Internalization of lucifer yellow in *Candida albicans* by fluid phase endocytosis

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Lucifer yellow (LY), an impermeable fluorescent dye used as a marker for fluid phase endocytosis, was internalized by *Candida albicans*. As observed by fluorescence microscopy, incubation of *C. albicans* with LY in potassium phosphate buffer (pH 6.0) and glucose (2%, w/v) resulted in localization of the dye inside vacuoles. Sodium azide and carbonyl cyanide *m*-chlorophenylhydrazine, which are inhibitors of energy metabolism, decreased the uptake of the dye. The optimum temperature for uptake was 30 °C; no internalization was observed at 0 °C. Quantification of cell-associated LY by fluorescence spectrometry showed an uptake linear with time and not saturable over a 400-fold range of concentration. Thus, *C. albicans* internalized LY into vacuoles by a non-saturable and time-, temperature- and energy-dependent process consistent with fluid phase endocytosis. Both the yeast and mould phase of this dimorphic fungus endocytosed LY. Growth in complex medium appeared to be required to enable the cells to internalize LY. However, addition of peptone or yeast extract to the phosphate buffer/glucose assay medium interfered with LY uptake by causing an apparent increase of exocytosis. These studies provide the first evidence of fluid phase endocytosis in *C. albicans* and may explain how some large molecules, such as toxins and cationic proteins, enter *C. albicans*.

Introduction

Cells internalize their own plasma membrane and macromolecules from the environment by a mechanism termed endocytosis (reviewed by Silverstein *et al.*, 1977; Steinman *et al.*, 1983). It is now generally accepted that endocytosis brings about the bidirectional flow of plasma membrane into and out of the cell. There are a wide array of substances that can be internalized by this process such as nutrients, toxins and effector molecules (growth factors, hormones, antibodies and enzymes). Two general types of endocytosis are recognized, namely, fluid phase (Silverstein *et al.*, 1977) and receptor mediated endocytosis (Goldstein *et al.*, 1979). Both processes are time-, temperature- and energy-dependent. In fluid phase endocytosis, molecules in the extracellular medium are incorporated by bulk transport into endocytic vesicles and uptake of the ligand is not saturable. However, in receptor mediated endocytosis the ligand to be internalized binds to the cell membrane before it is

incorporated into endocytic vesicles and uptake of the ligand is saturable.

Recent studies show that *Saccharomyces cerevisiae* can endocytose mating pheromones and lucifer yellow (LY) from the culture medium (Riezman, 1985). These results raised the possibility that other yeasts may also possess this type of uptake mechanism; our interest in peptide uptake and drug design in *Candida albicans* prompted us to investigate whether a similar process is operative in this dimorphic fungus, an important opportunistic pathogen (reviewed by Odds, 1988).

On the basis of studies determining the porosity of *S. cerevisiae* and *C. albicans* cell walls, endocytosis would not be expected. The porosity of the yeast cell wall has been studied using polyethylene glycols of different molecular sizes (Scherrer *et al.*, 1974; Cope, 1980) and the threshold of permeability was estimated to correspond to an M_r of 620 and an Einstein-Stokes hydrodynamic radius of 0.81 nm. However, the results of cell wall porosity contrast with the killing effects of several proteins much larger than the size limit proposed. The best studied examples are defensins (M_r 3500–4000), which are variably cationic, cysteine-rich antimicrobial

Abbreviations: ASM, amino acid synthetic medium; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; LY, lucifer yellow.

peptides (29–34 residues in length) and are major constituents of azurophil granules of polymorphonuclear neutrophils (Lehrer *et al.*, 1985). They are active *in vitro* against several micro-organisms, including certain fungi (Selsted *et al.*, 1985). The susceptibility of *C. albicans* to human defensins depends both on the ionic environment and on the metabolic state of the target cell (Lehrer *et al.*, 1988). The mechanism of action of defensins is not clear, although it has been proposed that they act through membrane perturbation (Selsted *et al.*, 1985). Killer toxins (M_r 15000–25000), secreted into the culture medium from selected killer yeasts, cause lysis of *C. albicans* (Polonelli *et al.*, 1985). Yphanitis *et al.* (1967) also reported that certain cationic proteins such as ribonuclease, protamine, bovine serum albumin, cytochrome *c* and myoglobin cause lysis of *S. cerevisiae* and *C. utilis*. All these studies point to the fact that these proteins may penetrate the cell wall to act directly on the cell by an undescribed mechanism.

