Rise in Intracellular pH is Concurrent with 'Start' Progression of Saccharomyces cerevisiae

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Intracellular pH (pHi) was determined during arrest and recovery of temperature sensitive-cell division cycle mutants of Saccharomyces cerevisiae. In all mutants, pHi decreased during arrest; but when the mutants were released from arrest a rapid increase in pHi ensued in only cdc28- and cdc37-arrested cells. Both of these mutations cause arrest at 'start', the sole regulatory point in the S. cerevisiae cell cycle. In cells with cdc4 or cdc7 mutations, which arrest past start, pHi remained constant and exhibited a decrease, respectively, upon recovery of growth. The activity of plasma membrane ATPase decreased during the first 30 min of recovery of cdc28-arrested cells, concomitant with the rise in pHi. During the same period, there was no significant change in activity in cdc4-bearing cells, whereas an increase was observed for cdc7-bearing cells. Increase in pHi may be used as a specific signal by S. cerevisiae for start traversal and commitment to a new cycle.

INTRODUCTION

The cell cycle of the budding yeast Saccharomyces cerevisiae is pleiotropically regulated in G1 at a point termed 'start' (Hartwell, 1974; Yanishevsky & Stein, 1981). This occurs in late G1 and is immediately followed by two divergent subcycles, one involving nuclear events of DNA synthesis and nuclear division, the other consisting of cytoplasmic events governing bud emergence and growth (Hartwell et al., 1974). Although much is known with regard to morphological and genetical aspects of start, (Hartwell et al., 1973; Reed, 1980; Bedard et al., 1981; Pringle et al., 1981) the underlying biochemistry is not so well defined. The gene product of CDC28 is a protein kinase (Reed et al., 1985b; Medenhall et al., 1987). The gene product of CDC7, a G1 gene whose sequential expression after CDC28 is essential for the initiation of DNA synthesis, is also a kinase (Patterson et al., 1986).

Recent reports indicate that a transient alkalinization of intracellular pH (pHi) precedes DNA synthesis when quiescent animal cells are allowed to proliferate in response to mitogens and growth factors (Roos & Boron, 1981; Schuldiner & Rozengurt, 1982; Bravo & Bravo, 1986; Hagag et al., 1987; Kakinuma et al., 1987; Ober & Pardee, 1987; Rotstein et al., 1987). In addition, increase in pHi is associated with fertilization of sea urchin eggs (Steinhardt & Mazia, 1973; Lopo & Vacquier, 1977), development of Dictyostelium and dimorphic transition of Candida albicans (Aerts et al., 1987; Stewart et al., 1988). In most cases control of pHi is accomplished, at least in part, by Na+/H+ antiport (Schuldiner & Rozengurt, 1982; Doppler et al., 1986; Hagag et al., 1987; Kakinuma et al., 1987; Ober & Pardee, 1987; Rotstein et al., 1987).

We have been studying the biochemical events associated with G1 to S transition in S. cerevisiae using temperature-sensitive cdc mutants (Dudani & Prasad, 1983, 1984a, b; Dudani et al., 1983; Anand & Prasad, 1987). The present study was undertaken in view of the importance of pHi in growth and differentiation of various cells.

Abbreviations: pHi, external pH; pHi, intracellular pH.

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METHODS

Strains and growth conditions. Various cdc mutants, H. 185. 3. 4 (cdc28-l), H. 135. 1. 1 (cdc4-3), 4008 (cdc7-4), SR. 661. 2 (cdc36-l6), SR665. 1 (cdc39-l), SR672 (cdc37-l), and the wild-type strain (A364A) were obtained from Yeast Genetic Stock Center, Berkeley, California, USA. The growth conditions and maintenance of all these strains have been described (Hartwell et al., 1973; Hereford & Hartwell, 1974; Reed, 1980). Arrest conditions were as described by Anand & Prasad (1987). All the strains were grown in YE PD medium at 23 °C. The mutants were synchronized by shifting them to their respective non-permissive temperatures: 36 °C for cdc36 and cdc4; 37 °C for cdc37 and cdc39; 38 °C for cdc28 and cdc7 (Hartwell et al., 1973; Reed, 1980). Cell synchrony was established microscopically by counting at least 200 cells and looking for terminal phenotype of individual cells and by following DNA synthesis (Hartwell, 1970). Cells were collected at indicated times, harvested and washed twice with distilled water at 1500 g. For pH measurements, 10% (w/v) cell suspensions were prepared in 100 mM-Tris/citrate buffer (pH 5.4).

Measurement of pHi. pH values were calculated by determining the steady state distribution of propionic acid across the plasma membrane (Cartwright et al., 1986). Assay conditions were similar to those described by Prasad & Hofer (1987). A typical reaction mixture containing 100 mM-Tris/citrate buffer (pH 5.4) and cells (10-15 mg dry wt ml⁻¹) was incubated at 30 °C. The distribution of propionic acid was initiated by the addition of 10 μM-sodium [2-¹⁴C]propionate (1.85 kBq ml⁻¹). In pilot experiments with wild-type cells (A364A) it was established that 3-4 min were required for propionic acid to equilibrate across the plasma membrane. Triplicate samples (0.3 ml) were removed after 4 min, rapidly filtered through Maxflow filter discs (0.45 μm), and washed four times with 1 ml ice-cold unlabelled 10 μM-propionic acid. Radioactivity retained was counted in a Beckman LS 1801 Beta liquid scintillation counter. pH, values were calculated as described by Prasad & Hofer (1987).

