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The *Candida albicans* Plasma Membrane and H^+-ATPase during Yeast Growth and Germ Tube Formation

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***PMA1* expression, plasma membrane H^+-ATPase enzyme kinetics, and the distribution of the ATPase have been studied in carbon-starved *Candida albicans* induced with glucose for yeast growth at pH 4.5 and for germ tube formation at pH 6.7. *PMA1* expression parallels expression of the constitutive *ADE2* gene, increasing up to sixfold during yeast growth and twofold during germ tube formation. Starved cells contain about half the concentration of plasma membrane ATPase of growing cells. The amount of plasma membrane ATPase is normalized prior to either budding or germ tube emergence by the insertion of additional ATPase molecules, while ATPase antigen appears uniformly distributed over the entire plasma membrane surface during both growth phases. Glucose addition rapidly activates the ATPase twofold regardless of the pH of induction. The turnover of substrate molecules per second by the enzyme in membranes from budding cells quickly declines, but the enzyme from germ tube-forming cells maintains its turnover of substrate molecules per second and a higher affinity for Mg-ATP. The plasma membrane ATPase of *C. albicans* is therefore regulated at several levels; by glucose metabolism/starvation-related factors acting on gene expression, by signals generated through glucose metabolism/starvation which are thought to covalently modify the carboxyl-terminal domain of the enzyme, and possibly by additional signals which may be specific to germ tube formation. The extended period of intracellular alkalinization associated with germ tube formation may result from regulation of proton-pumping ATPase activity coupled with higher ratios of cell surface to effective cytosolic volume.**

The human pathogen *Candida albicans* is a diploid fungus which exists in two principal forms (13, 31, 32): as budding yeast cells or as mycelia. In pathogenic states (22), during routine clinical analysis, and under certain experimental conditions (13, 31), the budding yeast cells can be induced to produce an intermediate state in which a narrow tube, designated a germ tube, emerges from the cell and grows directionally at a uniform rate (11). After one or more septations, which occur some distance from the base of the germ tube (11), the fungus adopts a mycelial habit. A major difference between budding cells and germ tube-forming cells is the mode of cell surface change: budding cells exhibit growth over the entire bud surface while germ tubes elongate by apical extension. Detection of molecular changes at the cell surface during germ tube formation may therefore help define fundamental determinants in fungal morphogenesis and could assist the development of new therapeutic approaches and prophylactic measures aimed at containing *C. albicans* infections.

Germ tube formation in *C. albicans* has been studied by several experimental approaches and with a range of induction systems. However, the key genetic or chemical regulators of germ tube formation have yet to be identified. During germ tube formation, specific cell wall structures are elaborated and modified (reviewed by Shepherd [30]), but little is known about the plasma membrane or the specific responses of genes specifying components of this organelle. One exception to this generalization may be an increase in the plasma membrane biosynthetic enzyme chitin synthase II in germ tube-forming cells (4). Given its essential role in maintaining intracellular pH, ion balance, and nutrient uptake (27, 28), several authors (25, 34) have suggested that the plasma membrane ATPase may be an important factor in morphogenesis. Manipulation of ATPase expression and activity in *Saccharomyces cerevisiae* demonstrates that the plasma membrane ATPase can determine the rate of cell growth and influence morphology during cell division (6, 24, 35). Evidence for the role of the ATPase in *C. albicans* morphogenesis comes from the study of Stewart et al. (34), who observed stage-specific changes in intracellular pH during yeast growth and germ tube formation. They also showed that the ATPase inhibitor diethylstilbestrol inhibits germ tube formation, and this occurs at concentrations similar to those required for inhibition of the ATPase in purified plasma membrane preparations (17a). Monk et al. (18) recently cloned and characterized the gene *PMA1* for the plasma membrane proton-pumping ATPase of *C. albicans*. The codon bias of this gene and the observation that the enzyme contributes in the range of 20 to 40% of plasma membrane protein during yeast growth suggest that the gene is highly expressed. The structure of the 5′ region of the gene supports this notion. A TATA box and a CT block (7, 17), a feature of several highly transcribed genes in filamentous fungi, are found within the 100 nucleotides upstream of the transcription initiation site. The coding region of the *C. albicans PMA1* is 83% homologous at the DNA level (in the region of overlap) and is 92% similar in deduced primary structure to its *S. cerevisiae* equivalent. As expected, antibodies raised against the 100-kDa *S. cerevisiae* plasma membrane ATPase cross-react with 98-kDa *C. albicans* plasma membrane ATPase (18, 19).

The availability of *PMA1*-specific DNA probes, specific antibodies capable of detecting the ATPase in situ, and the ability to prepare highly purified plasma membrane preparations from *C. albicans* have allowed studies of *PMA1* expression, analysis of ATPase enzyme kinetics, and a determination of the distribution of the ATPase during yeast

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growth and germ tube formation. This paper shows that the plasma membrane ATPase of *C. albicans* appears to be regulated at several levels: by glucose metabolism/starvation-related factors acting on gene expression, by signals generated through glucose metabolism/starvation which are thought to covalently modify the carboxyl-terminal domain of the enzyme, and possibly by additional signals which may be specific to germ tube formation.

MATERIALS AND METHODS

Cell culture, cell growth, and morphogenesis. *C. albicans* ATCC 10261 was maintained on solid YPD medium (1% yeast extract, 2% peptone, 2% glucose, 1.5% agar). For biochemical experiments, cells were grown overnight at 28°C to stationary phase in a semidefined medium consisting of 0.5% glucose–0.1% yeast extract in SB medium [15 mM KH_2PO_4, 7.6 mM $(NH_4)_2SO_4$, 0.2 mM $MgSO_4$, 0.34 mM $CaCl_2$, 60 μg of biotin per liter at pH 4.5]. The cells were harvested by centrifugation, washed once with distilled water, and resuspended at an A_{540} of 3 to 4 in SB medium adjusted to pH 6.7. The cells were gently shaken at 37°C in a water bath and aerated overnight with two spargers supplied by an air pump. The cells were harvested by centrifugation and resuspended in 500 ml of SB medium adjusted to either pH 4.5 or pH 6.7 and preequilibrated at 37°C in a gyratory shaker at 200 rpm for 2 h. The cultures were made 16 mM in glucose to initiate yeast growth at pH 4.5 or germ tube formation at pH 6.7. Yeast morphology was quantitated microscopically as previously described (31). Culture supernatants were recovered by centrifuging 1-ml samples of each culture at 5,000 × *g*. Duplicate 100-μl samples of the supernatants were used to determine the residual glucose concentration by the phenol-sulfuric method of Dubois et al. (10).

