High-affinity myo-inositol transport in Candida albicans: substrate specificity and pharmacology

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Inositol is considered a growth factor in yeast cells and it plays an important role in Candida as an essential precursor for phospholipomannan, a glycoprophatidylinositol (GPI)-anchored glycolipid on the cell surface of Candida which is involved in the pathogenicity of this opportunistic fungus and which binds to and stimulates human macrophages. In addition, inositol plays an essential role in the phosphatidylinositol signal transduction pathway, which controls many cell cycle events. Here, high-affinity myo-inositol uptake in Candida albicans has been characterized, with an apparent K_m value of 240 ± 15 μM, which appears to be active and energy-dependent as revealed by inhibition with azide and protonophores (FCCP, dinitrophenol). Candida myo-inositol transport was sodium-independent but proton-coupled with an apparent K_m value of 11.0 ± 1.1 nM for H^+, equal pH 7.96 ± 0.05, suggesting that the C. albicans myo-inositol–H^+ transporter is fully activated at physiological pH. C. albicans inositol transport was not affected by cytochalasin B, phloretin or phlorizin, an inhibitor of mammalian sodium-dependent inositol transport. Furthermore, myo-inositol transport showed high substrate specificity for inositol and was not significantly affected by hexose or pentose sugars as competitors, despite their structural similarity. Transport kinetics in the presence of eight different inositol isomers as competitors revealed that proton bonds between the C-2, C-3 and C-4 hydroxyl groups of myo-inositol and the transporter protein play a critical role for substrate recognition and binding. It is concluded that C. albicans myo-inositol–H^+ transport differs kinetically and pharmacologically from the human sodium-dependent myo-inositol transport system and constitutes an attractive target for delivery of cytotoxic inositol analogues in this pathogenic fungus.

INTRODUCTION

The yeast Candida albicans is one of the most commonly encountered human pathogens and is a normal component of the human endogenous microflora. As an important nosocomial and opportunistic fungus, C. albicans can cause a wide variety of infections ranging from mucosal infections in generally healthy persons to life-threatening systemic infections in individuals with impaired immune defence, cancer therapy, antibiotic treatment, diabetes or burn victims. Furthermore, the development of drug resistance and the limitations and severe side effects of drug treatment pose an increasing problem with regard to C. albicans infections (Vanden Bossche et al., 1998; Pfaller et al., 1998; Cowen et al., 2002).

Inositol is considered a growth factor in yeast cells and necessary for their optimum growth (Nikawa et al., 1982, 1991), despite the ability of some yeasts, including Saccharomyces cerevisiae, to also synthesize myo-inositol de novo from glucose 6-phosphate (Dean-Johnson & Henry, 1989) at the expense of glycolysis. Inositol plays an important role in Candida as an essential precursor for phospholipomannan, a family of glycoprophatidylinositol (GPI)-anchored glycolipids on the cell surface of Candida that is involved in the pathogenicity of this fungus and which binds to and stimulates human macrophages (Trinel et al., 1999). Thus, phospholipomannan is considered a virulence factor in Candida and has been shown to possess immunomodulatory properties such as TNF-α induction (Jouault et al., 1994). GPI-membrane anchors are of particular significance in lower eukaryotes and parasitic protozoa (McConville & Ferguson, 1993), and about 60 GPI-anchored membrane proteins were estimated in yeast from the Saccharomyces cerevisiae genome project (Sütterlin et al., 1997). Moreover, inhibition of GPI-anchor biosynthesis was shown to be lethal in Saccharomyces cerevisiae (Leidich et al., 1994). In addition to its role as an essential precursor for GPI-membrane anchors, inositol plays a central role also in the phosphatidylinositol signal transduction pathway, which controls many cell cycle events in eukaryotic cells. Hence, myo-inositol transport in C. albicans appears to be an attractive drug target to interfere with important

Abbreviations: FCCP, carbonylcyanide-4-(trifluoromethoxy)phenylhydrazone; GPI, glycoprophatidylinositol.
cellular and physiological functions of this fungus and for delivery of cytotoxic inositol analogues.