Preparation of crude plasma membrane fraction. The method was similar to that described by Tuduri et al. (1985). Washed cells (450 mg wet wt) were suspended in 1 ml grinding medium containing 250 mM-sucrose, 10 mM-Tris/HCl, pH 7.5, and 1 mM-phenylmethylsulphonyl fluoride (PMSF). To 900 μl of this suspension, 900 mg of glass beads (0.45 mm diameter) were added and the mixture was agitated nine times for 17 s with intervals of 3 s using a CO₂-refrigerated MSK Braun homogenizer with a micro adapter. The homogenate was centrifuged for 5 min at 1000 g and the pellet was rinsed by centrifugation at 1500 g for 5 min with 500 μl grinding medium. The combined supernatants were centrifuged again for 5 min at 1000 g. The resulting supernatant was centrifuged for 40 min at 15000 g. The pellet was suspended in 300 μl of 250 mM-sucrose, 10 mM-Tris/HCl, pH 7.5. This final suspension was called the 'crude membrane fraction'.

ATPase assay. 'Crude membrane fraction' (20 μl) was incubated for 10 min at 30 °C in 450 μl of 9 mM-MgCl₂, 50 mM-MES/KOH, pH 6/0, 10 mM-Na₂SO₄, After preincubation the reaction was initiated by the addition of 5 μM-ATP (vanadate free). After 8 min the reaction was stopped by the addition of 2 ml 2% (w/v) H₂SO₄, 0.5% ammonium molybdate and 0.5% SDS. The phosphomolybdate was reduced with 20 μl 10% (w/v) ascorbic acid and absorbance at 750 nm was read after 10 min. Protein was estimated according to the method of Bradford (1976).

RESULTS AND DISCUSSION

Synchronization and recovery of cells

The cdc mutants were arrested by shifting exponentially growing cultures to their respective non-permissive temperature. G1 arrest of these mutants was established by counting the cells of the respective terminal phenotype (Hartwell et al., 1973; Bedard et al., 1981). In all cases 90-95% synchrony was obtained after 7-7.5 h; this was confirmed by the incorporation of [³H]uracil (Hartwell, 1970). On return to the permissive temperature arrested cells are able to enter the S phase and subsequently complete a round of DNA synthesis without further protein synthesis (Hereford & Hartwell, 1974).

\( pHi \)

The pH of the assay medium for all the cells was 5.4, for two reasons. Firstly, the external pH (pHₐ) for maximum growth varied between 5.2 and 5.5. Secondly, the values from the steady state distribution of propionic acid demonstrated that pHi, was maintained fairly constant between pHₐ 3.5 and 5.5 (Fig. 1). However, it increased sharply with further increase in pH. It was evident that the cells maintained a constant pHi at or about pHₐ 5.4, which was also the pH of maximum growth rate (mid-exponential phase). There was not much change in pH during arrest and recovery of the different cells (data not shown).
Fig. 1. pH of A364A cells suspended in 100 mM-Tris/citrate buffer of the indicated pH, value. The conditions were as described in Methods. All data are means of three to four sets; bars represent SEM.

Fig. 2. pH during arrest and recovery of cdc mutants. The bars on each point represent SEM values.

Fig. 2 depicts the change in pH, during the arrest and recovery of the three G1 mutants bearing cdc28, cdc4 and cdc7 mutations. During the 7-7.5 h taken for arrest, there was a gradual decrease in pH, in all the cells. However, on recovery (shifting of cells to permissive temperature), the pH, pattern among various mutants varied greatly. In the cdc28-bearing mutant, there was a rapid increase in pH, within the first 30 min. This increase of 0.21 units was followed by a sharp decline up to 60 min, after which the pH, continued to rise again. There was no significant change in pH, in cells with the cdc4 mutation during the initial period of recovery, whereas cells carrying a cdc7 mutation exhibited an initial decrease in pH, on return to permissive temperature, followed by an increase around 90 min.

The pH, was also determined by exposure of unbuffered cell suspensions (2.5%, w/v) to nystatin (10 µg ml⁻¹) (Hauer et al., 1981). Nystatin interacts with sterols of the plasma membrane to produce unspecific pores, which permit equilibration of protons across the membrane. Since it does not interact with the subcellular organelle membranes the change in proton concentration of the unbuffered suspension gives values close to true pH, (Hauer et al., 1981). By this method the pattern and magnitude of changes in pH, observed were similar to those derived from propionate distribution, but the absolute values were slightly higher (data not shown). Although the pH, values reported here are lower than those reported by Gillies et al. (1981) using 31P NMR, they match with those of Cartwright et al. (1986), who also used the propionate distribution method. To rule out the possibility of a temperature effect on observed
pHi, it was measured in wild-type cells (A364A) subjected to a similar temperature shift-up and shift-down regimen (Fig. 3). Although a decrease in pHi similar to that of mutants occurred during the 7 h incubation at non-permissive temperature (36, 37 or 38 °C), on shift down to 23 °C, pHi was almost constant over a 120 min period. Therefore, the fluctuations in pHi observed in the cell cycle mutants are specific to their arrest and recovery.