In this paper we report on the characteristics of uptake of LY by the yeast and mould phases of *C. albicans* using fluorescence microscopy and fluorescence spectroscopy. LY, an impermeable fluorescent dye, has been used as a marker for fluid phase endocytosis in mammalian cells and *S. cerevisiae* (Swanson *et al.*, 1985; Reizman, 1985). This dye offers several advantages for use as a marker for fluid phase endocytosis (Miller *et al.*, 1983; Stewart, 1978, 1981): a very high quantum yield resulting in easily visualized fluorescence after uptake by cells; small size, allowing passage through the yeast cell wall; stability over a wide range of pH, from 2.0–10.0; negative charge at all pH values above 2.0 and, hence, not diffusable across the cell membrane; not toxic to cells; and fluorescence not quenchable by yeast cell extracts (Reizman, 1985).

Methods

Organism and growth conditions. *Candida albicans* H317, a well-characterized (Riggsby *et al.*, 1982) clinical isolate obtained from the Centers for Disease Control, Atlanta, Georgia, USA, was used in all experiments. The strain was maintained on YEPD agar [1%, w/v, yeast extract, 2%, w/v, bacto-peptone (Difco), 2%, w/v, glucose and 2%, w/v, agar] at 4 °C and transferred monthly.

For all experiments, unless mentioned otherwise, the yeast phase of growth was obtained in YEPD medium. Cells were inoculated at an initial cell density of 10^4 cells ml⁻¹ in 200 ml YEPD broth and incubated at 37 °C on a rotary shaker at 200 r.p.m. An exponentially growing culture at a cell density of $1-2 \times 10^7$ cells ml⁻¹, was used for all experiments. Cells were harvested by centrifugation at 1500 *g* for 5 min, washed once with distilled water and twice with 50 mM-potassium phosphate buffer, pH 6.0, and resuspended in phosphate buffer.

The mould phase of growth was obtained using two procedures, one a temperature shift and the other a nutritional shift. For the temperature shift procedure we used the amino acid synthetic medium (ASM) described by Lee *et al.* (1975) and the procedure of Logan *et al.* (1983).

Briefly, yeast phase cells were first grown to stationary phase in ASM, pH 6.8, at 25 °C. Cultures were stored at 4 °C for 12 h. Yeast phase cultures were obtained by diluting stationary phase cultures into fresh medium at 25 °C. Conversion to the mould phase was initiated by diluting (1:20) the stationary phase culture into fresh medium at 37 °C. The mould phase cultures (>90% conversion) were harvested 12 h after the initiation of conversion. For nutritional shift conversion (Joshi *et al.*, 1973), yeast cells were grown on Sabouraud dextrose agar for 48 h at 37 °C. Cells were scraped from the agar and resuspended in water at 1×10^6 cells ml⁻¹, then inoculated into prewarmed (37 °C) 1% (w/v) bacto-peptone. The mould phase was obtained after 4–5 h at a frequency of 50–60%.

