Low Concentrations of Trifluoperazine Arrest the Cell Division Cycle of *Saccharomyces cerevisiae* at Two Specific Stages

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Low concentrations of trifluoperazine (TFP) reversibly inhibited vegetative growth of *Saccharomyces cerevisiae*. The cell cycle was analysed by flow cytometry using haploid *a* cells synchronized by α -factor arrest and several temperature-sensitive cell division cycle mutants (*cdc*). Cells were pulse-labelled with fluorescein-labelled concanavalin A (ConA-FITC) to determine cell division or stained with propidium iodide to determine the stage of cell cycle arrest by TFP. Cell growth was estimated from the changes in the relative intensity of scattered light, and budding was determined microscopically. When TFP was added before Start on release from α -factor arrest, after release of *cdc28*-arrested cells, and at transition from stationary phase to vegetative growth, cell growth, budding and DNA synthesis were inhibited. When TFP was added after execution of spindle pole body duplication, cell growth, bud emergence and DNA synthesis were not inhibited but cell division was inhibited and the cells arrested with buds at G2 + M. Using *cdc* mutants, the second stage of arrest by TFP was determined to be just before medial-nuclear division.

INTRODUCTION

In higher eukaryotic cells, Ca^{2+} ions are considered as regulators of a diverse array of cellular functions, many of which are mediated by interaction with the Ca^{2+} binding protein calmodulin (Cheung, 1982; Klee *et al.*, 1980; Means *et al.*, 1982). In the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, calmodulin is an essential protein (Davis *et al.*, 1986; Takeda & Yamamoto, 1987) but the functions regulated by calmodulin are unknown. Several Ca^{2+} -dependent mutants were isolated from *S. cerevisiae* by Ohya *et al.* (1984, 1986). The *cal1-1* mutation causes arrest at the restrictive temperature and in low concentrations of Ca^{2+} at a specific stage of the cell cycle. A pseudorevertant dependent on trifluoperazine (TFP) (an inhibitor of calmodulin; Weiss *et al.*, 1980) was obtained that showed suppression of *cal1-1* in the presence of TFP. This led to the suggestion that Ca^{2+} ions and a calmodulin-like protein may be important in regulation of the cell division cycle in yeasts.

Studies in our laboratory (Eilam, 1983, 1984; Eilam *et al.*, 1985) have shown that TFP and other phenothiazines have the following effects on yeast cells: (1) inhibition of H^+ -ATPase in isolated membrane preparations; (2) K^+ efflux induced by activation of a K^+ extrusion mechanism; (3) membrane hyperpolarization resulting from K^+ efflux; (4) increased Ca^{2+} influx driven by the increase in membrane potential (Eilam & Chernichovsky, 1987). We also observed that low concentrations of phenothiazines inhibit growth of *S. cerevisiae* and other yeasts (Eilam *et al.*, 1987). This inhibition was not caused by the membrane changes described above since at the concentrations of TFP which inhibited growth, K^+ efflux was not induced when cells were suspended in a broth which contained a considerable concentration of cations (Eilam, 1983). Inhibition of H^+ -ATPase required higher concentrations of TFP than did growth arrest (Eilam, 1984). In order to elucidate the mechanism of growth inhibition by TFP we

Abbreviations: ConA-FITC, fluorescein-isothiocyanate-labelled concanavalin A; PI, propidium iodide.

examined whether low concentrations of TFP caused arrest at specific stages of the cell division cycle and compared the results with the stages of arrest induced by low Ca^{2+} concentrations in the *cal1-1* mutant (Ohya *et al.*, 1984).

METHODS

Organism and culture conditions. The following strains of *Saccharomyces cerevisiae* were obtained from G. Simchen, Department of Genetics, Hebrew University, Jerusalem, Israel. Diploid N123 (*MATa/MAT α his1-1/his1-1*); haploid 834 (*MATa, his1-1*). Strains 435-5, 429-33, 404, 204-4, 202-2 and 443-1 are diploid (*MATa/MAT α*), and each is heterozygous for *ade1, ura1, lys2, tyr1, his7* and *metx*, heteroallelic for *ade2/ade2-R8* and homozygous for the temperature-sensitive cell division cycle (*cdc*) mutation as follows: 435-5, *cdc2-1*; 429-33, *cdc9-2*; 404, *cdc4-1*; 204-4, *cdc7-4*; 202-2, *cdc21*; 443-1, *cdc28*. Strain 324 is haploid [*MATa cdc10 ura1* (and/or 4) *ade1* (or 2) *lys2-2 trp1 his4* (and/or 7)].