Biochemical methods. Sucrose-gradient-purified plasma membranes were prepared from cells as described by Monk et al. (18) except that samples of cells (100 to 200 ml) at each time point were pelleted by centrifugation and resuspended in 5 ml of ice-cold homogenization buffer supplemented with 16 mM glucose. Glucose was routinely included in the homogenization medium to avoid the possibility of rapid ATPase inactivation which has been demonstrated for *S. cerevisiae* (29). Conversely, ATPase activation was not observed unless the cells were treated with glucose prior to resuspension in the ice-cold homogenization medium, as shown by the kinetic profiles of Table 1. Glass beads (8 g, 0.45-mm diameter; B. Braun Melsungen AG, Melsungen, Germany) were added, and the samples were vortexed four times for 1 min, with storage on ice for 1 min between each vortexing step. The cell homogenate was recovered by decantation and by twice washing the beads with 5 ml of homogenization buffer. ATPase assays were conducted in microtiter plates as described by Monk et al. (18). Affinity-purified antibodies directed against native plasma membrane ATPase (α-ATPase) were prepared as described by Monk et al. (19), except that fivefold-diluted rabbit α-ATPase in phosphate-buffered saline (PBS) was preabsorbed against 15 A_{540} units of stationary-phase *S. cerevisiae* Y55 for 2 h at room temperature and the yeast cells were removed by centrifugation. Preimmune serum was similarly preabsorbed. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted in a Minigel system (Bio-Rad Laboratories, Richmond, Calif.), and proteins were transferred to nitrocellulose as previously described (19). Blots were briefly stained with 1% Ponceau S in 0.1% acetic acid, and nonbound stain was removed by washing with 0.1% acetic acid. Ponceau S-stained blots were blocked with 0.2% gelatin–0.1% Tween 20 in PBS, which also removed the bound Ponceau S, and probed with antibodies or concanavalin A (12). The second antibody used was a swine α-rabbit immunoglobulin (Ig)-peroxidase complex (Dako Corporation, Carpinteria, Calif.). Antibody antigen and concanavalin A-horseradish peroxidase complexes were detected with 4-chloronaphthol and hydrogen peroxide as substrates (12). Densitometry of autoradiographs, 35-mm negatives, and SDS-polyacrylamide gels was conducted with an LKB laser scanning densitometer. Protein was estimated by the Bio-Rad microassay (1) adapted for microtiter plates.

TABLE 1. Kinetic properties of the plasma membrane ATPase during yeast growth and germ tube formation[a]

Time and presence of glucose	Yeast growth at pH 4.5			Germ tube formation at pH 6.7		
	V_{max}	$K_{0.5}$	k_{cat}	V_{max}	$K_{0.5}$	k_{cat}
T_0						
Without glucose	0.8	3.0	9.7	0.6	2.9	6.8
With glucose	1.5	2.0	17.9	1.3	2.0	17.1
T_{3h}						
With glucose	1.5	2.8	10.9	3.0	1.9	21.5

[a] The ATPase activity sensitive to 100 μM Na vanadate in each preparation was determined in replicate experiments as described in Materials and Methods and with a double dilution series of Mg^{2+}-ATP in the range of 0.24 to 15 mM at pH 6.5. In these experiments, duplicates of datum points, which were corrected for nonenzymatic hydrolysis of ATP, differed by less than 10%. The ATPase is saturated at high concentrations of Mg^{2+}-ATP but does not give a standard Michaelis-Menten kinetic profile at low Mg^{2+}-ATP. $K_{0.5}$ was therefore determined as the millimolar concentration of Mg^{2+}-ATP required to give 1/2 V_{max}. The units for V_{max} are micromoles of Mg^{2+}-ATP hydrolyzed per minute per milligram of protein. The k_{cat} was determined as the moles of Mg^{2+}-ATP hydrolyzed per second per mole of ATPase, assuming a molecular mass of 98 kDa, and represents the turnover of substrate molecules by the ATPase per second. The relative contribution of the 98-kDa band to plasma membrane protein was determined by densitometric analysis and was in the range of 12.3 to 14.6% of plasma membrane protein for T_0 samples and 21.5 to 22.3% of plasma membrane protein for T_{3h} samples.

Immunofluorescence microscopy. Samples (4 ml) of glucose-induced cells growing as yeast cells or forming germ tubes were harvested by centrifugation, washed once with 0.1 M sodium phosphate buffer (pH 6.5), and incubated in 4 ml of the same buffer containing 3.7% formaldehyde (Analar) for 90 min at room temperature. The fixed cells were washed three times with phosphate buffer and resuspended in 4 ml of medium containing 1 M sorbitol–20 mM sodium phosphate buffer, pH 6.5, supplemented with 0.5% 2-mercaptoethanol–5,000 U of snail gut glucuronidase (Sigma Chemical Company, St. Louis, Mo.). After 45 min of incubation at 30°C, the cells were washed twice with 1 M sorbitol in 20 mM sodium phosphate buffer, treated for 5 min with 5 ml of methanol at −20°C, and washed with 5 ml of acetone and then 10 ml of PBS at room temperature. The cells were resuspended in 2 ml of PBS containing 2% filter-sterilized heat-inactivated bovine serum and blocked overnight at 0 to 4°C. Samples of the cells (250 μl) were then incubated with 2% bovine serum plus the indicated amounts of presorbed preimmune serum or presorbed affinity-purified α-ATPase serum for 1.5 h at room temperature and overnight at 0 to 4°C. The cells were washed three times with PBS, resuspended in 250 μl of the same buffer, and treated with 2% bovine serum and 10 μl of fluorescein isothiocyanate-labeled goat anti-rabbit IgG serum (Sigma Chemical Company), which had been precentrifuged at 13,000 × *g* for 10 min to

remove insoluble antibody conjugate. After 3 h of incubation in the dark at room temperature, the cells were washed four times with PBS and resuspended in 25 μl of 80% glycerol. The cells were viewed in a Nikon microscope equipped with a 75-W xenon lamp. Transmission images were obtained with an oil-immersion 100× objective with numerical aperture 1.3. Fluorescent images were obtained with epi-illumination at 490 nm (narrow pass interference filter), and a dichroic filter at 510 nm was also employed. Emitted fluorescence was observed at 520 nm and captured with a KS701 image intensifier with a Cohu CCD camera. Information was transferred to an IBM PCAT equipped with a Matrox board. Images were contrast enhanced and balanced with Imagepro software.