Visualization of cell-associated lucifer yellow. The procedure for the assay was similar to that described previously for *S. cerevisiae* (Reizman, 1985) with a few variations. *C. albicans* was grown overnight as described above. The assay was done with 2×10^7 cells in 50 mM-potassium phosphate buffer, pH 6.0. Glucose or other additions were made to the cell suspension and, after 15 min at 30 °C, lucifer yellow CH (Molecular Probes, Eugene, Oregon, USA) was added to a final concentration of 4 mg ml⁻¹. After the indicated time of incubation cells were diluted with 5 ml of ice-cold buffer A (50 mM-succinic acid/NaOH, pH 5.0, containing 100 mM-NaCl, 10 mM-MgCl₂, 20 mM-Na₂N₃), collected by vacuum filtration on nitrocellulose filters (GN-6, 0.46 µm) washed four times with 2 ml buffer A and once with 2 ml 50 mM-Tris/HCl, pH 7.25. The cells were eluted off the filter with the Tris/HCl buffer and the suspension was kept on ice. For visualization of cell-associated LY, the cells were mounted on a slide precoated with 20 µl 1 mg concanavalin A ml⁻¹, which immobilized the yeast cells. Fluorescence was observed with a Leitz Orthoplan microscope with a xenon mercury lamp equipped with epifluorescence using an excitation filter BP 450–490, a beam splitting mirror TK 495 and a suppression filter K530. Photographs were taken using Ektachrome 200 ASA with exposure of 50–60 s.

Quantification of cell-associated lucifer yellow. For quantification of cell-associated LY, cells were incubated in assay medium containing 2% (w/v) glucose and various concentrations of LY in 50 mM-potassium phosphate buffer, pH 6.0. A portion of the cell suspension was removed at various time points and the cells were washed as described above. Appropriate dilutions of the cells were used to quantify cell-associated LY using a luminescence spectrometer model LS-5B (Perkin Elmer), with excitation at 426 nm and emission at 535 nm (slit widths 10 and 20 nm, respectively); a standard curve was produced using known amounts of LY. Haemocytometer counts of each sample were determined to account for non-uniform elution of cells from the nitrocellulose filters. Each experiment was repeated at least three times with values not differing by more than 10% of each other.

Exocytosis of lucifer yellow. *C. albicans* cells grown in YEPD medium were harvested in the exponential phase, washed, incubated in assay medium containing 2% (w/v) glucose and LY (1 mg ml⁻¹) in 50 mM-potassium phosphate buffer, pH 6.0, at 30 °C for 90 min, collected and washed on filters as described above, and eluted from filters into phosphate buffer. Cells were then re-incubated either in phosphate buffer or in 2% (w/v) peptone at 30 °C. A portion of the cell suspension was removed at various time points and centrifuged. Fluorescence associated with the cells and in the supernatant was quantified as described above.

Column chromatography. Column chromatography of LY and a mixture of LY and peptone was done using Sephadex G-25, dry bead diameter 50–150 µm. The column (1.5 × 8 cm) was equilibrated with 50 mM-potassium phosphate buffer, pH 6.0. The downward flow elution rate during the packing was controlled by a peristaltic pump to give a flow rate of 1 ml min⁻¹. A sample (200 µl) containing LY (37.5 µg

ml⁻¹) was applied to the column, 1 ml fractions were collected and fluorescence was determined using the fluorescence spectrometer. A second sample (200 µl) containing LY (37.5 µg ml⁻¹) plus peptone (500 µg ml⁻¹) was incubated at 30 °C for 90 min and then applied to the column. Fractions were collected and fluorescence and protein of each fraction was determined. The protein in each fraction was measured using a dye binding protein assay (Bio-Rad) with bovine serum albumin as standard.

Results

Effect of temperature and energy inhibition on uptake of lucifer yellow by the yeast phase of C. albicans

An optimum temperature of 30 °C for the uptake of LY by *C. albicans* was determined in buffered glucose medium during a 90 min incubation; 80–90% of the cells internalized LY at 30 °C. Cells incubated at 22, 37 and 42 °C internalized LY at levels 50, 22 and 75% less, respectively, than the levels internalized at 30 °C. Intracellular fluorescence appeared in vacuoles; some cells showed many small vacuoles, while others had two or three large vacuoles (Fig. 1a, b). There was no internalization at 0 °C. A few cells showed entrapment of the dye between the cell wall and cell membrane at all the temperatures studied (Fig. 1c).

