Magnesium and the Regulation of Germ-tube Formation in *Candida albicans*

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Candida albicans requires Mg²⁺ for germ-tube formation. Mg-deficient media, metal ion chelators and the ionophore A23187 inhibited germ-tube formation. Cell Mg content during exponential yeast-phase growth remained constant but increased throughout germ-tube formation. The onset of germ-tube formation coincided with a sharp peak in Mg concentration within the cells. Yeast-phase cells of strain CA2, which did not form germ-tubes, had a lower Mg content and failed to accumulate Mg when incubated under conditions for germ-tube formation. Mg also increased the uptake and incorporation of *N*-acetylglucosamine. These findings point to a central regulatory role for Mg in *C. albicans* morphogenesis.

INTRODUCTION

Candida albicans, the causative agent of mycotic infections collectively known as candidosis, is a convenient organism for studying the regulation of differentiation. It is dimorphic and grows either by forming buds (as in most laboratory media) or by elongating as hyphae (Odds, 1979). The induction of germ-tube formation in *C. albicans* is the initial stage in the yeast-to-mycelial transition (Cassone *et al.*, 1973), and a number of environmental factors have been described which effect this transition (Odds, 1979). Previous studies have pointed indirectly to the involvement of magnesium ions in *C. albicans* morphogenesis (Widra, 1964; Simonetti *et al.*, 1974; Gooday, 1978), but this study represents the first attempt to identify a regulatory role for magnesium in the initiation of germ-tube formation in this organism.

METHODS

Organisms and culture conditions. The strains of *C. albicans* used in this study were ATCC 10261, A72 and CA2. Strains A72 and CA2 were provided by Dr A. Cassone, Istituto Superiore di Sanito, Rome, Italy. A72 has been designated a 'high-responder' with respect to germ-tube formation and CA2 a non-germ-tube former (Mattia *et al.*, 1982). Conditions for propagation of the organism have been described previously (Shepherd & Sullivan, 1976). Cell numbers (the mean of 4 counts of ~100 cells) were determined using a haemocytometer. Yeast cells were scored as two when the daughter bud had reached two-thirds the size of the mother cell.

Germ-tube induction. The pH shift method of Lee *et al.* (1975) and the *N*-acetylglucosamine (GlcNAc) induction system of Simonetti *et al.* (1974) were used to induce formation of germ-tubes in *C. albicans*. Both methods require a temperature of 37 °C. With strain A72, the starvation/aeration pretreatment used by Shepherd *et al.* (1980) was not deemed necessary to produce a high proportion of germ-tubes in the GlcNAc induction system. The percentage of germinating cells was routinely assessed using a haemocytometer.

Incorporation of *N*-acetylglucosamine. *N*-Acetyl-D-[1-¹⁴C]glucosamine [0.05 µCi (1.85 kBq); 0.2 µmol; Amersham] was added (50 µl) to yeast cultures (50 ml) and duplicate 1 ml samples were removed periodically. Each sample was washed on a Whatman GF/C glass-fibre filter three times with distilled water and twice with ethanol.

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Abbreviation: GlcNAc, *N*-acetylglucosamine.

The filters were placed in vials containing Triton/toluene scintillant and radioactivity was determined in an LKB 1215 Rackbeta II liquid scintillation counter.

Magnesium analysis. Cell magnesium content was determined by flameless atomic absorption spectrophotometry (Walker & Duffus, 1980) after microcentrifuging cells through silicon oil of appropriate specific gravity (Walker & Zeuthen, 1980).

Chemicals. Concentrated stock solutions of EDTA, EGTA and sodium pyrophosphate were prepared by dissolving the chemicals in water and adjusting the pH to 6.6. Concentrated stock solutions of the ionophore A23187 (from Calbiochem) were prepared by dissolving it in acetone and ethanol. Appropriate quantities of these stock reagents were added directly to cultures to give the final desired concentration. Mg, Mn, Ca and Zn were added directly as their chloride salts to give the final desired concentrations. All solutions were prepared in water which had been distilled and further purified by passage through a Milli-Q system (Millipore).