Cells were maintained on YPD-agar slopes and grown in YPD-broth (Bacto yeast extract, 10 g l⁻¹; Bacto peptone 20 g l⁻¹, glucose, 20 g l⁻¹), or in YNB broth (Difco yeast nitrogen base containing histidine, methionine and tryptophan, 6.7 g l⁻¹; HEPES or MES buffer at the pH indicated, 20 mM; glucose, 100 mM; and adenine 40 µg ml⁻¹ for experiments with *cdc* mutants). For experiments with strain 324, uracil (10 µg ml⁻¹) and lysine (40 µg ml⁻¹) were also added.

Cell synchronization. Strain 834 was grown overnight in YPD broth, centrifuged and suspended at a density of 6×10^6 cells ml⁻¹ in YNB broth at pH 5.8 which contained α -factor (1.25 µg ml⁻¹). Cells were incubated at 30 °C for 3.5 h. At the end of this incubation, cells appeared unbudded with G1 nuclei, as determined by flow cytometry of cells stained with propidium iodide (PI). Cells were released from α -factor arrest by centrifugation and resuspension in YNB broth at pH 7.2, without α -factor, at a density of 6×10^6 cells ml⁻¹.

Pulse-labelling of cells with ConA-FITC. After synchronization cells were centrifuged and suspended (2×10^8 cells ml⁻¹) in YNB broth, pH 7.2, containing ConA-FITC (20 µg ml⁻¹) and incubated at 25 °C for 5 min. The cells were then sedimented, washed once with sterile distilled water and resuspended in YNB broth, pH 7.2, for additional incubation as indicated. At the end of the required period of incubation, cells were collected and fixed with a solution of formaldehyde (3.7%, v/v) and NaCl (0.15 M). Before examination with a cell sorter, cells were sonicated for 15 s at maximum power (MSE 150 W ultrasonic disintegrator) to disperse clumps.

Propidium iodide staining procedure. Cells were harvested, washed with distilled water and suspended in 3 ml of water; methanol (4 ml) was then added. After 10 min incubation at 23 °C the cells were collected on a Millipore filter, resuspended in water, centrifuged and resuspended in a solution of bovine pancreas RNAase (DNAase-free) (200 µg ml⁻¹). Cells were incubated at 30 °C for 3 h, collected by centrifugation, resuspended in PI solution (50 µg ml⁻¹), Tris/HCl buffer (pH 7.0, 10 mM) and MgCl₂ (5 mM) at a density of 1.5×10^6 cells ml⁻¹ and incubated at room temperature for 75 min (Deitch *et al.*, 1982).

Flow cytometry. Cells stained with PI or labelled with ConA-FITC were examined in a Becton Dickinson FACS 440 cell sorter equipped with a Spectra Physics 164-05 argon ion laser. An output of 0.3 W of 488 nm light was used for excitation and fluorescence was determined at wavelengths above 530 nm for ConA-FITC and above 620 nm for PI. Calculation of the percentages of cell population at G1, S and G2 + M was done according to Deitch *et al.* (1982) using the fluorescence histograms of PI-stained cells.

Uracil uptake. Incorporation of [¹⁴C]uracil into RNA and DNA was determined by standard procedures (Hartwell, 1970). Rates of [¹⁴C]uracil uptake were determined by incubating the cells with [¹⁴C]uracil for 5 min at 30 °C and then filtering the cells on Millipore filters and washing with water. The filters were dried and the radioactivity was determined.

Microscopy. The number of nuclei per cell was determined by examining PI-stained cells with a Zeiss Universal epifluorescence microscope using a BP 546/12 nm excitation filter, FT 580 deflector and LP 590 barrier filter.