The energy-dependence of uptake of LY by *C. albicans* was studied during a 90 min incubation at 30 °C. Cells incubated in 2% (w/v) glucose internalized LY into vacuoles as described above. Cells incubated with 2% (w/v) glucose in the presence of 20 mM-NaN₃, an energy inhibitor, did not internalize the dye into vacuoles. However, 1–2% of cells appeared to have the dye diffuse throughout the cytoplasm and not localized in discrete vacuoles. Cells incubated with 20 mM-NaN₃ in the absence of glucose did not internalize the dye. Only 10–20% of the cells incubated with 2% glucose and 25 µM-CCCP, an uncoupler of H⁺ gradients (Harold, 1986), accumulated LY into apparently discrete vacuoles. Cellular fluorescence was not reduced when cells were incubated with 2% glucose and 8 mM-sodium orthovanadate, which acts on membrane-associated ATPases (Hubbard *et al.*, 1985, 1986).

Quantitative assays for lucifer yellow uptake

We quantified cell-associated LY to determine if the uptake of the dye by the yeast phase of *C. albicans* was time-dependent and saturable. Cells were incubated for various periods of time in assay medium containing LY (0.1, 1, 2 and 4 mg ml⁻¹). Quantification of cell-associated LY for each concentration of LY at different time points suggested that the uptake of the dye by *C. albicans* increased over a 2 h incubation period (Fig. 2). The uptake of LY was not saturable over a 400-fold range of concentration tested (inset Fig. 2).

Internalization of lucifer yellow by the mould phase of C. albicans

Studies with the yeast phase of *C. albicans* prompted us to investigate if the mould phase of this dimorphic fungus internalized LY. The mould phase was obtained by a nutritional shift and assays were done for 90 min at both 30 °C, the optimum temperature for internalization of LY by the yeast phase, and also 37 °C, the temperature at which the mould phase was obtained. The mould phase internalized LY at both 30 and 37 °C and the dye was localized either in several small vacuoles or in large vacuoles distributed all along the length of the mycelium (Fig. 1d, e).

Effect of growth medium on the uptake of lucifer yellow by the yeast and mould phases of C. albicans

Yeast and mould phases obtained by growth in ASM were assayed for LY uptake during a 90 min incubation in medium containing 4 mg LY ml⁻¹. Yeast cells obtained by growth at 25 °C did not internalize LY at either 25 or 30 °C, the optimum temperature for LY internalization by the yeast phase grown in YEPD. Also, the mould phase generated by a temperature shift procedure in ASM did not internalize LY when assays were done at 30 or 37 °C.

Effect of peptone and yeast extract on the uptake of lucifer yellow by C. albicans

The observation that the yeast phase or mould phase grown in complex medium internalized LY, whereas both phases grown in ASM did not, led us to determine if peptone or yeast extract affected LY internalization. Cells were grown in complex medium and assays were done at 30 °C for 90 min. Only 10–20% of the cells internalized LY in the presence of 0.5–2% peptone or yeast extract, whereas 80–90% of the cells internalized LY without peptone or yeast extract addition. In order to investigate if peptone complexed with LY and thus made it unavailable for endocytosis, we performed column chromatography of LY and an LY-peptone mixture using Sephadex G-25. The elution profile of LY was not affected by co-chromatography with peptone when applied as a mixture of peptone and LY. The peptone components were distributed over all the fractions collected.