Northern (RNA) blots. Total RNA was prepared from *C. albicans* yeast and germ tube-forming cells by the method of Schmitt et al. (26). Ethidium bromide-treated RNA samples containing approximately equal amounts of 26S rRNA were solubilized and separated by electrophoresis in 1.2% agarose gels containing formaldehyde, transferred to Hybond N by vacuum blotting, and fixed with UV as described by Kroczek and Seibert (15). Blots were photographed during UV illumination to determine the relative levels of rRNAs successfully transferred in each track. The blots were also treated with 50 mM NaOH for 5 min and rinsed in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Prehybridization and hybridization were carried out at 65°C in the solutions described by Church and Gilbert (5), supplemented with 8% dextran sulfate. Blots were hybridized overnight with the *PMA1* probe and washed twice with 1× SSC containing 0.1% SDS for 15 min at room temperature and with 0.1× SSC containing 0.1% SDS for 1 h at 65°C. The wet blot was sealed in plastic wrap and exposed to X-ray film at −80°C. After suitable exposure, the blot was stripped by twice exposing the blot to 0.1× SSC in 0.5% SDS at 90°C for 20 min and reprobed for the *ADE2* transcript. The probe for *C. albicans PMA1* was prepared with the Amersham Multiprime kit with 10 μCi of [α-^{32}P]ATP and 25 ng of agarose gel-purified 1.2-kb *Ava*I-*Eco*RV template from plasmid pJAM25 (18). The template includes the amino-terminal coding region of the gene and the nontranslated leader of the transcript. The probe for the *ADE2* transcript was similarly prepared with 25 ng of an agarose gel-purified 2.35-kb *Eco*RV fragment of the plasmid pSM7 (16). The probes were recovered from the labeling mixes by passage through a 1-ml Sephadex G-50 spin column equilibrated with sonicated herring sperm DNA.

RESULTS

Induction and kinetics of budding and germ tube formation. Quantities of material sufficient for biochemical analysis of germ tube formation were obtained by initially growing yeast cells to stationary phase at 28°C in the presence of GSB medium (13) supplemented with 0.1% yeast extract. The washed cells, resuspended at a cell density of A_{540} = 3 to 4.5, were starved of carbon source but not nitrogen by vigorous aeration overnight at 37°C in SB medium at pH 6.7. The starved cells were harvested, preequilibrated for 2 h at 37°C in the same volume of either pH 4.5 or pH 6.7 SB medium, and then induced for yeast growth at pH 4.5 or germ tube formation at pH 6.7 by the addition of glucose to 16 mM.

Growth of cells in semidefined rather than GSB medium gave much higher cell densities at stationary phase (A_{540} = 8 to 10 rather than 1 to 2) and allowed reproducible glucose induction of germ tubes from 500-ml starved cultures at cell densities in the range of A_{540} = 3 to 4. The kinetics of germ tube formation in this large-scale system are shown in Fig. 1A. Within 3 h of glucose addition, at least 80% of the cells at pH 6.7 formed germ tubes, while none were observed at pH 4.5. The formation of germ tubes was relatively synchronous, with the bulk of germ tube emergence and bud formation both occurring between 60 and 120 min and the absorbance of both induced cultures following essentially identical courses. Only about 5% of the cells at either pH were pseudomycelia. The extracellular glucose was rapidly utilized at the same rate by both budding cells and germ tube-forming cultures and was essentially exhausted from the medium after 2.5 h of growth (Fig. 1B). With this system, cells were induced to form germ tubes with either *N*-acetylglucosamine or galactose (Fig. 1) but not when proline was used as inducer (data not shown). The formation of germ tubes with *N*-acetylglucosamine was slightly delayed (by about 20 min) compared with glucose induction and occurred at both pH 6.7 and pH 4.5. Germ tube induction with galactose was delayed by about 1 h compared with induction

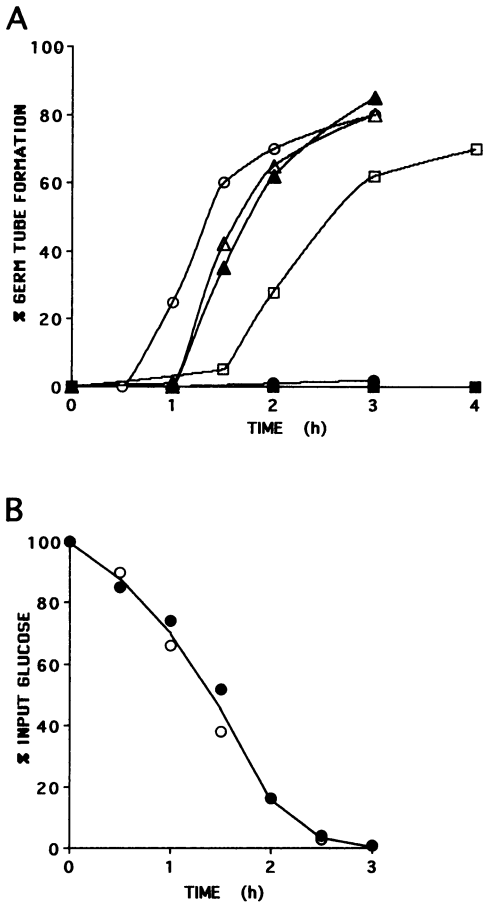

FIG. 1. Kinetics of germ tube formation and glucose utilization. (A) Kinetics of germ tube formation and yeast growth. Starved cells at 37°C were induced with 16 mM glucose (○ and ●), *N*-acetylglucosamine (△ and ▲), or galactose (□ and ■) at pH 4.5 (closed symbols) and at pH 6.7 (open symbols) and quantitated as described in Materials and Methods. (B) Kinetics of glucose utilization. Glucose levels detected in supernatants recovered by centrifuging cells at the indicated times after glucose induction were determined as described in Materials and Methods. Symbols are as described for panel A.