RESULTS

Low concentrations of TFP reversibly inhibited cell division in yeasts grown on YNB medium; washing the cells after 10 h of incubation with TFP restored normal cell division (see below). We examined whether the inhibition of growth occurred at specific stages of the cell division cycle. Haploid α cells were synchronized by α -factor arrest. On release from arrest cells were pulse-labelled with ConA-FITC, and at different times thereafter were fixed and analysed using a fluorescence-activated cell sorter (Steinkamp, 1984). Thus we could record cell growth semiquantitatively (since scattered light intensity per particle increases with particle size). It was impossible, however, to distinguish between total cell growth and bud growth. Cell division could be recorded, since cells which underwent cell division had a lower fluorescence intensity

Fig. 1. Flow cytometry profile of cells pulse-labelled with ConA-FITC. Haploid *a* cells were arrested by α -factor and pulse-labelled with ConA-FITC at the time of the release from arrest. The numbers in (a) and (b) indicate the time (min) after release from α -factor at which the cells were fixed for examination. The numbers in (c) indicate the time after release from α -factor at which TFP (20 μ M) was added. In (c) the cells were fixed and examined 150 min after release from arrest.

per cell. It was also possible to determine which part of the cell population underwent one or more cell divisions. Between 0 and 60 min after release from α -factor arrest, there was a synchronous increase in the intensity of scattered light indicating growth (Fig. 1*a*). Microscopic examination revealed that on release from arrest, 96% of the cells were unbudded. After 30 min, 74% of the cells had small buds and after 60 min, 72% had medium to large buds. The right-hand panel of Fig. 1*a* shows that during this time, there was no change in the intensity of fluorescence per cell indicating that there was no cell division (the cell count confirmed this result). A subpopulation underwent cell division 100 min after release from arrest, as indicated in Fig. 1*b* (right) by the appearance of a peak of lower fluorescence intensity. The fluorescence per cell in this peak was half the original fluorescence. The pattern of scattered light intensity indicated the appearance of small cells. At 150 min most of the cells had completed cell division (Fig. 1*b*).

In order to detect stages of inhibition by TFP, haploid *a* cells were arrested by α -factor and pulse-labelled with ConA-FITC. TFP (20 μ M) was added at different times after release from α -factor arrest. All cells were fixed and examined 150 min after release from arrest, at the time when cells in the control had completed one cell cycle. When TFP was added at the time of release from arrest, growth was inhibited as indicated from the relative intensity of the scattered light. Microscopic examination revealed unbudded cells (92%). Fluorescence intensity indicated that cell division did not occur (Fig. 1*c*). When TFP was added 30 or 60 min later cell growth was not inhibited as indicated by the higher intensity of the scattered light, but cell division was inhibited as indicated by the constant intensity of fluorescence (Fig. 1*c*). Microscopic examination showed that 65 and 69% of the cells were budded when TFP was added at 30 and 60 min respectively.

Fig. 2. Flow cytometry profile of cells stained with PI. (a) Diploid cells removed from a refrigerated stock (indicated 0) or incubated for 10 h at 23 °C were stained with PI as described in Methods and examined with the cell sorter. Arrows indicate the peak positions of haploid and diploid stationary phase control cells. (b) TFP (20 μ M) was added immediately after suspending the cells in medium. Cells were fixed, stained with PI and examined after 10 h incubation at 23 °C.

The next group of experiments was intended to determine the effect of TFP on DNA synthesis. TFP caused inhibition of [14 C]uracil incorporation into DNA and RNA (not shown), but it was found that the uptake of uracil was also inhibited by TFP, so this method could not be used to determine DNA synthesis. Therefore DNA synthesis was measured by using PI, which interacts with DNA quantitatively in the absence of RNA (Deitch *et al.*, 1982) (Fig. 2). G1 and G2 + M stages of the cell cycle were recognized by the fluorescence intensity; the peak due to G2 + M cells showed twice the intensity of the G1 peak. Each experiment included haploid and diploid control cells taken from refrigerated stocks. The diploid cells always showed twice the fluorescence intensity of the haploid cells.