Exocytosis of lucifer yellow

Since peptone in the assay medium reduced LY internalization, we determined if peptone affected exocytosis of internalized LY from the yeast phase of *C. albicans*. Cells were incubated in assay medium contain-

Fig. 1. Internalization of LY by *C. albicans*. Cells were incubated in assay medium containing 2% (w/v) glucose and 4 mg LY ml⁻¹ in 50 mM-potassium phosphate buffer, pH 6.0, at 30 °C for 90 min. Cells were washed and visualized by phase contrast and fluorescence microscopy. (a) Phase contrast micrograph. (b) Fluorescence micrograph of the same field as in (a) showing uptake of the dye inside vacuoles. (c) Fluorescence micrograph showing entrapment of the dye as indicated by fluorescence around the cellular periphery. (d) Phase contrast micrograph of the mould phase. (e) Fluorescence micrograph of the same field as in (d) showing intracellular accumulation of the dye inside vacuoles. Bars: (a), (b), (c), 1 µm; (d), (e), 5 µm.

Fig. 2. Time course of uptake of LY by *C. albicans*. Cells were incubated with various concentrations of LY ($\diamond$, 0.1 mg ml^{-1} ; $\triangle$, 1 mg ml^{-1} ; $\square$, 2 mg ml^{-1} ; $\circ$, 4 mg ml^{-1}) in assay medium containing 2% (w/v) glucose in 50 mM-potassium phosphate buffer, pH 6.0, at 30°C . Cell-associated LY was quantified using a fluorescence spectrometer. Inset, nonsaturable uptake of LY. Cell-associated LY values for the 90 min time point ($\bullet$) were replotted for each concentration of LY.

Fig. 3. Exocytosis of LY. *C. albicans* cells incubated with 2% glucose and 1 mg LY ml^{-1} in 50 mM-potassium phosphate buffer, pH 6.0, at 30°C for 90 min, were washed and re-incubated in phosphate buffer (a), or 2% (w/v) peptone (b), for various time periods at 30°C . Fluorescence associated with the cells ($\triangle$, $\circ$) and supernatant ($\blacktriangle$, $\bullet$), was measured.

ing 1 mg LY ml^{-1} at 30°C for 90 min, washed, then re-incubated in the presence or absence of 2% (w/v) peptone. Results of this experiment (Fig. 3) show that yeast cells re-incubated with peptone lost nearly 60% of cell-associated LY within 50 min with a concomitant

increase in the fluorescence of the supernatant. Cells re-incubated in phosphate buffer retained about 80% of the cell-associated LY even after re-incubation for 2 h.

Viability of lucifer yellow treated cells

C. albicans cells were incubated in a medium containing 4 mg LY ml^{-1} at 30°C for 90 min. After examining cells under the fluorescence microscope appropriate dilutions of cells were plated on YEPD agar plates. Control cells incubated without LY were also plated and the counts were compared. After incubation at 37°C for 48 h, 98% of the control cells were viable as compared to 88% of the LY treated cells (values are means of three experiments). Thus, uptake of LY did not greatly affect cell viability.

Discussion

In this report, we have shown that LY is internalized by *C. albicans* in a non-saturable and time-, temperature- and energy-dependent, process consistent with the characteristics of fluid phase endocytosis. The dye appeared to be concentrated inside vacuoles similar to those observed in *S. cerevisiae* (Riezman, 1985). A genetic basis for the involvement of vacuoles in endocytosis has been shown in *S. cerevisiae*. A temperature sensitive mutant, which lacked a morphologically recognizable vacuole, was defective for vacuolar enzyme biogenesis and was unable to internalize lucifer yellow (Chvatchko *et al.*, 1986; Reizman *et al.*, 1986; Dulic & Riezman, 1989).

Energy-dependence of uptake of the dye is reflected by the finding that glucose in the assay medium allowed the internalization of LY, while inhibitors of energy metabolism decreased the internalization of the dye. NaN_3 , in the absence of glucose, probably blocks even the basal level of metabolism and, hence, no internalization was observed. The diffuse fluorescence in the presence of glucose and NaN_3 might indicate a non-endocytic uptake into poisoned cells, as NaN_3 has been reported to interact with the yeast cell membrane causing an increase in membrane permeability (Riemersma, 1968). Sodium orthovanadate, which inhibits fungal plasma membrane ATPases (Hubbard *et al.*, 1985, 1986; Goffeau & Slayman, 1981; Willsky, 1979), might be expected to reduce cellular fluorescence of LY due to the central role of ATPases in energy metabolism. However, in both *S. cerevisiae* (Riezman, 1985) and *C. albicans*, intracellular fluorescence does not seem to be reduced in the presence of sodium orthovanadate. It is possible that the membrane-associated ATPases are dispensable for endocytosis in *C. albicans* and *S. cerevisiae*, or that

