) **Pulse-labeling.** Spheroplasts were cultured as described above with either 10 or 20 μ g of histidine per ml and 40 μ g of tryptophan per ml for 60 min at 25°C. Toxin or toxin-related proteins were then added in 1/10 the culture volume, and incubation was continued for another 3.5 h at 25°C. A 1/20 volume of 1 M sorbitol containing 400 μ Ci of [3 H]histidine per ml was added, and incubation was again continued for 30 (see Fig. 2) or 60 (see Fig. 3) min before the incubation was terminated by chilling and addition of trichloroacetic acid as described above.

RESULTS

Action of diphtheria toxin on intact *S. cerevisiae* cells and spheroplasts. We have been unable to find growth conditions under which diphtheria toxin exhibits a detectable inhibition of intact *S. cerevisiae* yeast cell growth. *S. cerevisiae* spheroplasts, on the other hand, were inhibited by toxin. The nature of this inhibition and the characteristics of spheroplasts as compared with

FIG. 1. Action of diphtheria toxin on viability, cell division, and protein synthesis of intact *S. cerevisiae* cells and spheroplasts. (A) A spheroplast preparation in regeneration medium was divided into two aliquots, and at time zero, diphtheria toxin was added to one sample. The samples were incubated with agitation at 25°C. At the times indicated, two portions of each sample were removed. To measure the ability of the spheroplasts to form colonies, one portion was diluted by greater than 10,000-fold in osmotically stabilizing regeneration medium and then plated on agar in an osmotically stabilizing agar overlay. The second portion was diluted greater than 100-fold with sterile water and plated directly on an agar surface to measure the number of intact cells capable of forming colonies. Symbols: Spheroplasts in the absence (○) and presence (●) of diphtheria toxin; osmotically stable intact cells in the absence (Δ) and presence (▲) of diphtheria toxin. (B) and (C) Cells were grown in the presence of [³H]histidine for more than five generations until they reached a density of 7×10^5 cells per ml. A sample was removed, made into spheroplasts, and suspended in regeneration medium containing [³H]histidine as before. At time zero, both the spheroplast culture and the intact cell culture were divided into two portions, and diphtheria toxin was added to one of each pair. Incubation was continued at 25°C, and at the times indicated samples were removed for analysis. (B) Analysis of cell or spheroplast number observed microscopically. Because the spheroplasts form clumps, each spherical body or bud was counted as a distinct spheroplast or cell. (C) Analysis of incorporation of [³H]histidine into protein. Symbols: Intact cells in the absence (Δ) or presence (▲) of diphtheria toxin; spheroplasts in the absence (○) or presence (●) of diphtheria toxin.

intact yeast cells are shown in Fig. 1. Figure 1A demonstrates the effect of toxin on the ability of spheroplasts to form colonies on an agar plate. In the absence of toxin the number of colony formants increased very little over this time period because the dividing spheroplasts formed

clumps which did not separate before plating. The addition of diphtheria toxin to spheroplast cultures (Fig. 1A) rapidly reduced the colony formants to ca. 1% of their former level. Osmotically resistant intact cells present in the spheroplast preparation (Fig. 1A) initially accounted for about 0.01% of the colony formants. These whole cells continued to divide and were of course resistant to toxin. Thus, spheroplasts but not intact yeast cells are killed by diphtheria toxin.

We also measured the effect of toxin on cell or spheroplast division by microscopically counting cells or spheroplasts after application of toxin (Fig. 1B). In this experiment, a culture of exponentially growing cells was divided into two samples, and one was made into spheroplasts. The sample containing the intact cells continued to divide exponentially in the absence or presence of toxin as expected. On the other hand, toxin-treated spheroplasts failed to divide, whereas the untreated spheroplasts, after a lag of approximately 2 h, began to divide with a division time roughly one-third that of intact cells. The lag period and longer doubling time are characteristic of spheroplasts as compared with whole cells (S. Murakami and D. M. Livingston, submitted for publication). The results shown in Fig. 1A and B thus demonstrate that *S. cerevisiae* spheroplasts, but not intact cells, are prevented from dividing by diphtheria toxin.

Because diphtheria toxin kills sensitive animal cells by inhibiting protein synthesis, the effect of toxin on protein synthesis was examined. Figure 1C shows the incorporation of [³H]histidine with time into protein by intact cells and spheroplasts. For this experiment, cells were grown for more than five generations in medium containing [³H]histidine, the culture was divided, and one portion was made into spheroplasts. After preparation, the spheroplasts and intact cells were suspended in medium with [³H]histidine of the same specific activity. The pattern of protein synthesis in untreated spheroplasts, like the time course of spheroplast division, showed an early lag followed by an increase in protein at approximately one-third the rate exhibited by intact cells. The rate of protein synthesis in both intact cells and spheroplasts was proportional to the division time of each (Fig. 1B). Thus, toxin inhibition of spheroplast protein synthesis probably accounts for its ability to kill *S. cerevisiae* spheroplasts.