There is evidence that controls on initiation of a new cell cycle take place in steps that precede and include the CDC28-mediated step, and that passing start leads to commitment to the cell cycle (Hartwell, 1974; Yanishevsky & Stein, 1981). Moreover, CDC4 and CDC7 are expressed sequentially after CDC28, but prior to DNA synthesis (Hereford & Hartwell, 1974). Therefore, our observation that there is an elevation in pHi specifically when start-arrested cells are allowed to re-enter cell cycling suggests that, like animal cells, S. cerevisiae cells can also use pHi as a signal or regulatory factor for entering a new cell cycle. Gillies et al. (1981), using glucose-deprived synchronous cultures of S. cerevisiae, have also suggested that alkalinization of pHi may accompany traversal of start. Although, because of low values of pHi observed by us in comparison to 31P NMR measurements, the rise in pHi at start does not appear to be alkaline, nonetheless if one compares the change in pHi with the progression of different mutants (observed by two independent methods), there is a definite pattern of increase in pHi which is concurrent with start.

In order to confirm whether the observed increase in pHi is start specific, three other strains, carrying cdc36, cdc37 and cdc39 mutations, were also studied. These mutants arrest with terminal phenotypes similar to the strain bearing cdc28, but they carry different mutations. Based on their conjugational efficiency and degree of viability at the restrictive temperature, Reed (1980) has suggested that the physiological state of cdc28- and cdc37-arrested cells is different from that of cdc36- and cdc39-arrested cells. Genetic evidence also suggests that functions of the CDC28 and CDC37 gene products may be related (Reed et al., 1985a). The observed pHi pattern strengthens these findings (Fig. 4). In SR672 (cdc37), as with H. 185. 3. 4 (cdc28), there was a rapid increase in pHi within 30 min of recovery from arrest. In the cdc36-bearing strain there was almost no change and strain SR665.1 (cdc39) exhibited a decrease in pHi on release from arrest, followed by a gradual rise.

**ATPase activity**

In animal cells, an increase in pHi is accompanied by the activation of Na+/H+ antiport (Roos & Boron, 1981; Schuldiner & Rozengurt, 1982; Doppler et al., 1986; Hagag et al., 1987; Kakinuma et al., 1987; Ober & Pardee, 1987; Rotstein et al., 1987). The movement of protons across the plasma membrane of yeast cells is controlled by a H+-translocating ATPase which
Intracellular pH and 'start' progression

Fig. 4. pHᵢ during arrest and recovery in 'start' mutants. The bars on each point represent SEM values.

Fig. 5. Plasma membrane ATPase activity during arrest and recovery of cdc mutants. ATPase was assayed as described in Methods. The graph in each panel is representative of a typical experiment.

expels protons electrogenically (Goffeau & Slayman, 1981; Goffeau & Boutry, 1986; Serrano, 1988). In order to correlate the observed changes in pHᵢ, ATPase was assayed in crude membrane fractions isolated at the same time as for pHᵢ measurements (Fig. 5). The ATPase activity decreased as cdc28-carrying cells were allowed to recover. This coincided with the rapid increase in pHᵢ during that time. The activity increased during the next 30 min, with a simultaneous decline in pHᵢ. Similar to the pattern of pHᵢ values. ATPase activity in cdc4-bearing cells also exhibited no significant changes during recovery from arrest. However, in cdc7-bearing mutants ATPase activity increased at the time of shift to permissive temperature until 90 min, which was concomitant with a decrease in pHᵢ. The activity fell to a normal level during the next 30 min, with a simultaneous rise in pHᵢ (Fig. 5).

Since the changes in ATPase activity are specific to arrest at particular stages of the cell cycle, the temperature effect is ruled out. These changes correlate inversely with pHᵢ: whenever there is an increase in pHᵢ, ATPase activity goes down, and vice versa. This suggests that the plasma membrane ATPase activity, or in other words the rate of H⁺ efflux, may be self-controlled by an internal H⁺ regulatory site. A similar situation exists for the Na⁺/K⁺ ATPase in animal cells, which reportedly shuts off when the pHᵢ rises above a critical value and leads to the activation of Na⁺/H⁺ antiport (Pouyssegur, 1985). Such an antiport is not known in yeasts; however the possibility of a K⁺/H⁺ antiport exists, since flux of potassium has been associated as a counter-ion for ATPase-linked proton efflux (Eilam et al., 1984; Blatt & Slayman, 1987; Glaser & Hofer, 1987). It is possible that S. cerevisiae has a functional K⁺/H⁺ antiport for rapid and fine-tuning of intracellular pH.
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