vanadate is metabolized to vanadyl ions leading to stimulatory effects on yeast cells (Dubyak & Kleinzeller, 1980; Willsky *et al.*, 1984).

C. albicans is a dimorphic fungus which undergoes yeast to mould phase conversion when provided with appropriate environmental conditions. Lee *et al.* (1975) developed a synthetic medium composed of amino acids, salts, glucose and biotin which maintained this fungus in the yeast form at 25 °C and in the mould form at 37 °C. Surprisingly, we found that neither the mould phase nor the yeast phase grown in synthetic medium internalized LY even though distinct vacuoles could be seen in the cells by phase contrast microscopy. However, both phases grown in complex medium and assayed for LY uptake in buffered glucose medium internalized LY into vacuoles. These results suggest that growth in a complex medium triggers the process of fluid phase endocytosis.

The influence of growth conditions on uptake processes in *C. albicans* has been investigated by several workers. Recently, Skowronski & Feldman (1989) reported that significant binding of oestrogen to oestrogen-binding protein in *C. albicans* could only be found if yeast cells were grown in complex medium whereas negligible binding was seen when cells were grown in some synthetic media. Page & Odds (1988) reported that the growth medium affects binding of human albumin, fibrinogen and transferrin to *C. albicans* and found that the mould phase obtained by growth in a synthetic medium bound less protein. Logan *et al.* (1979) have shown that the nitrogen source for growth affected peptide transport in *C. albicans*.

Since growth in complex media appeared to be required to enable cells to internalize LY, we investigated the effect of addition of peptone and yeast extract to the assay medium. Our results showed that peptone and yeast extract in the assay medium reduced the number of fluorescent cells, and that this was not due to complex formation between peptone and LY. Rather, peptone caused a rapid increase in efflux of internalized LY. Exocytosis of endocytosed material has been shown to be stimulated by extracellular calcium (Besterman *et al.*, 1981) and chloroquine (Kotal & Kotyk, 1988). Further experiments are required to elucidate the mechanisms operating in these processes.

Studies on fluid phase endocytosis in *C. albicans* may explain how some large molecules such as defensins (Lehrer *et al.*, 1985), toxins (Polonelli *et al.*, 1985) and cationic proteins (Yphanitis *et al.*, 1967; Olson *et al.*, 1977) cause cell damage. Since the characterized oligopeptide permeases are not known to transport such large proteins (Becker & Naider, 1980), and since no receptors have been reported for these large molecules, it is possible that they enter cells by fluid phase endocytosis.

A relationship between endocytosis and secretion has been reported for *S. cerevisiae* (Riezman, 1985; Dulic & Riezman, 1989), as several yeast secretory mutants were defective in internalization of LY. It is possible, therefore, that studies on endocytosis may increase our understanding on how proteinases are secreted from *C. albicans*. Secretion of proteinase (M_r 40 000–50 000) is an example of a protein crossing the cell wall barrier despite the apparent M_r limit of 600 proposed for cell wall porosity (Scherrer *et al.*, 1974; Cope, 1980). The secreted proteinase of *C. albicans* has been implicated as a virulence factor by several workers (reviewed in Douglas, 1988), although reports to the contrary also exist (Burnie *et al.*, 1985). It will be interesting to investigate if the endocytic and secretory pathways of *C. albicans* are coupled by studying LY endocytosis in *C. albicans* proteinase deficient mutants (Kwon-Chung *et al.*, 1985).

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