In another experiment (not shown), we measured both DNA and RNA synthesis in toxin-inhibited spheroplasts. We found that toxin totally inhibits protein synthesis within 1 to 2 h, whereas the same concentration of toxin does not inhibit DNA or RNA synthesis, as measured by the method of Hartwell (10), until it has been

applied for nearly 4 h. Thus, the inhibition of DNA and RNA synthesis is probably the result of protein synthesis inhibition by toxin.

Effects of varying concentrations of toxin on the rate of protein synthesis. The effect of varying concentrations of toxin on the rate of spheroplast protein synthesis was measured. The rate was measured by giving a 30-min pulse of [3 H]histidine to spheroplasts in regeneration medium at varying times after the addition of toxin. In uninhibited spheroplasts the rate of protein synthesis increased rapidly for 3 h after spheroplast preparation and then increased slowly (Fig. 2). This pattern is a reflection of the fact that spheroplasts undergo a lag of approximately 2 h before reaching a steady-state rate of growth (Fig. 1C). Application of a high level of toxin reduced the rate of protein synthesis 40-fold in 2 h in comparison to uninhibited spheroplasts. Addition of lower concentrations of toxin had a less drastic but measurable effect. These results show that the inhibition in rate of spheroplast protein synthesis is dependent on toxin concentration.

Action of proteolytic fragments of toxin and mutant toxin proteins on *S. cerevisiae* spheroplasts. Diphtheria toxin is a single polypeptide chain of 62,000 daltons (3, 6) which, in addition to its enzymatic activity, ADP ribosylation of

protein synthesis EF-2, possesses the ability to bind and enter sensitive mammalian cells. The polypeptide can be proteolytically hydrolyzed and reduced to yield an enzymatically active A fragment (5, 7) and a hydrophobic B fragment which provides the binding and entry capacities (2, 19). We therefore tested the ability of fragment A alone to kill *S. cerevisiae* spheroplasts and found that it had no detectable effect, even at a concentration as high as 2×10^{-5} M (data not shown). This result suggested that all or part of the hydrophobic fragment B is needed for entry.

Consequently, we examined the mutant form of toxin, CRM45, which is shorter than intact toxin but longer than fragment A. CRM45 retains enzymatic activity in vitro (20), but because it contains only a small portion of the hydrophobic fragment B, it lacks the ability to bind and enter sensitive mammalian cells (2). The action of CRM45 on the viability and protein-synthesizing ability of *S. cerevisiae* spheroplasts is shown in Fig. 3A and B. Even at high levels of CRM45, the spheroplasts are not killed, nor is their synthesis of protein inhibited.

To further test the necessity of the fragment B binding region for entry into spheroplast, we examined the action of CRM197 (18). CRM197 is a full-length polypeptide which has a single amino acid replacement in fragment A that results in catalytic inactivity (19). It retains the binding region of fragment B and therefore can compete with active toxin for binding sites on sensitive mammalian cell membranes (2, 19). CRM197 by itself does not inhibit yeast spheroplast protein synthesis, but it is capable of inhibiting the toxic effect of active toxin (Fig. 3C). In agreement with this result we have observed (data not shown) that ATP inhibits toxin action on spheroplasts, as has been found for sensitive mammalian cells (12, 13). These results suggest that yeast spheroplasts contain a saturable number of entry sites which interact with the toxin fragment B binding region.

DISCUSSION

This study shows that *S. cerevisiae* spheroplasts, but not the intact cells, are killed by diphtheria toxin. The primary site of toxin action appears to be the protein-synthesizing machinery of the cell. Intact toxin protein, containing both the ADP-ribosylating catalytic activity and the mammalian membrane binding region, is needed for toxin entry into the spheroplasts.

The concentration of toxin required to kill *S. cerevisiae* spheroplasts is at least 1,000-fold greater than that needed to kill sensitive mammalian cells (11). Many factors might account for this observation. First, the efficiency of toxin

FIG. 2. Effect of varying concentrations of diphtheria toxin on the rate of protein synthesis in *S. cerevisiae* spheroplasts. The rate of protein synthesis in spheroplasts was measured by pulse-labeling as described in the text. Symbols: Protein synthesis in untreated spheroplasts (○); protein synthesis in spheroplasts treated with 8×10^{-7} M (●), 8×10^{-8} M (▲), and 8×10^{-9} M (■) diphtheria toxin.