Inositol transport in yeast cells was characterized in an earlier study in the fission yeast *Schizosaccharomyces pombe* (Cheneval et al., 1970) and in more detail in baker’s yeast *Saccharomyces cerevisiae* (Nikawa et al., 1982, 1991; Lai & McGraw, 1994). For *Schizosaccharomyces pombe*, myo-inositol transport was shown to be energy-dependent and upregulated upon depletion of inositol from culture medium (Cheneval et al., 1970). For *Saccharomyces cerevisiae*, myo-inositol transport was shown to be energy-dependent and sodium-independent (Nikawa et al., 1982, 1991), but no further investigations about its ion specificity, substrate selectivity or pharmacology have been described. The apparent *Km* values reported for myo-inositol transport were 0.26 ± 0.04 mM in *Schizosaccharomyces pombe* (Cheneval et al., 1970) and ranged from 0.1 mM (Nikawa et al., 1982, 1991) to 0.47 mM (Lai & McGraw, 1994) in *Saccharomyces cerevisiae*. Furthermore, Nikawa and co-workers were able to isolate two distinct myo-inositol transporter genes from *Saccharomyces cerevisiae*, designated ITR1 and ITR2 (Nikawa et al., 1991), which belong to the large sugar transporter superfamily that also includes the red cell glucose transporter GLUT1 and the protozoan myo-inositol–H+ transporter MIT from *Leishmania donovani* (Baldwin, 1993; Seyfang & Landfear, 2000). Targeted gene disruption revealed that the ITR1 transporter was the major permease for myo-inositol in *Saccharomyces cerevisiae*, while the ITR2 transporter contributed only 0–4–3% of total inositol transport capacity in yeast cells and ITR2 transcripts were hardly detectable in regular Northern blots where ITR1 transcripts gave strong hybridization signals (Nikawa et al., 1991). Regulation of ITR1 function and repression by external inositol was demonstrated at the transcriptional level (Nikawa et al., 1993) and the protein level, involving inositol-induced changes in permease stability (Lai & McGraw, 1994; Lai et al., 1995), and ITR1 is part of a complex network of inositol/choline regulation that controls phospholipid biosynthesis in *Saccharomyces cerevisiae* [reviewed by Carman & Henry (1989) and Greenberg & Lopes (1996)].

While baker’s yeast *Saccharomyces cerevisiae* is able to synthesize myo-inositol de novo from glucose 6-phosphate, the closely related fission yeast *Schizosaccharomyces pombe* appears to be strictly auxotrophic for exogenous inositol owing to the lack of a functional inositol-1-phosphate synthase, INO1 (Fernandez et al., 1986; Ingavale & Bachhawat, 1999), which converts glucose 6-phosphate to myo-inositol 1-phosphate. Interestingly, the subsequent enzyme in inositol biosynthesis, inositol monophosphatase INM1 (Murray & Greenberg, 2000), which dephosphorylates myo-inositol 1-phosphate to myo-inositol, is still functional in *Schizosaccharomyces pombe* (Ingavale & Bachhawat, 1999). While an INO1 gene homologue has been identified in *C. albicans* (Klig et al., 1991), the complete biosynthetic pathway and a functional inositol monophosphatase have not been described in *Candida*. Hence, either synthesis or transport of myo-inositol (or both) is essential for yeast viability.

The plasma membrane and especially membrane proteins with important physiological functions mediate the interaction of the pathogenic fungus with its host. Hence, the membrane properties allow the fungal pathogen to successfully colonize the gastrointestinal tract and a variety of other tissues in the human host and are important for causing disease. Membrane transporters are of particular interest since they offer rational drug design to (i) inhibit their physiological functions or (ii) deliver cytotoxic substrate analogues. Inositol plays an important role in *C. albicans* as an essential precursor for GPI-membrane anchors of protective and immunomodulatory surface molecules, and nothing is known about the mechanism of myo-inositol uptake in *Candida*. In this study, we have identified and characterized in detail high-affinity myo-inositol transport in *C. albicans*, which was proton-dependent and differed both kinetically and pharmacologically from the human sodium-dependent myo-inositol transport system in the intestine and kidney. Furthermore, we have probed the substrate recognition and binding of the *C. albicans* inositol transport system as an important step to better understand its remarkable substrate specificity.

**METHODS**

**Strains and growth conditions.** The wild-type *Candida albicans* strain SC5314 was used throughout this study (kindly provided by Dr Nancy A. Federspiel, Stanford DNA Sequencing & Technology Center, Stanford University, Palo Alto, USA). This strain was maintained on yeast extract/peptone/dextrose (YPD) agar (Bio 101) and subcultured onto fresh medium when needed. *C. albicans* cultures were grown at 30°C in YPD medium (1% yeast extract, 2% peptone, 2% glucose; Bio 101) with rotary shaking at 200 r.p.m. in baffled flasks.

**Reagents.** myo-[2-3H]inositol (specific activity of 21–24 Ci mmol⁻¹; 777–888 GBq mmol⁻¹) was purchased from NEN/PerkinElmer Life Sciences. Inositol isomers and derivatives were obtained from Sigma (alpha-, D-chiro-, L-chiro-, epi-, muco-, myo-inositol; phytic acid), Aldrich (neo-inositol; quebrachitol), Calbiochem (sphingo-inositol; 3-fluoro-3-deoxy-myco-inositol) or Industrial Research Ltd (Lower Hutt, New Zealand) (L-chiro-inositol; ononitol, pinitol, viburnitol). Monosaccharides, ionophores and inhibitors were purchased from Sigma and were of the highest grade available.