RESULTS AND DISCUSSION

If stationary-phase cells of *C. albicans* were inoculated in an amino acid-rich medium at pH 7.3 and at 37 °C (Lee *et al.*, 1975), they synchronously formed germ-tubes (see also Mitchell & Soll, 1979). However, such cells failed to germinate if they were inoculated into Mg-free media (data not shown). Similarly, cells failed to germinate in the GlcNAc induction system made deficient in Mg^{2+} (Fig. 1). The buffer system normally uses Mn^{2+} (Simonetti *et al.*, 1974), but we consistently found that if Mn^{2+} was replaced by Mg^{2+} , a significantly higher percentage of germ-tubes was obtained (Fig. 1). We also found that the proportion of germ-tubes obtained in Mn-containing buffer was dependent on the concentration of contaminating Mg^{2+} (data not shown). Ca^{2+} and Zn^{2+} failed to substitute for Mg^{2+} in producing a high percentage of germ tubes (Fig. 1).

The dependence of germ-tube formation on Mg^{2+} concentration was confirmed (Fig. 2) by inhibiting germination with agents known to affect Mg^{2+} availability, such as the chelators EDTA and sodium pyrophosphate and the ionophore A23187. EGTA, a chelator with a greater affinity for Ca^{2+} than Mg^{2+} , failed to produce the same inhibiting effects as Mg^{2+} chelators.

Figure 3 shows the concentration of cell-Mg in cultures of *C. albicans* 10261 which were induced to grow either by yeast-phase budding or by germ-tube elongation. The different modes of development were established by manipulating the pH of the medium (Lee *et al.*, 1975; Mitchell & Soll, 1979). The cell Mg content remained constant during exponential growth of yeast-phase cultures (pH 4.5), whereas during germ-tube formation the Mg content per cell doubled over the 4 h incubation period. If Mg values were to be expressed on a dry weight basis, cell Mg would remain relatively constant since the dry weight of *C. albicans* mass increases by 100% over this period (Shepherd *et al.*, 1980). However, the gradual increase was interrupted by a peak of Mg content per cell which coincided with the onset of germ-tube formation. This peak represents a three-fold increase indicating a transient, but net, increase in cell Mg at this time. We have observed similar qualitative changes in cell Mg (including a Mg peak) in cultures induced for germ-tube formation in the GlcNAc system (data not shown). This Mg influx may represent a controlling element in yeast-to-mycelial transition in *C. albicans*, perhaps by activating enzymes for chitin synthesis or by governing chitin deposition. Both processes are required for mycelial cell wall extension. With regard to chitin synthesis, it is of interest that the specific activity of chitin synthase increases three-fold during the initial period of germ-tube formation (Chiew *et al.*, 1980) and this enzyme is Mg-dependent. Gooday (1978) has previously suggested that Mg may play an *in vivo* role in regulating this key enzyme in *C. albicans*. With regard to chitin deposition, it is likely that cytoplasmic microtubules are involved in this process since these organelles are known to govern cell wall extension in fungi (Howard & Aist, 1980). Walker & Duffus (1983) have recently proposed that Mg controls the integrity of microtubules *in vivo*; Mg may, therefore, affect *C. albicans* morphology at the level of the cytoskeleton.

Strains A72 and CA2 are distinguished by their differing capabilities to form germ-tubes: A72 has been designated a 'high-responder' and CA2 a non-germ-tube former (Mattia *et al.*, 1982). In an effort to determine if these strains also differed in their cell Mg status and in their ability to take up Mg^{2+} , both strains were incubated at 37 °C in buffered GlcNAc and the percentage germ-tubes and cell Mg were monitored over 4 h. With strain CA2 the Mg content remained

Fig. 1

Fig. 2

Fig. 1. Influence of divalent metal cations on germ-tube formation in *C. albicans* A72. Cells were propagated for 18 h in a glucose/salts/biotin medium (Mitchell & Soll, 1979), harvested, washed and resuspended to 10^8 cells ml^{-1} in glass-distilled water. Samples of these suspensions were added to buffered GlcNAc (Shepherd *et al.*, 1980), but minus the Mn source, and incubated with shaking at 37°C . Appropriate divalent metal cations were added to the buffer system to give final concentrations of 0.5 mM . ■, Control (no ions added); ●, Mg; ○, Mn; △, Ca; □, Zn.