Cells at stationary phase, taken from refrigerated stocks, were stained with PI and examined with the cell sorter. One peak of fluorescence corresponding to G1 phase was observed, and the scattered light intensity pattern indicated small cells. Microscopic examination showed that only 6% of the cells were budded. The cells from the refrigerated stocks were transferred to YNB medium containing 0 or 20 μ g TFP, grown at 23 °C for 10 h, then counted, stained with PI and examined with the cell sorter. After 10 h of growth without TFP, the number of cells increased from 4×10^6 to 1.5×10^7 cells ml^{-1} , 55% of the cells were budded and the fluorescence intensity analysis indicated distribution between G1, G2 + M, and S stages typical of an exponential phase culture (Fig. 2a). In cultures incubated with TFP (20 μ M) there was no increase in cell number and the percentage of budded cells remained low (6%). The pattern of scattered light intensity, which was similar to that in stationary phase cells, indicated that cell growth was inhibited. The cells remained in the G1 phase as determined by the fluorescence intensity (Fig. 2b).

The reversibility of the TFP effect was examined by washing the cells after 10 h of incubation with TFP and incubation for a further 4 h without TFP at 30 °C. During this incubation, the cell number increased from 4×10^6 to 1×10^7 cells ml^{-1} and the fluorescence intensity pattern was typical of exponential phase cultures. In the control, cell number increased from 1.5×10^7 to 5×10^7 cells ml^{-1} . This shows that the effect of TFP at low concentrations was fully reversed.

The effect of time of addition of TFP on DNA synthesis was examined by using synchronized cultures. TFP was added at the time of release from α -factor arrest. After 150 min incubation at

Table 1. *Effect of TFP on α -factor synchronized cells*

The number of cells at the time of the release from arrest was $6.1 \times 10^6 \text{ ml}^{-1}$. Cells were incubated in YNB medium at 30 °C. TFP was added at different time intervals, and the cells were fixed for cell count and flow cytometry 150 min after release from arrest.

Time of addition of TFP (after release from α -factor arrest) (min)	No. of cells ml^{-1}	Percentage of cells in:		
		G1	S	G2 + M
No addition	1.1×10^7	74	8	18
0	6.1×10^6	100	—	—
30	6.2×10^6	7	2	91
60	6.4×10^6	8	3	89

Table 2. *Effect of TFP on cdc mutants*

TFP was added at the time of transition from the restrictive to the permissive temperature. Measurements were done after 4 h incubation at 23 °C. The experiments were initiated at cell densities of $6 \times 10^6 \pm 0.3 \times 10^6 \text{ ml}^{-1}$.

Mutant	TFP (20 μM)	No. of cells ml^{-1}	Percentage of cells:			
			Budded	in G1	in S	in G1 + M
<i>cdc28</i>	—	9.5×10^6	56	38	12	50
	+	5.9×10^6	—	100	—	—
<i>cdc4</i>	—	1.1×10^7	57	35	11	54
	+	5.8×10^6	82	12	—	88
<i>cdc7</i>	—	1.0×10^7	56	32	11	57
	+	6.2×10^6	84	10	4	86
<i>cdc21</i>	—	9.5×10^7	63	30	9	61
	+	6.3×10^6	79	14	3	83
<i>cdc2</i>	—	1.1×10^7	71	26	9	65
	+	9×10^6	29	66	3	31
<i>cdc9</i>	—	1.2×10^7	68	28	10	62
	+	9×10^6	31	64	2	34
<i>cdc10</i>	—	1.2×10^7	70	27	9	64
	+	1.0×10^7	22	72	4	24

30 °C cells were counted, stained with PI and examined by the cell sorter. Cell number remained unchanged ($6 \times 10^6 \text{ cells ml}^{-1}$) and the histogram of fluorescence intensity indicated that these cells were in the G1 phase. In the control, cell number increased to $1.1 \times 10^7 \text{ cells ml}^{-1}$. When TFP was added 30 or 60 min after release from arrest, cell division was inhibited and the cells were arrested with G2 + M nuclei (Table 1). Microscopic examination revealed one nucleus per cell.