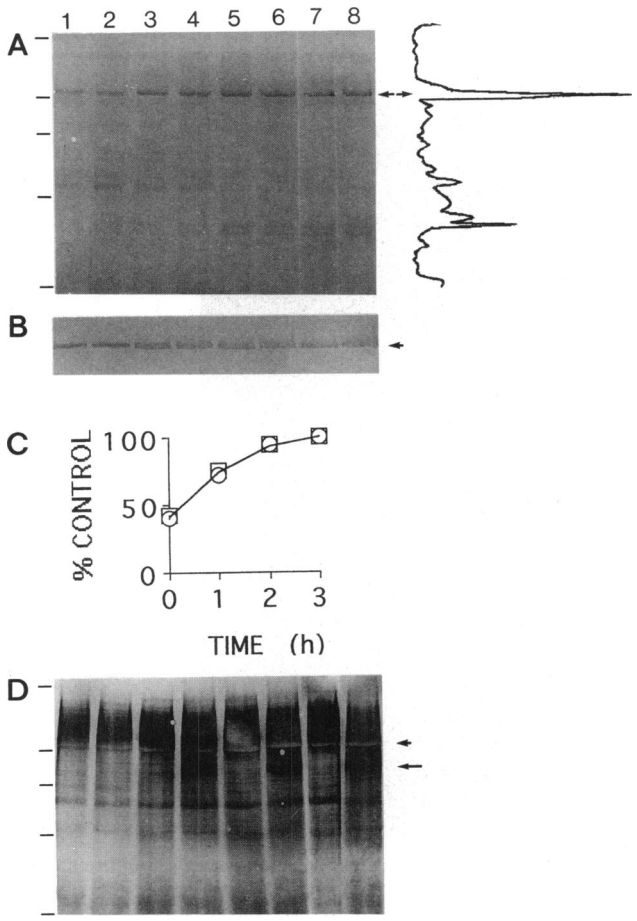

FIG. 2. Plasma membrane composition during germ tube formation and yeast growth. (A) Coomassie blue-stained polypeptide profiles of purified plasma membranes. Plasma membranes were purified as described in Materials and Methods from cells induced with glucose for yeast growth and germ tube formation as in Fig. 1A. The 2-μg samples of the membranes were separated by SDS-PAGE and stained with Coomassie blue. The small arrow indicates the 98-kDa plasma membrane H+-ATPase. Lane 1, T_0 (pH 4.5); lane 2, T_0 (pH 6.7); lane 3, 1 h (pH 4.5); lane 4, 1 h (pH 6.7); lane 5, 2 h (pH 4.5); lane 6, 2 h (pH 6.7); lane 7, 3 h (pH 4.5); lane 8, 3 h (pH 6.7). The migrations (short lines) indicated are of prestained molecular weight standards from Bethesda Research Laboratories comprising myosin (205 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), and carbonic anhydrase (28 kDa). The insert shows a densitometric profile of 2 μg of Coomassie blue-stained SDS-PAGE-separated plasma membranes obtained from cells after 3 h of induction for germ tube formation. (B) Western blot of plasma membranes. Samples (1.5 μg) of plasma membranes separated by SDS-PAGE as in panel A were electrotransferred to nitrocellulose and probed with a 1/100 dilution of presorbed affinity-purified α-ATPase serum as described in Materials and Methods. The small arrow indicates the location of the 98-kDa band. Preimmune serum gives no signal with the preparation. (C) Contribution of the 98-kDa band to plasma membranes. Shown is the percent contribution of the 98-kDa band in each Coomassie blue-stained profile (panel A) during yeast growth at pH 4.5 (□) and during germ tube formation at pH 6.7 (○) as estimated by laser densitometry and compared with the control level of this band (25% of Coomassie blue-stained plasma membrane protein) in the plasma membranes from yeast cells after 3 h of growth in the presence of glucose. (D) Concanavalin A blot of plasma membranes. Samples of 1.5 μg of plasma membranes separated by SDS-PAGE were electrotransferred to nitrocellulose and probed with concanavalin A and then horseradish peroxidase as described in Materials and Methods. The large arrow indicates the ~80-kDa glycoconjugate, and the small arrow indicates the 98-kDa H+-ATPase, while the short lines indicate the migration of molecular weight markers as in panel A. Lanes are as defined for panel A.

by glucose and occurred only at pH 6.7. This group of substrates (inducers) and their kinetic properties may prove useful in detecting molecules that are specific to germ tube formation against the background of nonspecific changes caused by metabolic induction or pH difference (21).

Plasma membrane composition during budding and germ tube formation. The polypeptide compositions of plasma membranes from cells undergoing glucose-induced yeast growth or germ tube formation were compared in order to detect growth-related and/or morphogenesis-related changes. The plasma membrane proteins were separated by SDS-PAGE and analyzed either by Coomassie blue staining or on Western blots (immunoblots) probed with Ponceau S, affinity-purified α-ATPase serum, and concanavalin A. The plasma membranes, apart from the quantitative differences detailed below, gave essentially identical Coomassie blue-stained polypeptide profiles during both 3-h time courses (Fig. 2A). Each preparation contained five major polypeptide bands in the region of >28 kDa plus a background of minor bands. Silver staining confirmed this observation and in addition revealed about 20 minor bands (data not shown). The major Coomassie blue-stained band, which accounted for 11 to 25% of stained material in the profiles (Fig. 2A, insert), ran with a molecular mass of 98 kDa and corresponded with the single band detected on the α-ATPase IgG-probed Western blot (Fig. 2B). The total amount of α-ATPase IgG bound (quantitated by densitometric analysis) was in proportion to the amount of both the 98-kDa Coomassie blue-stained band detected on gels and the 98-kDa Ponceau S-stained band on Western transfers (data not shown). These results identified the 98-kDa band as the plasma membrane ATPase and confirmed the high purity of the plasma membrane preparations. Electron microscopic examination of the negatively stained plasma membranes also indicated that they were homogeneous and indistinguishable from purified plasma membrane preparations from *S. cerevisiae* (data not shown). Densitometric analysis of the Coomassie blue-stained profiles of the plasma membrane preparations showed that the 98-kDa band at the beginning of glucose induction was about half the quantity found after 3 h of either growth or induction of germ tubes (Fig. 2C). This indicates that extended (overnight) starvation for carbon reduces the amount of the ATPase in plasma membranes of *C. albicans*. The pH of glucose induction made no significant difference in the numbers of ATPase molecules found in membranes either prior to glucose addition (T_0) or at T_{3h}. In addition, ATPase molecules were added to plasma membranes at the same rate in both germ tube-forming and budding cells (Fig. 2C). This implies identical rates of biosynthesis and/or membrane integration of the ATPase during germ tube formation and budding. The increasing quantities of the ATPase detected in plasma membranes prior to either bud or germ tube emergence (i.e., by 1 h) demonstrate that the mother cell inserts additional ATPase molecules into the plasma membrane during recovery from starvation. Probing of blotted membranes with concanavalin A (Fig. 2D) showed several minor conserved glycoprotein bands which were poorly stained with Coomassie blue. However, an ~80-kDa glycoconjugate was found in the membranes from cells forming germ tubes and not in the