**Inositol uptake assays.** For transport assays in *C. albicans*, overnight cultures (6 × 10⁶ to 10 × 10⁶ cells ml⁻¹) were diluted 10-fold in fresh YPD medium and grown to mid-exponential phase (1.5 × 10⁶ to 2 × 10⁶ cells ml⁻¹). Cells were harvested by centrifugation at 2000 g for 10 min, washed twice in PBS (pH 7.4) at 4°C and resuspended in PBS, unless otherwise stated. After 5 min pre-incubation in a water bath at 25°C, uptake of radiolabelled myo-inositol was measured at 25°C and initiated by adding 100 µl of cell suspension (5 × 10⁶) to 100 µl of myo-[2-3H]inositol (3 µCi ml⁻¹; 110 kBq ml⁻¹) at 100 µM final concentration in PBS. At various time points (1, 3, 5, 7 and 10 min), uptake was terminated by spinning the cells through an oil cushion of 100 µl of dibutyl phthalate (density = 1.04 g cm⁻³; Sigma) in 0.63 ml microcentrifuge tubes for 45 s, followed by immediate snap-freezing of the tube in a dry
ice/ethanol bath (Seyfang et al., 1997). Subsequently, the tip of the tube with the frozen cell pellet was clipped off into 200 μl of 1 % SDS in a scintillation vial, sonicated for 15 min in a sonication water bath and mixed with 3 ml of ScintiVerse BD (Fisher Scientific) for liquid scintillation counting. From these data, the uptake of radiolabelled myo-inositol at each time point was calculated and plotted as a function of incubation time. The initial myo-inositol uptake rate was determined by linear regression analysis of the five plotted data points for each assay, which lay within the linear uptake range during the first 10 min of myo-inositol uptake, and regression coefficients of 0.970 or higher were obtained for regression analyses.

For determination of the ion specificity of myo-inositol transport in C. albicans, cells were washed three times by centrifugation in the various uptake buffers with or without sodium (140 mM NaCl, KCl or choline chloride, respectively, with 25 mM HEPES, pH 7-3) prior to uptake assays in the respective buffer.

Substrate saturation kinetics. Substrate saturation kinetics for myo-inositol were determined in PBS, pH 7-4, within the linear uptake range of each concentration. For myo-inositol concentrations between 10 and 500 μM, the data points thus included 1, 3, 5, 7 and 10 min of incubation time; for 1 and 1·5 mM myo-inositol, the time points were 1, 2, 3, 5 and 7 min, respectively. The initial myo-inositol uptake rate was determined for each substrate concentration by linear regression analysis of the time-course of the five data points of each assay. Proton-dependent saturation kinetics were determined in PBS supplemented with 10 mM Tris, which was adjusted to individual pH values of pH 9-5, 9-0, 8-5, 8-0, 7-5, 7-0, 6-7 or 6-5, respectively. Cells were washed twice in the pH-specific uptake buffer prior to starting the uptake assay. For the substrate saturation kinetics of myo-inositol and H⁺, the apparent Kₘ and Vₘₐₓ values were determined by least squares fitting of the data to the Michaelis–Menten equation: $V = \frac{V_{\text{max}} [S]}{K_{\text{m}} + [S]}$, where $S$ represents either myo-inositol or proton, employing the Levenberg–Marquardt algorithm (KALEIDOGRAPH program; Synergy Software) (Seyfang & Landfear, 2000). The regression coefficient for Michaelis–Menten equation fit was 0.995 for myo-inositol kinetics and 0.998 for proton kinetics. Linearization of the substrate saturation kinetics was obtained by Hanes plot transformation of the data through plotting of $[S]/V$ versus $[S]$, which prevents the distortion of error limits for small substrate concentrations as found for linearization methods that use reciprocal substrate values like Lineweaver–Burk plot ($1/V$ versus $1/[S]$) or Eadie–Hofstee plot ($V$ versus $[S]/V$) (Bisswanger, 2002).

Inhibitor and substrate specificity studies. For inhibitor studies, protonophores or other potential inhibitors were applied from stock solutions in ethanol (FCCP, dinitrophenol, cytochalasin B, phloretin, phlorizin) or PBS (sodium azide) and yeast cells were pre-incubated with the drugs for 10 min at 25 °C prior to the initiation of uptake assays. Cells incubated with 0·5 % ethanol served as a control and did not show any effect of the ethanol on inositol uptake activity. For substrate specificity studies, potential competitors like hexose and pentose sugars (Fig. 4) or inositol isomers and derivatives (Fig. 5) were added to the radiolabelled myo-inositol cocktail from stock solutions in PBS. Statistical analysis of the data was performed by the paired-sample t-test with two-tailed $P$ values (Zar, 1984), and data were considered significantly different from the control if $P < 0·05$.

RESULTS

Kinetics of high-affinity myo-inositol transport

We determined the kinetic parameters of myo-inositol uptake by C. albicans using a rapid oil-spinning technique (Seyfang et al., 1997), which enabled the separation of cells and radiolabelled substrate within less than 10 s by spinning through an oil cushion and subsequent flash-freezing in a dry ice/ethanol bath. The accuracy of this method was significantly improved over filtration methods for uptake assay termination, by reducing background levels and variability that would result from trapped moisture in the filters and/or additional washing steps. Furthermore, parallel uptake assays could be terminated simultaneously in the same centrifugation step and thus reduced the time between harvesting of the yeast cells and completion of uptake assays.