FIG. 3. Effect of mutant toxin proteins on viability and protein synthesis in *S. cerevisiae* spheroplasts. The rate of protein synthesis in spheroplasts was measured by pulse-labeling as described in the text. Viability was measured after a 3.5-h exposure to toxin-related proteins. (A) Effect of the concentration of CRM45 on spheroplast viability. Symbols: Regeneration of toxin-treated (○) and CRM45-treated (●) spheroplasts. (B) Effect of the concentration of CRM45 on spheroplast protein synthesis. Symbols: Protein synthesis in spheroplasts treated with toxin (○) or with CRM45 (●). (C) Effect of the concentration of CRM197 on protein synthesis in toxin-treated or uninhibited *S. cerevisiae* spheroplasts. Symbols: Protein synthesis in spheroplasts treated with toxin (○), CRM197 (▲), or 4×10^{-7} M active toxin and various amounts of CRM197 (●).

entry into *S. cerevisiae* spheroplasts might be low in comparison to entry into sensitive mammalian cells. Second, the unknown relationship between (i) the rate of ADP-ribosylating activity of EF-2 by toxin and by the turnover of yeast cells and (ii) the absolute rate of EF-2 synthesis must be an important factor in determining the sensitivity of yeast cells to toxin. The fact that *S. cerevisiae* spheroplasts divide approximately

every 4 to 6 h, whereas cultured mammalian cells have a much longer division time, might result in a higher lethal dose for the spheroplasts. Finally, yeast cells are known to secrete proteases, and these may destroy much of the toxin before it can successfully penetrate the spheroplast membrane. In fact, we have found that greater than 90% of the toxin activity is lost within 2 h when incubated with yeast spheroplasts. Whether this represents proteolysis or irreversible binding to the cell membrane of toxin molecules is unknown. Because of the many unknown parameters which affect toxin killing of cells, we do not believe the high lethal dose needed to kill *S. cerevisiae* spheroplasts can be construed to mean that the mechanism of toxin killing of these spheroplasts is significantly different from the mechanism of toxin killing of sensitive mammalian cells.

The requirement for the complete toxin polypeptide chain is surprising. The fact that toxin fragment A or CRM45 mutant protein, both of which are catalytically active *in vitro*, cannot kill *S. cerevisiae* spheroplasts indicates that the complete hydrophobic B region of the toxin polypeptide is needed for entry. This conclusion is confirmed by the observation that CRM197, which contains an intact B region, successfully competes with intact toxin to prevent the killing of *S. cerevisiae* spheroplasts. These results indicate that a specific, saturable element on the *S. cerevisiae* membrane interacts with the portion of the B region needed for binding to sensitive mammalian cells. The nature of the receptor on mammalian cells is unknown.

Our stated interest in the action of diphtheria toxin on *S. cerevisiae* spheroplasts is the possibility of generating yeast mutants insensitive to toxin. Such mutants should not only help to elucidate the role and biosynthetic pathway of the unusual amino acid, diphthamide, found in EF-2, but might also help in the study of toxin entry into cells. We believe that the possibility of rapidly producing and genetically characterizing yeast mutants will complement the ongoing work on mammalian cell mutants (4, 8, 9, 14-17). We are encouraged by the fact that greater than 99% of an *S. cerevisiae* spheroplast culture can be quickly killed by toxin. Studies are currently under way to develop a successful selection scheme.

ACKNOWLEDGMENTS

We are grateful to A. M. Pappenheimer, Jr. for supplying us with mutant toxin proteins. We thank Patricia Dunlop for unrelenting yet valuable comments throughout the course of this work.

These studies were supported by Public Health Service grant GM 26832 from the National Institutes of Health to J.W.B. and by grant PCM 80-21893 from the National Science Foundation to D.M.L. S.M. is on leave from the Biophysics

Division, Cancer Research Institute, Kanazawa University, Japan, and is supported by a stipend from the Japanese Ministry of Education.