Fig. 2. Inhibition of germ-tube formation in *C. albicans* A72 by chelating agents and the ionophore A23187. Cells were prepared as for Fig. 1 and incubated in buffered GlcNAc plus 0.5 mM-Mg^{2+} with chelating agents at final concentrations of 40 mM . The final concentration of the ionophore A23187 was $10\text{ }\mu\text{g ml}^{-1}$. ●, Control (no additions); ○, EGTA; ■, sodium pyrophosphate; □, EDTA; ▲, A23187.

constant (16 ± 1.5 fg per cell) whereas with strain A72 the initial Mg content was 36 fg per cell which increased steadily (with no detectable Mg peak) to 80 fg per cell during germ-tube formation. These findings clearly indicate that *C. albicans* mutants which are defective in germ-tube formation also possess altered capacities to accumulate Mg^{2+} .

Widra (1964) has previously shown that yeast or mycelial forms of *C. albicans* can be induced by manipulating the phosphate supply in the growth medium. This was interpreted by Widra (1964) on the basis of the binding of Mg^{2+} by polyphosphate within the cell. Thus, in high phosphate conditions, excess polyphosphate forms to bind high levels of Mg^{2+} which results in production of mycelial forms. Conversely, in low phosphate conditions, Mg^{2+} remains unbound and this results in production of yeast forms. In the present work, we can only speculate whether changes in free Mg^{2+} govern yeast-to-mycelial transitions because our measurements of total cell Mg give no indication of the proportion of free and bound Mg within the cell. Consequently, we are unable to support or refute the postulate put forward by Widra (1964).

Mg enhanced the incorporation of [^{14}C]GlcNAc into yeast cells incubated at 28°C for 2 h (Fig. 4). When the Mg-supplemented and Mg-deprived cultures were transferred to 37°C , GlcNAc uptake continued initially at the same rates, but only the former produced germ-tubes. The rates of incorporation decreased with continued incubation because the exogenous GlcNAc became depleted (Sullivan *et al.*, 1983). The ionophore A23187 immediately inhibited germ-tube formation and GlcNAc incorporation in the Mg^{2+} -supplemented culture (Fig. 4). In further experiments in which Mg-deprived cells were induced for GlcNAc uptake at 28°C (2 h), as described in Fig. 4, the addition of Mg^{2+} after the transfer to 37°C increased the rate of GlcNAc uptake and triggered germ-tube formation. Direct measurement of GlcNAc uptake rates (Sullivan & Shepherd, 1982) after 4 h incubation at 28°C gave values of 40 and 60 $\text{nmol min}^{-1} (\text{mg dry wt})^{-1}$ for the Mg-deprived and Mg-supplemented cells, respectively. In support of the role proposed above for Mg^{2+} in regulating chitin synthesis and deposition, these data demonstrate a direct link between the incorporation of *N*-acetylglucosamine (which activates chitin synthase) and Mg^{2+} availability.

In conclusion, we propose that Mg^{2+} plays a central role in regulating dimorphism in *C. albicans* through a combination of its effects on enzyme activation and cytoskeletal organization.

Fig. 3

Fig. 4

Fig. 3. Changes in cell magnesium content during yeast growth and germ-tube formation of *C. albicans* 10261. Cells were cultivated in an amino acid-rich medium (Lee *et al.*, 1975) and induced to grow either by yeast-phase budding at pH 4.5 (open symbols) or by forming germ-tubes at pH 7.3 (filled symbols).

Fig. 4. Influence of magnesium on germ-tube formation and GlcNAc incorporation. Yeast cells of *C. albicans* A72 were prepared as for Fig. 1 and resuspended in buffered GlcNAc containing *N*-acetyl-D-[1-¹⁴C]glucosamine with or without Mg²⁺. After 2 h incubation at 28 °C, the cultures were shifted to 37 °C. In one experiment the ionophore A23187 (10 µg ml⁻¹) was added to the Mg-supplemented culture: ■, ●, with Mg; □, ○, without Mg; △, with the ionophore A23187.

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