myo-[2-³H]inositol uptake in C. albicans was time-dependent and saturable, and substrate saturation curves obeyed Michaelis–Menten kinetics (Fig. 1), as was expected for saturable carrier-mediated transport. From the initial, linear uptake rate at each substrate concentration an apparent Michaelis constant of $K_{\text{m}} = 240 \pm 15 \mu$M myo-inositol concentration and a $V_{\text{max}}$ value of $131 \pm 6$ pmol min⁻¹ per $5 \times 10^7$ cells could be determined. This high-affinity inositol transport in C. albicans is well comparable with the apparent substrate affinities determined for inositol transport in Schizosaccharomyces pombe ($K_{\text{m}} = 0.26 \pm 0.04$ mM; Cheneval et al., 1970) and Saccharomyces cerevisiae ($K_{\text{m}} = 0.1$ to 0.47 mM; Nikawa et al., 1982, 1991; Lai & McGraw, 1994), or the protozoan flagellate Leishmania donovani ($K_{\text{m}} = 0.25 \pm 0.05$ mM; Drew et al., 1995). Subsequent linearization of the data by Hanes plot transformation

![Fig. 1. Substrate saturation kinetics of myo-inositol uptake in C. albicans. Uptake of myo-[2-³H]inositol in Candida yeast cells was monitored between 10 μM and 1·5 mM myo-inositol concentration at pH 7·4 in PBS, using the rapid oil-spinning technique. From the initial, linear uptake rate at each substrate concentration an apparent Michaelis constant of $K_{\text{m}} = 240 \pm 15 \mu$M myo-inositol was determined (mean ± SD, $n = 3$). Inset, Hanes plot transformation of the data suggested that myo-inositol uptake in C. albicans occurs by a single, high-affinity transport system.](https://mic.sgmjournals.org)
(Fig. 1, inset) revealed a single straight line for the entire substrate range tested (10 μM to 1.5 mM myo-inositol), which demonstrated one single apparent $K_m$ and $V_{max}$ value each for the transport kinetics within the substrate range. These results suggest that myo-inositol uptake in C. albicans occurs primarily by a single (or dominating) high-affinity transport system. Nevertheless, it cannot be ruled out that additional, minor inositol transport system(s) with low transport capacity of less than 5% of Candida’s total inositol transport capacity are hidden beneath the transport kinetics of one dominating transport system.

**Pharmacology and ion specificity of myo-inositol transport in C. albicans differs from mammalian myo-inositol transport in the intestine**

Inositol uptake in the intestine and kidney of the human host is performed by the sodium-dependent myo-inositol transporter SMIT (Kwon et al., 1992), which belongs to the sodium-dependent solute transporter family (Reizer et al., 1994; Turk & Wright, 1997) and utilizes the plasma membrane Na$^+$ gradient to concentrate inositol within epithelial cells. In the protozoan parasite Leishmania donovani, in contrast, inositol uptake is performed by the myo-inositol–H$^+$ transporter MIT which belongs to the large and ubiquitous sugar transporter superfamily (Henderson, 1991; Baldwin, 1993) and couples the transport of protons down a membrane electrochemical gradient to drive the concentration of inositol within the cell (Drew et al., 1995; Seyfang et al., 1997). In addition to these active transporters that utilize sodium or proton ion gradients across the cell membrane to drive concentrative uptake of substrate, many transporters of the sugar transporter superfamily are facilitative transporters, like the red cell glucose transporter GLUT1, include the fungal metabolite cytochalasin B (300 μM) or the intestinal SMIT inositol transport inhibitor phlorizin (300 μM). Inositol transport was sodium-independent and NaCl in the uptake buffer (140 mM NaCl, 25 mM HEPES, pH 7.3) could be replaced by sodium-free uptake buffer (140 mM KCl or 140 mM choline chloride, respectively, 25 mM HEPES, pH 7.3) without loss of myo-inositol transport activity. In contrast, inositol transport was strongly pH-dependent and a 77% reduction of transport activity was observed when the proton concentration of the uptake buffer was reduced from pH 6.5 to pH 8.5. Bars are given as mean±SD of three independent experiments and inositol uptake is expressed as a percentage relative to control uptake (34.3±2.5 pmol min$^{-1}$ per 5x10$^7$ cells). Statistical significance of inhibition compared with the control was found for FCCP ($P<$0.05), dinitrophenol ($P<$0.002) and sodium azide ($P<$0.005), while cytochalasin B, phloretin and phlorizin values were not significantly different from the control ($P>$0.10; paired-sample $t$-test).

Fig. 2. Pharmacology and ion specificity of myo-inositol transport in C. albicans. myo-[2-3H]inositol uptake was measured in the absence (control) or presence of various inhibitors such as protonophores (FCCP, 10 μM; dinitrophenol, 1 mM), sodium azide (1 mM), the red cell GLUT1 glucose transport inhibitors cytochalasin B (300 μM) and phloretin (100 μM) or the intestinal SMIT inositol transport inhibitor phlorizin (300 μM). Inositol transport was sodium-independent and NaCl in the uptake buffer (140 mM NaCl, 25 mM HEPES, pH 7-3) could be replaced by sodium-free uptake buffer (140 mM KCl or 140 mM choline chloride, respectively, 25 mM HEPES, pH 7-3) without loss of myo-inositol transport activity. In contrast, inositol transport was strongly pH-dependent and a 77% reduction of transport activity was observed when the proton concentration of the uptake buffer was reduced from pH 6.5 to pH 8.5. Bars are given as mean±SD of three independent experiments and inositol uptake is expressed as a percentage relative to control uptake (34.3±2.5 pmol min$^{-1}$ per 5x10$^7$ cells). Statistical significance of inhibition compared with the control was found for FCCP ($P<$0.05), dinitrophenol ($P<$0.002) and sodium azide ($P<$0.005), while cytochalasin B, phloretin and phlorizin values were not significantly different from the control ($P>$0.10; paired-sample $t$-test).