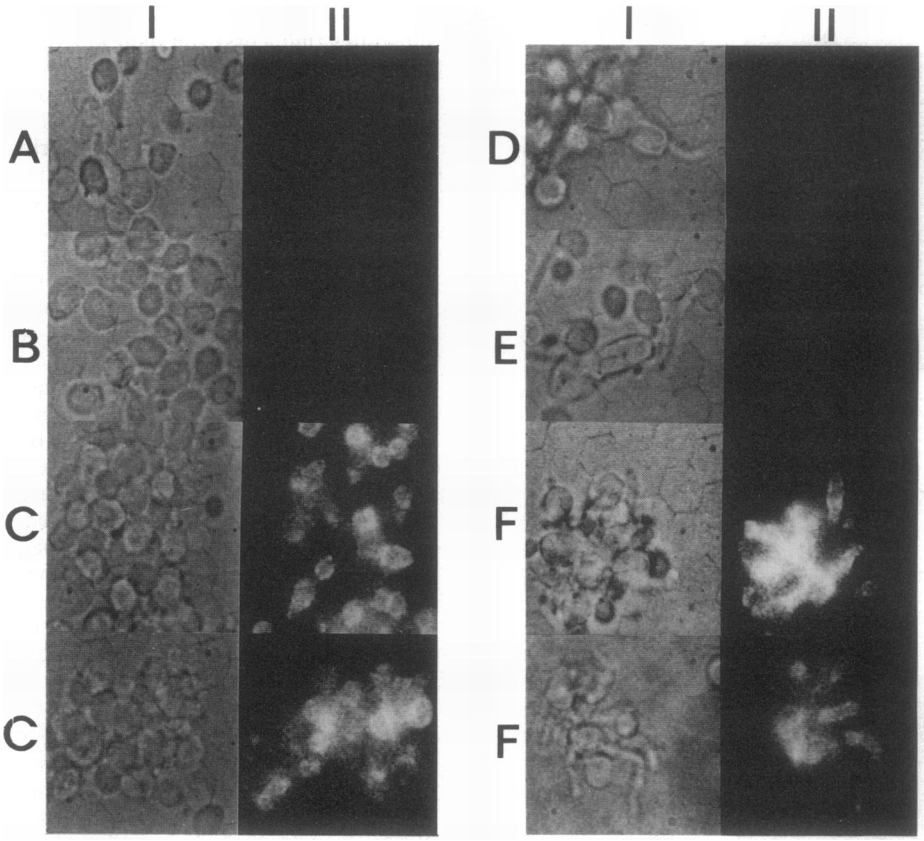

FIG. 3. Fluorescence microscopy of yeast cells and germ tube-forming cells. Cells induced for yeast growth or germ tube formation for 3 h at 37°C with 16 mM glucose were harvested and treated for 90 min with 3.7% formaldehyde at room temperature; cell walls were removed, and the material was processed for immunofluorescence microscopy as described in Materials and Methods. (A) Yeast cells with primary serum omitted; (B) yeast cells incubated with presorbed preimmune serum; (C) yeast cells incubated with presorbed affinity-purified α-ATPase serum; (D) germ tube-forming cells with primary serum omitted; (E) germ tube-forming cells incubated with presorbed preimmune serum; (F) germ tube-forming cells incubated with presorbed affinity-purified α-ATPase serum. I, transmitted light images; II, fluorescent images.

other membrane preparations. This band was detected after 1 h of induction, and slightly increased amounts appeared in membrane preparations obtained after germ tube emergence, i.e., during germ tube elongation. These results indicate that either the biosynthesis, membrane integration, or unmasking of concanavalin A binding sites of the ~80-kDa glycoconjugate is associated with germ tube emergence and that the glycoconjugate may be a component of the germ tube plasma membrane.

ATPase antigen distribution. The distribution of the plasma membrane ATPase in cells during growth and morphogenesis was evaluated by immunofluorescence microscopy with presorbed affinity-purified α-ATPase IgG as a probe. Presorbed preimmune serum from the same rabbit, adjusted to the IgG concentration of the affinity-purified antibody, provided the necessary negative control to assure specificity. Fluorescein isothiocyanate-labeled goat α-rabbit IgG was used to detect the binding of IgG to formaldehyde prefixed snail gut α-glucuronidase-treated protoplasts. The preimmune serum IgG did not increase the low background of nonspecific signal from binding of the fluorescein isothiocyanate-labeled probe antibody to either yeast cells or germ tube-forming cells (Fig. 3A, B, D, and E). The presorbed affinity-purified α-ATPase gave an intense signal which specifically associated with the plasma membranes of both yeast (Fig. 3C) and germ tube-forming (Fig. 3F) cells. The antibody bound uniformly to the plasma membrane of yeast cells, giving ovals of membrane-associated fluorescence. The antibody also bound uniformly to the mother cell and along the entire length of germ tubes. The plasma membrane, observed during the complete yeast cell cycle or during germ tube formation (data not shown), bound the antibody uniformly over its entire surface and at a concentration equivalent to that of the mother cell. This result implies that the plasma membrane ATPase is uniformly distributed over the entire cell surface and is not concentrated in regions of cell membrane expansion or apical membrane growth. This conclusion rests on the assumption that the treatment of cells with 3% formaldehyde blocks the lateral diffusion of ATPase molecules in the plane of the plasma membrane and traps ATPase molecules in their endogenous distribution.