Next, we attempted to determine the point in the cell cycle after which addition of TFP would not inhibit subsequent nuclear and cellular division. Different *cdc* mutants growing exponentially in YNB medium at 23 °C were shifted to the restrictive temperature (36 °C) for 90 min; TFP (20 μM) was then added and the cultures were shifted-down to the permissive temperature (23 °C) for an additional 4 h. Cell division was determined by cell count and by the ConA-FITC pulse-labelling method, and budded cells were determined by microscopic examination. Cells were stained with PI and examined by flow cytometry to determine the percentages of cell populations at G1, S and G2 + M. The rationale of the experiment was that when the stage of commitment for the TFP effect was executed during incubation at the restrictive temperature without TFP, first cell cycle division would not be inhibited by subsequent incubation with TFP at permissive temperature. In all *cdc* mutants inhibition at 36 °C was not completely reversed by shift-down to the permissive temperature. Therefore, results were always compared with controls incubated without TFP. In *cdc28*-bearing cells, which arrest before spindle pole body duplication, addition of TFP at the permissive temperature caused inhibition of cell division and the cells remained with G1 nuclei. The relative intensity of scattered light was similar to that measured in arrested cells (not shown).

This indicates that cell growth was inhibited. The control culture showed continuing cell division and typical exponential-phase distribution. In strains bearing the mutations *cdc4* (which arrest at the restrictive temperature before spindle pole body separation), *cdc7* (which arrest at the initiation of DNA synthesis) and *cdc21* (which arrest during DNA synthesis), first cycle division was inhibited and most of the cells were arrested by TFP with buds and a single G2 + M nucleus. In strains with mutations *cdc2* and *cdc9* (which arrest at medial nuclear division) and *cdc10* (which arrest after nuclear division and before cytokinesis), first cycle division was not inhibited but high percentages of the cells were found at G1, indicating arrest at 'Start' of the second cycle (Table 2). The results indicate that the second stage of arrest by TFP is late in the cell cycle, just before the stage arrested by the *cdc9* and *cdc2* mutations.

DISCUSSION

We found that a low concentration of TFP caused a reversible inhibition of the cell cycle in *S. cerevisiae*. The stage of inhibition was dependent on the time at which TFP was added. When TFP was added before Start on release from α -factor arrest, at transition from stationary stage to vegetative growth or after release of *cdc28*-arrested cells, the cells had no buds, contained G1 nuclei, and cell growth and DNA synthesis were inhibited. When TFP was added later, growth and DNA synthesis were not inhibited, and cells arrested with buds and single G2 + M nuclei. The first three methods of synchronization are not identical. Stationary phase cells are arrested by starvation and sometimes are considered to be in the G₀ stage. They are smaller in size, not growing and require a period of growth before proceeding through Start. Cells arrested by α -factor or by *cdc28* are larger than the minimal size and are therefore ready to proceed through Start immediately upon release from arrest. Although the methods of synchronization used are known to disrupt the normal physiological balance of the cells, and therefore artifacts may be introduced, the fact that three different methods yielded similar results indicates that the probability of obtaining an artifact is low.

An interesting observation was that cells treated with TFP immediately after release from arrest by starvation, α -factor or *cdc28* showed no increase in the intensity of scattered light. This indicates inhibition of cell growth. When TFP was added 30 min later, an increase in the scattered light intensity was observed indicating that growth had occurred. Thus TFP exerted a cell-cycle-specific inhibitory effect on cell growth manifested only before, or at Start. The inhibition of bud formation may be secondary to growth inhibition. Cell-cycle-specific inhibition of growth has not been reported before.

The second stage of inhibition by TFP is at nuclear division. The execution of this stage is late in the cell cycle, just before medial nuclear division (stage arrested by *cdc9*). The phenotype of the cells arrested at nuclear division by a low concentration of TFP is very similar to that in a Ca²⁺-dependent mutant of *S. cerevisiae* (*cal1-1*) grown in low Ca²⁺ medium (Ohya, 1984) and in wild-type *S. cerevisiae* cells grown in the presence of methylbenzimidazole-2-yl-carbamate (MBC), a chemical that disrupts microtubule functions (Quinlan *et al.*, 1980). In these experiments, cells arrested with buds and a single G2 + M nucleus. The similarity in the stage of arrest may suggest that the inhibition exerted by TFP and by low Ca²⁺ in *cal1-1* may be connected with disruption of the normal function of the microtubules.

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