A quantification of the inhibitor profile and ion specificity of the C. albicans inositol transport system is given in Fig. 2. Cells were pre-incubated for 10 min with the various drugs prior to initiation of uptake assays to allow the drugs to enter the cells and develop their specific pharmacological activity. The protonophores FCCP and dinitrophenol, which collapse transmembrane proton gradients, showed a pronounced inhibitory effect on myo-inositol transport in C. albicans. A transport inhibition of 41±10% and 88±4%, respectively, was observed for the two proton uncouplers and strongly suggested that myo-inositol transport in C. albicans is proton-coupled. Furthermore, sodium azide showed 69±6% inhibition of transport activity and indicated that Candida myo-inositol transport is energy-dependent. Azide is a poison of oxidative phosphorylation and thus reduces cellular ATP levels and the processes that rely on ATP hydrolysis, including ion pumps that maintain a proton-electrochemical gradient across the cell membrane. Typical inhibitors of transporters that operate by facilitated diffusion, such as the erythrocyte glucose transporter GLUT1, include the fungal metabolite cytochalasin B and the drug phloretin (Garcia et al., 1992; Walmsley et al., 1998; Seyfang & Duszenko, 1991), neither of which inhibited C. albicans inositol uptake even at
concentrations of 300 and 100 μM, respectively. For comparison, in another lower eukaryote, the protozoan flagellate Trypanosoma brucei, the facilitated diffusion transporter for glucose was inhibited 76 and 60%, respectively (Seyfang & Duszenko, 1991), by the same concentrations of cytochalasin B and phloretin that did not affect the inositol transport system of Candida (Fig. 2). Sodium-dependent inositol transport by SMIT in the human intestine and kidney is strongly inhibited by the glucoside phlorizin with a $K_i$ value of 64–76 μM (Hager et al., 1995; Coady et al., 2002).

In C. albicans, however, no inhibition of inositol transport was observed even at 300 μM phlorizin concentration (Fig. 2). The sodium independence of C. albicans myo-inositol transport was unambiguously shown by uptake in sodium-free buffer and replacement of NaCl by KCl or choline chloride without loss of transport activity. In contrast, inositol transport in C. albicans was strongly pH-dependent and a 77% reduction of transport activity was observed upon reducing the uptake buffer’s proton concentration from 316 nM H$^+$ (pH 6.5) to 3.2 nM H$^+$ (pH 8.5) (Fig. 2). This further supported the presence of a proton-coupled myo-inositol transport system in C. albicans, as already shown by the strong inhibitory effect of the two protonophores FCCP and dinitrophenol above.

**Proton dependence of C. albicans myo-inositol transport**

Following our finding that C. albicans myo-inositol transport is strongly pH-dependent and apparently proton-coupled (Fig. 2), we determined substrate saturation kinetics as a function of various proton concentrations. Uptake of radiolabelled myo-inositol was monitored at eight different proton concentrations for the linear uptake range at 100 μM inositol concentration. Proton saturation kinetics obeyed classical Michaelis–Menten kinetics and an apparent $K_m$ value of 11·0 ± 1·1 nM H$^+$, equal pH 7·96 ± 0·05, was determined from the data (Fig. 3). This remarkably high proton affinity and $K_m$ in the alkaline pH range revealed that at physiological pH around pH 7·4 the C. albicans myo-inositol transporter is already fully activated by H$^+$ binding and apparently operating at near-$V_{max}$ conditions for proton binding. For sodium-coupled inositol transport by the human SMIT, a sigmoid sodium saturation curve is observed, characteristic for the subsequent binding of two sodium ions with a positive co-operativity effect between two sodium binding sites (Matskevitch et al., 1998; Coady et al., 2002). Thus, care was taken to obtain a good data point coverage for proton concentrations below or equal the $K_m$ value at the very beginning of the saturation curve and no sigmoidal course could be observed for proton saturation kinetics of C. albicans myo-inositol transport (Fig. 3), suggesting that there is one proton binding site and no co-operativity effect between multiple protons bound subsequently to multiple binding sites of the transporter. Linearization of the data by Hanes plot transformation further supported this observation with a single straight line (Fig. 3, inset).

**High substrate specificity of C. albicans myo-inositol transport and no competition in the presence of high sugar levels**

Substrate specificity is one of the fundamental features that characterizes different transporters and of particular interest when it comes to rational drug design or the delivery of cytotoxic substrate analogues via uptake by a specific permease. For inositol transport, monosaccharides like hexose or pentose sugars are of particular interest since, as pyranoses, they constitute the structurally closest molecules to the cyclic polyalcohol inositol. Furthermore, sugars can be found at often high concentrations in the intestine or tissues that are colonized by Candida and they constitute a principal carbon source for yeasts. Substrate selectivity of myo-inositol transport in C. albicans was probed by measuring the uptake of radiolabelled myo-inositol in the absence (control) or presence of 100-fold excess of unlabelled sugars as potential competitors (Fig. 4).