LITERATURE CITED

1. Beggs, J. D. 1978. Transformation of yeast by a replicating hybrid plasmid. *Nature (London)* **275**:104-109.
2. Boquet, P., and A. M. Pappenheimer, Jr. 1976. Interaction of diphtheria toxin with mammalian cell. *J. Biol. Chem.* **251**:5770-5778.
3. Collier, R. J., and J. Kandel. 1971. Interaction of diphtheria toxin. I. Thiol-dependent dissociation of a fraction of toxin into enzymatically active and inactive fragments. *J. Biol. Chem.* **246**:1496-1503.
4. Draper, R. K., D. Chin, D. Eurey-Owens, I. E. Scheffler, and M. I. Simon. 1979. Biochemical and genetic characterization of three hamster cell mutants resistant to diphtheria toxin. *J. Cell Biol.* **83**:116-125.
5. Drazin, R., J. Kandel, and R. J. Collier. 1971. Structure and activity of diphtheria toxin. II. Attack by trypsin at a specific site within the intact toxin molecule. *J. Biol. Chem.* **246**:1504-1510.
6. Gill, D. M., and L. L. Dinius. 1971. Observations on the structure of diphtheria toxin. *J. Biol. Chem.* **246**:1485-1491.
7. Gill, D. M., and A. M. Pappenheimer, Jr. 1971. Structure-activity relationships in diphtheria toxin. *J. Biol. Chem.* **246**:1492-1495.
8. Gupta, R. S., and L. Siminovitch. 1978. Isolation and characterization of mutants of human diploid fibroblasts resistant to diphtheria toxin. *Proc. Natl. Acad. Sci. U.S.A.* **75**:3337-3340.
9. Gupta, R. S., and L. Siminovitch. 1978. Diphtheria-toxin-resistant mutants of CHO cells altered in protein synthesis: a novel phenotype. *Somatic Cell Genet.* **4**:553-571.
10. Hartwell, L. H. 1967. Macromolecule synthesis in temperature-sensitive mutants of yeast. *J. Bacteriol.* **93**:1662-1670.
11. Middlebrook, J. L., and R. B. Dorland. 1977. Response of cultured mammalian cells to the exotoxins of *Pseudomonas aeruginosa* and *Corynebacterium diphtheriae*: differential cytotoxicity. *Can. J. Microbiol.* **23**:183-189.
12. Middlebrook, J. L., and R. B. Dorland. 1979. Protection of mammalian cells from diphtheria toxin by exogenous nucleotides. *Can. J. Microbiol.* **25**:285-290.
13. Middlebrook, J. L., R. B. Dorland, and S. H. Leppla. 1978. Association of diphtheria toxin with Vero cells: demonstration of a receptor. *J. Biol. Chem.* **253**:7325-7330.
14. Moehring, J. M., and T. J. Moehring. 1979. Characterization of the diphtheria toxin-resistance system in Chinese hamster ovary cells. *Somatic Cell Genet.* **5**:453-468.
15. Moehring, J. M., T. J. Moehring, and D. E. Danley. 1980. Posttranslational modification of elongation factor 2 in diphtheria toxin-resistant mutants of CHO-K1 cells. *Proc. Natl. Acad. Sci. U.S.A.* **77**:1010-1014.
16. Moehring, T. J., D. E. Danley, and J. M. Moehring. 1979. Codominant translational mutants of Chinese hamster ovary cells selected with diphtheria toxin. *Somatic Cell Genet.* **5**:469-480.
17. Moehring, T. J., and J. M. Moehring. 1977. Selection and characterization of cells resistant to diphtheria toxin and *Pseudomonas* exotoxin A: presumptive translational mutants. *Cell* **11**:447-454.
18. Uchida, T., A. M. Pappenheimer, Jr., and A. A. Harper. 1973. Diphtheria toxin and related proteins. I. Isolation and properties of mutant proteins serologically related to diphtheria toxin. *J. Biol. Chem.* **248**:3838-3844.
19. Uchida, T., A. M. Pappenheimer, Jr., and A. A. Harper. 1973. Diphtheria toxin and related proteins. II. Kinetics studies on intoxication of HeLa cells of diphtheria toxin and related proteins. *J. Biol. Chem.* **248**:3845-3850.
20. Uchida, T., A. M. Pappenheimer, Jr., and A. A. Harper. 1973. Diphtheria toxin and related proteins. III. Reconstitution of hybrid "Diphtheria toxin" from nontoxic mutant proteins. *J. Biol. Chem.* **248**:3851-3854.
21. Van Ness, B. G., J. B. Howard, and J. W. Bodley. 1978. Isolation and properties of the trypsin-derived ADP-ribosyl peptide from Diphtheria toxin modified yeast elongation factor 2. *J. Biol. Chem.* **253**:8687-8690.
22. Van Ness, B. G., J. B. Howard, and J. W. Bodley. 1980. ADP-ribosylation of elongation factor 2 by diphtheria toxin: NMR spectra and proposed structures of ribosyl-diphthamide and its hydrolysis products. *J. Biol. Chem.* **255**:10710-10716.
23. Van Ness, B. G., J. B. Howard, and J. W. Bodley. 1980. ADP-ribosylation of elongation factor 2 by diphtheria toxin: isolation and properties of the novel ribosyl-amino acid and its hydrolysis products. *J. Biol. Chem.* **255**:10717-10720.