Plasma membrane ATPase enzyme kinetics. In *S. cerevisiae*, the activity of the plasma membrane ATPase is activated/deactivated up to 10-fold by the glucose metabolism/starvation. The activation mechanism is thought to involve covalent modification of the carboxyl-terminal domain of the ATPase by a calmodulin-dependent protein kinase (29). Comparative analysis of the *PMA1* sequences suggests that the structural elements required for the enzyme to respond

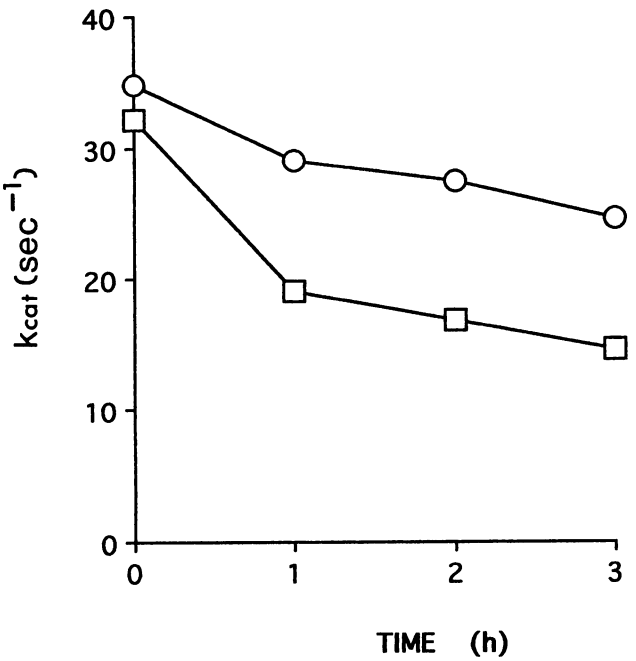

FIG. 4. k_{cat} of the plasma membrane ATPase during yeast growth (□) and germ tube formation (○). Duplicate samples (1 μg) of the plasma membranes purified from the samples described in the legend to Fig. 2A were assayed for vanadate-sensitive ATPase activity at 37°C as described in Materials and Methods in the presence of 15 mM Mg-ATP. The activities for individual duplicates varied by less than 10%, and all activities were sensitive to 100 μM sodium vanadate.

to glucose metabolism/starvation are maintained in the carboxyl-terminal domain of the *C. albicans* ATPase (18). The in vitro enzymatic properties of the *C. albicans* ATPase in plasma membranes of carbon-starved cells from cultures at pH 6.7 and 4.5 were compared with preparations from these cells either treated briefly with glucose or induced to undergo yeast growth or germ tube formation for 3 h. The results of these ATPase activity measurements are shown in Table 1. The ATPase activity of all the fractions measured was at least 95% sensitive to 100 μM sodium vanadate, each fraction giving a K_i for vanadate in the range of 4 to 6 μM. Addition of glucose to starved cells at either pH 4.5 or pH 6.7 gave about a twofold increase in plasma membrane ATPase-specific activity in each case and a 33% decrease in the millimolar concentration of Mg^{2+}-ATP required to give 1/2 V_{max} ($K_{0.5}$) for ATP from 3 to 2 mM. In yeast cells, the increased ATPase specific activity of the plasma membranes was maintained throughout the experiment but the $K_{0.5}$ for ATP increased by 50% from 2 to almost 3 mM after 3 h of growth. In germ tube-forming cells, ATPase specific activity ultimately increased a further twofold and the $K_{0.5}$ for ATP of 2 mM was maintained for a further 3 h. Determination of the turnover of substrate molecules per second by the ATPase (k_{cat}) shows that with glucose exhaustion the turnover number of the enzyme in budded cells declined to preinduction levels, while the enzyme from cells which have formed germ tubes remained activated.

In a complementary experiment, a temporal difference between the ATPase activity of germ tube-forming cells and that of budding cells was found by estimating the k_{cat} of plasma membrane ATPase molecules (Fig. 4). The high k_{cat} attained in response to glucose addition declined slowly during germ tube formation while the k_{cat} declined rapidly during yeast cell formation, with most of the ATPase activation lost prior to budding. Kaur and Mishra (14) have reported differential dimorphism-associated changes in overall H^+-ATPase activity in *C. albicans* with a whole-cell assay which are consistent with the present results.

***PMA1* expression.** Northern blots of mRNA prepared from yeast and germ tube-forming cells gave a semiquantitative measure of the *PMA1* transcript which specifies the plasma membrane proton-pumping ATPase. Expression of *PMA1* was compared on the same blots with expression of the constitutive *ADE2* gene, which encodes the enzyme adenylosuccinate lyase. The *PMA1* message was detected as a single transcript of ~4,000 nucleotides within 5 h of autoradiography (Fig. 5B, insert) following hybridization with a probe generated from a 1.2-kb *Ava*I-*Eco*RV 5′ fragment of the *PMA1* gene. This probe was previously used to detect specifically *PMA1* expression (18). A probe generated from the entire *ADE2* gene as template required 30-fold-longer autoradiography (Fig. 5A, bottom insert) to give a comparable exposure of a single transcript of about 2,000 bases. The hybridization of the *PMA1* and *ADE2* transcripts was normalized by reference to the amount of ethidium bromide-stained 26S RNA (Fig. 5A, top insert) detected in each lane. This approach corrects for differences in RNA recoveries between samples. Figure 5A shows the different patterns of *ADE2* expression in yeast cells and germ tube-forming cells. During yeast growth, the *ADE2* transcript/26S RNA ratio increased about sixfold in the first hour of glucose induction. This ratio then rapidly declined over the next 2 h. In germ tube-forming cells, the *ADE2* transcript/26S RNA ratio initially doubled but then declined by about 40% prior to germ tube emergence. After the majority of germ tube emergence (2 h), the message was again detected at about twice the relative amount observed in the starved cells. The *PMA1* gene and *ADE2* genes showed identical patterns of biphasic expression during yeast growth and triphasic expression during germ tube formation (Fig. 5A and B). Although *PMA1* seemed much more highly expressed than *ADE2*, the ratio of transcripts from the two genes remained approximately constant during both yeast growth and germ tube formation (Fig. 5C). More precise quantitation of the two transcripts will require the RNase protection techniques or polymerase chain reaction-based amplification of reverse transcriptase-generated *PMA1* cDNA. However, the present results demonstrate that *PMA1* is constitutively expressed during both phases of growth.