None of the four hexose sugars D-glucose, D-mannose, D-galactose or D-fructose showed any inhibition of myo-[2$^3$H]inositol uptake even at 100-fold excess, compared to 98% inhibition by unlabelled myo-inositol as an additional positive control (Fig. 4). Of interest is the 78 and 87% stimulation of inositol transport by D-glucose and

![Image](http://mic.sgmjournals.org)
was observed for xylose or any of the four pentoses as either transport (cf. inhibition by the cytochrome inhibitor azide, membrane gradient and stimulates proton-coupled membrane activity and ATP supply that supports the proton-electrochemical membrane gradient and thereby stimulates proton-coupled membrane transport. This explanation is also supported by the inhibitory effect of the cytochrome inhibitor azide (Fig. 2), which reduces intracellular ATP levels. The pentose sugar D-xylose is the structurally closest potential competitor to myo-inositol (see Fig. 6), but no inhibition was observed for xylose or any of the four pentoses as either D- or L-stereoisomer. The pyranose L-fucose (= 6-deoxy-L-galactose or 5-methyl-D-arabinose), which is increased in patients with diabetes (Sima et al., 1997), is a strong competitive inhibitor and substrate for the human SMIT (Hager et al., 1995) but did not affect C. albicans myo-inositol transport. Hence, the Candida transport system is characterized by a particularly high substrate specificity, which appears to be unaffected by the presence of high levels of sugars, in remarkable contrast to the human sodium-coupled transport system SMIT in the intestine and kidney which also recognizes L-fucose and L-xylose as substrates (Hager et al., 1995). A second sodium-coupled myo-inositol transporter isof orm, SMIT2, recently described in rabbit and human also shows a lower substrate specificity and recognizes myo-inositol, D-glucose and D-xylose as substrates (Coady et al., 2002).

**C-2, C-3 and C-4 hydroxyl groups of myo-inositol are critical for substrate recognition by the C. albicans myo-inositol transport system**

With this remarkably high substrate specificity of the C. albicans myo-inositol transporter, we were prompted to probe the substrate recognition of this fungal inositol permease in more detail. Radiolabelled myo-inositol uptake was measured in the absence (control) or presence of 100-fold excess of eight different unlabelled inositol isomers, which differ only in the position of their hydroxyl groups above (cis) or below (trans) the carbon ring plane. Moreover, several of these inositol isomers differed in the position of only one single hydroxyl group (scyllo-, D-chiro-, neo- and epi-inositol; Fig. 5) and thus were ideally suited to probe the significance of a specific hydroxyl group position for substrate recognition and binding by the fungal permease.

myo-Inositol was by far the best substrate and competitor with 97 ± 2 % inhibition for uptake of radiolabelled myo-inositol in C. albicans (Fig. 5). No inhibition of myo-[2-3H]inositol uptake in Candida, even at 100-fold excess, was observed for scyllo-inositol or D-chiro-inositol, which differ from the myo-inositol isomer only in the position of the hydroxyl group at C-2 or C-3, respectively (Fig. 5), suggesting that both C-2 and C-3 hydroxyl are critical for substrate recognition and binding to the fungal permease. Accordingly, L-chiro-inositol (P > 0.05) and muco-inositol (P < 0.05) showed no significant or only very weak inhibition, respectively, since they both carry a C-3 hydroxyl in *trans* position while myo-inositol as the optimum substrate has a C-3 hydroxyl in *cis* position. Furthermore, C-4 hydroxyl in *trans* position appeared to be important for transporter binding since epi-inositol showed only very weak inhibition of 13 ± 7 % (P < 0.05) and allo-inositol did not show any inhibition at all (P > 0.50). neo-Inositol, with the orientation of the C-5 hydroxyl as the only difference to myo-inositol, showed strong inhibition of 55 ± 3 %, suggesting that C-5 hydroxyl alone is not essential for substrate recognition. Remarkable is the pronounced inhibition of 95 ± 1 % by 3-fluoro-3-deoxy-my o-inositol, which carries a fluoride at the same position as the C-3...
hydroxyl group of myo-inositol in cis orientation (Fig. 6). This showed that the C-3-fluorinated myo-inositol analogue has a similar affinity for the transporter as myo-inositol. Furthermore, it strongly suggests that the C-3 hydroxyl oxygen acts as an electron donor to form a proton bond with a positive charge of the Candida permease, a function that could be fully restored by replacement with the negative fluoride ion.

Five additional inositol derivatives were tested for their inhibitory effect on C. albicans inositol transport. The O-methylated chiro-inositol derivatives L-quebrachitol (2-O-methyl-L-chiro-inositol) and D-pinitol (3-O-methyl-D-chiro-inositol) showed little or no inhibition of myo-inositol transport, as did the chiro-inositol isomers from which they were derived. Interesting is the 69 ± 3% inhibition by viburnitol (1-D-3-deoxy-my-o-inositol), which lacks a hydroxyl group at C-3, suggesting that steric hindrance of the C-3 hydroxyl in trans position of D-chiro-inositol may also contribute to the inability of this isomer to bind to the Candida transporter. Similarly, steric hindrance may contribute to the loss of substrate affinity in epimyoinositol, where the C-4 hydroxyl is orientated in the opposite direction to that in myo-inositol, whereas D-ononitol (1-D-4-O-methyl-my-o-inositol) showed 58 ± 4% inhibition with a C-4 O-methyl group in the same direction as the C-4 hydroxyl group in myo-inositol. Phytic acid (myo-inositol-1,2,3,4,5,6-hexakisphosphate) is the major inositol derivative in plants owing to its function as the primary phosphorus store in plant material, particularly in seeds. Since phytic acid is also found in the human vegetarian diet, it was interesting to see its 44 ± 4% inhibition of myo-inositol transport in Candida, which may be exposed to high levels of phytic acid in the intestine depending on the diet of its human host. Since the six phosphate groups of phytic acid face the same direction as the hydroxyl groups of myo-inositol, it may be possible that phoshide ions can replace the function of the oxygen ions of myo-inositol as potential electron donors for binding to the fungal permease. Our inhibitor studies did not answer the question, however, if phytic acid only inhibited myo-inositol uptake or if in addition it was also transported by the C. albicans myo-inositol uptake system. An unambiguous answer to this question is awaiting the molecular cloning and heterologous expression of a C. albicans myo-inositol transporter in Xenopus laevis oocytes or ITR1-less Saccharomyces cerevisiae.

**DISCUSSION**

In eukaryotic organisms myo-inositol is an essential constituent of at least three different classes of molecules and cellular functions, many of which have been identified in Saccharomyces cerevisiae as a model yeast. First, it is a component of phosphatidylinositol as one of the four principal phospholipids of biological membranes and
myo-Inositol is a vitamin-like growth factor and important for optimum growth of yeast cells (Nikawa et al., 1982, 1991). Hence, inositol deprivation of cells can result in the phenomenon of ‘inositol-less death’, and membrane damage and osmotic imbalance have been reported for inositol-starved inositol auxotrophs of Saccharomyces cerevisiae (Atkinson et al., 1977; Ulaşewski et al., 1978). Furthermore, for the fission yeast Schizosaccharomyces pombe partial inositol starvation resulted in elongated hyphae-type or swollen cell shape, a morphology that could also be induced by isomylitol (2-C-methyl-myoinositol), a competitive inhibitor of myo-inositol uptake and inositol phospholipid biosynthesis in Schizosaccharomyces pombe (Deshusses et al., 1969). Moreover, another myo-inositol derivative, 2-C-methylene-myoinositol oxide, induced the formation of pseudohyphae in Schizosaccharomyces pombe (Schopfer et al., 1962; Deshusses et al., 1969). This remarkable observation in the usually monomorphic yeast Schizosaccharomyces pombe is of particular interest for the morphogenesis, phosphoinositol 3-kinase has been shown to be important for hyphae formation and virulence (Cutler, 1991; Soll, 2002). Of the multiple signalling pathways that regulate C. albicans morphogenesis, phosphoinositol 3-kinase has been shown to be important for hyphae formation and virulence (Bruckmann et al., 2000), as an example of how myo-inositol and phosphoinositide signalling can affect Candida hyphae formation and virulence. Furthermore, conserved signalling pathways have been identified that are shared amongst different yeast species and affect the formation of hyphae in C. albicans and pseudohyphae in Saccharomyces cerevisiae (Brown, 2002), which underscores the potential significance of the pseudohyphae-inducing myo-inositol derivatives described for Schizosaccharomyces pombe.

C. albicans has the opportunity to salvage inositol directly from the human diet when it colonizes the intestine and oral mucosa, but when the intestinal benign commensal becomes an opportunist pathogen Candida can also

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**Fig. 6.** Schematic model for the substrate recognition and binding of the C. albicans myo-inositol transport system. (a) From the competition studies with inositol isomers and derivatives (Fig. 5) it was revealed that the C-2, C-3 and C-4 hydroxyl groups of myo-inositol play a critical role in substrate recognition and binding of the transporter. Thus, alteration of the position of a single hydroxyl in the recognition and binding of the transporter. Hence, the electron donor function of the C-3 hydroxyl oxygen could be fully restored by a fluoride ion at this critical position. (b) Structures of D-xylose, the most similar sugar to myo-inositol, and L-fucose, neither of which are recognized by the C. albicans myo-inositol transporter but are good substrates of the human sodium-coupled myo-inositol transport system.