DISCUSSION

The *C. albicans* plasma membrane ATPase is the dominant protein component of the plasma membrane (18) in all phases of growth thus far examined: stationary-phase cells, starved cells, yeast growth, and germ tube formation. However, the proportion of ATPase molecules in the plasma membrane can differ by about twofold and plasma membrane ATPase specific activity can vary by up to fourfold among preparations from starved cells, budding yeast cells, and germ tube-forming cells. In these contexts, the *PMA1* gene, which encodes the ATPase, is constitutively transcribed. Thus, *PMA1* expression, while severalfold higher in apparent amount and specifying an enzyme associated with the cell surface, surprisingly closely parallels and maintains an essentially constant ratio of expression to that of the constitutive *ADE2* gene, which encodes the cytosolic en-

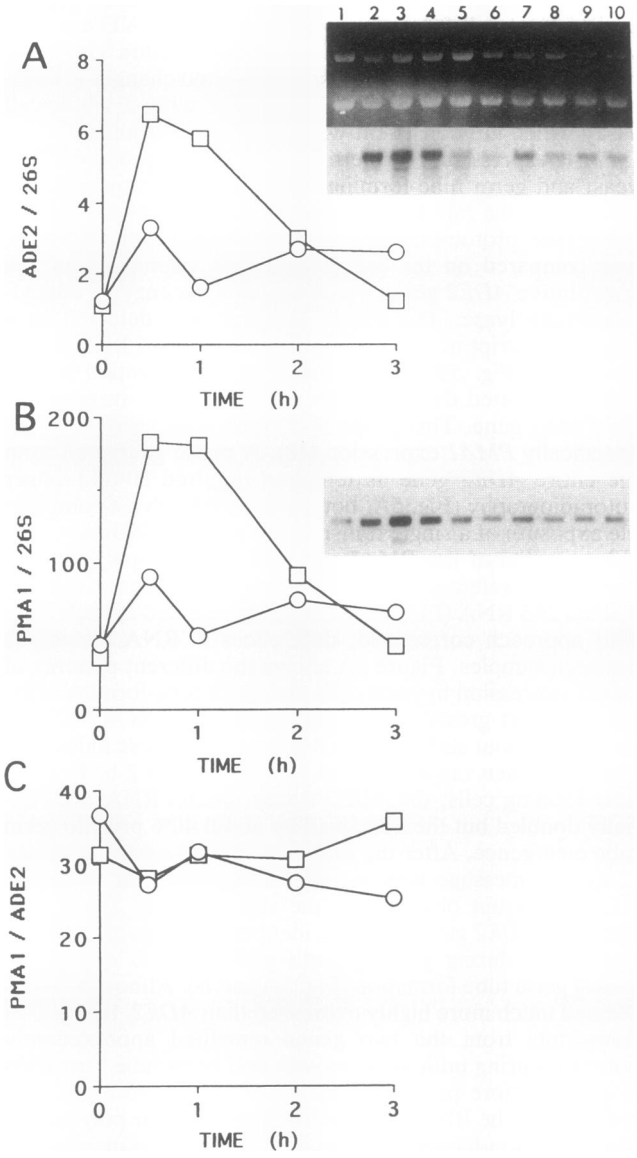

FIG. 5. Northern blot analysis of RNA from cells during glucose-induced yeast growth and germ tube formation. Starved cells were induced with glucose for yeast growth and germ tube formation as described in Materials and Methods. These cells showed morphogenetic kinetics essentially identical to those shown in Fig. 1. (A) Expression of *ADE2*. Ethidium bromide-stained profiles of RNA blots are shown in the top insert while autoradiographs of binding of the *ADE2* probe are shown in the bottom insert. Lanes 1 and 6, T_0; lanes 2 and 7, 30-min glucose induction; lanes 3 and 8, 60-min glucose induction; lanes 4 and 9, 120-min glucose induction; lanes 5 and 10, 180-min glucose induction. Lanes 1 to 5 were induced at pH 4.5 (yeast growth [□]) while lanes 6 to 10 were induced at pH 6.7 (germ tube formation [○]). The ratios of *ADE2*/26S RNA are arbitrary numbers defined by densitometric analysis of the probe autoradiograms, the exposure time required for the probe, and the relative amounts of 26S RNA detected by scanning negatives of the ethidium bromide-stained RNA profiles. (B) Expression of *PMA1*. The insert shows the autoradiograph of the binding of the *PMA1* probe to the blot. The lane numbers, symbols, and the calculation of *PMA1*/26S ratios are as described for panel A. (C) Relative expression of *PMA1* and *ADE2*. Symbols are as in panel A.

zyme adenylosuccinate lyase. Other results recently obtained in this laboratory indicate that a gene encoding a vacuolar protease follows a similar pattern of expression during yeast growth and germ tube formation at 37°C (20a).

Glucose-induced expression of *PMA1* reflects both recovery from starvation (with integration of additional ATPase into the plasma membrane occurring before either budding or significant germ tube emergence) and the membrane biosynthesis de novo needed for cell surface expansion during both budding and germ tube formation. The patterns of *PMA1* mRNA expression are consistent with the bulk of membrane expansion in budding cells taking place within 2 h of synchronous release from starvation while membrane expansion occurs more slowly and at a steady rate during germ tube formation. Thus, the *PMA1* mRNA/26S RNA ratios peak within about 30 min of glucose induction, the occupancy of the membrane by the ATPase is restored to near normal levels within 60 min, and then budding and germ tube emergence are observed. In *S. cerevisiae*, *PMA1* expression is moderately regulated by glucose, in a mechanism thought to be dependent on an upstream TUF element (2). In that system, the amount of the plasma membrane ATPase increases by only about 20% on the addition of glucose. A more dramatic effect is seen in *C. albicans*: the concentration of ATPase detected in the plasma membranes almost doubles within the first hour after glucose addition at both pH 4.5 and pH 6.7. This response is accommodated by a doubling of *PMA1* transcript/26S RNA ratio during germ tube formation at pH 6.7 and up to a sixfold increase during yeast growth at pH 4.5. The subsequent rapid decline in apparent *PMA1* transcript ratio in budded cells suggests that the *PMA1* transcript has a half-life of less than 60 min. In budding cells, the cell surface and cytoplasmic volume expand commensurately and cell division occurs between 1 and 2 h after glucose addition. During the first 3 h of glucose-induced germ tube formation, the cell surface expands slightly (~40%) more than during bud formation and in a linear fashion after germ tube emergence. In contrast to budding cells, the biosynthetic volume of the cytosol in germ tube-forming cells remains relatively constant because extensive vacuolation of the mother cell occupies much of the increased intracellular volume (11). This implies that in cells extending germ tubes the effective concentration of *PMA1* transcripts may be more comparable to those of budding cells than suggested by the observed *PMA1* transcript/26S RNA ratios. These geometric and kinetic factors, plus slow turnover of the fungal ATPase molecules compared with the division time of the organism, may explain the apparent disparity between the amounts of *PMA1* message detected during budding and germ tube formation while an essentially constant ratio of expression is maintained with the constitutive *ADE2* gene and similar concentrations of ATPase molecules are inserted into the plasma membranes during both phases of development. In addition, glucose metabolism/starvation acts on the membrane-integrated ATPase to fine-tune enzyme activity.

During growth of *S. cerevisiae*, the activity of the proton-pumping ATPase is primarily regulated by a glucose metabolism/starvation-dependent modification of the ATPase carboxyl-terminal domain, with the V_{max} of the ATPase varying by as much as 10-fold in response to glucose starvation/metabolism (19, 29). While the carboxyl-terminal domain of the *C. albicans* ATPase is similar in primary structure to the equivalent region in the *S. cerevisiae* enzyme, including a consensus sequence required for covalent phosphorylation by a calmodulin-dependent protein kinase, the kinetic prop-

erties of the *C. albicans* enzyme in exponentially growing yeast cultures were only modestly affected by glucose metabolism/starvation (18). A larger change in kinetic properties occurs on release from more exhaustive carbon starvation. In particular, the k_{cat} of the ATPase rapidly doubled and the $K_{0.5}$ for Mg-ATP simultaneously decreased on the addition of glucose at either pH 4.5 or pH 6.7, implying activation of the enzyme by glucose metabolism. In cells programmed to bud, the subsequent rapid reduction in the ATPase k_{cat} towards that of starved cells reflects either the observed decline in glucose availability or the diversion of glucose metabolites into pathways which no longer stimulate the ATPase. The glucose in culture supernatants is depleted at similar rates and is essentially exhausted after 2.5 h (Fig. 1B), suggesting similar rates of metabolic flux during both budding and germ tube formation. The maintenance of an elevated k_{cat} and a higher affinity for ATP in germ tube-forming cells indicates that the enzyme remains activated independently of the glucose concentration of the medium. Whether this involves novel signaling mechanisms specific to germ tube formation or the fine-tuning of a calmodulin-dependent protein kinase pathway has yet to be determined. Consistent with the latter hypothesis, Paranjape et al. (23) detected at least twice as much calmodulin activity, measured by the capacity of extracts to stimulate cyclic AMP-phosphodiesterase, in germ tube-forming/hyphal cells as in yeast cells. Since the germ tube may facilitate nutrient scavenging through directional growth, limiting ATPase activity by ATP availability ($K_{0.5}$ for Mg-ATP is 2 mM) rather than by the product of a single metabolic substrate such as glucose may be an advantage. Regardless of the molecular basis of regulation, the present study is consistent with the previously observed transient alkalinization of budding cells and the extended alkalinization of germ tube-forming cells (34). Cells elaborating germ tubes for 3 h have a small apical cytosolic slug, show a >40% higher ratio of cell surface area/total intracellular volume, and maintain an ATPase specific activity about twofold higher than that of budding cells. This suggests a minimal three- to fivefold-higher ratio of total ATPase activity/cytosolic volume in germ tube-forming cells compared with budding cells. These features may assist the ATPase in maintaining cytosolic pH during hyphal growth and generate local ionic and biosynthetic gradients.

In the course of our work, we have also observed a plasma membrane concanavalin A-binding glycoconjugate of 80 kDa which seems specific to cells undergoing germ tube formation. It is found in the plasma membrane fraction either prior to or about the time of germ tube emergence and during germ tube elongation increases slightly in quantity or concanavalin A reactivity, suggesting that it might be a component of the germ tube membrane. Further research will be required to localize and characterize this glycoconjugate and to determine whether it is biosynthesized during germ tube formation or whether it is detected because of posttranslational modification specific to germ tube formation but not budding. The identity of the ~80-kDa glycoconjugate remains to be established, but candidates include chitin synthase II (4), a group of hyphally specific mannoproteins (3), or a component required for the cell-cell aggregation which commences prior to germ tube emergence and continues during germ tube elongation.

While plasma membrane ATPase-related cytoplasmic alkalinization may be needed for the continued development of germ tubes and aid in the polarization of morphogenesis-related biosynthetic processes, this phenomenon is unlikely to be the primary determinant in morphogenesis. The commitment to one morphogenic form over the other does not occur until after germ tube emergence in *C. albicans* and coincides with filament ring formation (33). The differential ability of hyphal cells to maintain an elevated intracellular pH may be a perturbing factor that helps lock in a morphogenic choice initially established through site selection and the finely regulated development of subcortical structures which determine membrane vesicle interaction with the plasma membrane (8, 9, 20, 30). Our observations on the *C. albicans* ATPase provide some additional insights into the physiology of germ tube formation and suggest further regulatory molecular targets that might be exploited in the design of antifungal agents.

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