Upon phosphorylation to phosphatidylmyo-inositol 3-phosphate is involved in exocytosis, cytoskeletal reorganization and intracellular membrane trafficking (Wiedemann & Cockcroft, 1998; Simonsen et al., 2001). Second, phosphatidylmyo-inositol 4,5-bisphosphate plays a central role in the phosphatidylmyo-inositol signal transduction pathways that control many cell cycle events and vital cell functions, which are triggered by cleavage of phosphatidylmyo-inositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate and diacylglycerol (Berridge, 1993). Third, myo-inositol is an essential precursor of GPI-membrane anchors, which are used to anchor membrane proteins or membrane glycolipids to the cell surface. These GPI-membrane anchors are of particular significance and abundance in lower eukaryotes and parasitic protozoa where they can be two to three orders of magnitude more concentrated than in mammalian cells (McConville & Ferguson, 1993). Thus, in *Candida* the GPI-anchored glycolipid phospholipomannan is of particular significance for the pathogenicity of this opportunistic fungus through immunomodulatory functions like stimulation of and binding to macrophages and induction of TNF-α (Trinel et al., 1999). In addition, some soil bacteria such as *Pseudomonas* and Klebsiella spp. (Reber et al., 1977; Deshusses & Reber, 1977) and the pathogenic fungus *Cryptococcus neoformans* (Molina et al., 1999) can utilize myo-inositol as a major carbon source, thus taking advantage of the abundance of inositols in soil and plant materials.

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*C. albicans* has the opportunity to salvage inositol directly from the human diet when it colonizes the intestine and oral mucosa, but when the intestinal benign commensal becomes an opportunistic pathogen *Candida* can also
salvage inositol from the serum, lymphatic fluid or human tissue as it disseminates through the blood stream and colonizes various tissues in the body. *myo*-inositol intake from the average diet is about 1 g per day in the human host, with phytic acid as the major dietary form that is readily dephosphorylated in the intestine to deliver free *myo*-inositol for intestinal absorption. Other dietary inositol sources are *myo*-inositol-containing phospholipids from animal and plant sources. Hence, *C. albicans* can rely on a steady supply of *myo*-inositol in its intestinal environment. In the disseminated form, *C. albicans* could salvage inositol from a human serum concentration of 15–70 μM *myo*-inositol (Ostlund et al., 1993; Kouzuma et al., 2001), which increases further in liver tissue and lymphatic fluid and can reach a concentration of 200–270 μM *myo*-inositol in cerebrospinal fluid (Lentner, 1981). One of the complications associated with diabetes mellitus can be an increased rate of urogenital candidiasis (Goswami et al., 2000; de Leon et al., 2002). At the same time, a 10-fold increase of the urinary *myo*-inositol concentration of diabetics has been reported (Ostlund et al., 1993; Kouzuma et al., 2001), and it appears possible that elevated urinary levels of *myo*-inositol as a growth factor for yeast may contribute to the increased *Candida* growth in diabetes mellitus patients.

Few classes of drugs are effective against these fungal infections, and all of them have limitations with regard to efficacy, side effects and the development of drug resistance. In contrast to the well-studied permeases for nutrient salvage and transport in the human host, remarkably little is known about *Candida* membrane transport proteins, despite their biological and pharmacological significance. In this study, we have analysed the special transport kinetics, the substrate specificity and the pharmacology of *myo*-inositol transport in *C. albicans*, which differed significantly from the human inositol transport system. *Candida* *myo*-inositol transport showed a substrate affinity of $K_m = 240$ μM, which is about 4–20 times higher than the substrate affinities with millimolar $K_m$ values of mammalian sugar transporters like the GLUT1 glucose transporter in erythrocytes (Baldwin, 1993). Nevertheless, the fungal high-affinity *myo*-inositol uptake system has to compete with a high-affinity sodium-coupled inositol transport system SMI in the human host, which has a $K_m$ of 50–120 μM (Hager et al., 1995; Coady et al., 2002). Both transport systems are active and energy-dependent, but our inhibitor and ion specificity studies revealed that *myo*-inositol transport in *C. albicans* was proton-coupled and sodium-independent with a proton affinity of $K_m = 11$ nM H$^+$, in contrast to human sodium-coupled *myo*-inositol transport by the two SMIT isomers with a sodium affinity of $K_m = 13–76$ mM Na$^+$ (Hager et al., 1995; Coady et al., 2002). Remarkable is the high substrate specificity of *C. albicans* *myo*-inositol transport, which is of particular significance for the direct competition between fungal and human uptake systems. While none of the 14 different hexose and pentose sugars tested had any inhibitory effect on *Candida* *myo*-inositol uptake even at 100-fold excess, the human SMI transport system has a considerably lower substrate specificity and also transports various sugars like glucose, xylose and fucose (Hager et al., 1995; Coady et al., 2002). Since sugars can be found in abundance in the human diet, intestine and body fluids, *C. albicans* *myo*-inositol transport appears to have a significant advantage over the human inositol transport system with its lower substrate specificity. Recently, an unusual proton-coupled *myo*-inositol transporter HMIT has been described in intracellular vesicles and cytoplasmic structures of mammalian brain cells (Uldry et al., 2001); the physiological function of HMIT remains unclear since it appears to be absent from the plasma membrane and required the mutation of seven amino acids in three different motifs for functional characterization. Because of its limited tissue distribution predominantly in the brain and its intracellular localization, HMIT does not appear to affect *myo*-inositol transport in *C. albicans*.

The remarkably high substrate specificity of the *Candida* *myo*-inositol transport system prompted us to probe the apparent structural requirements for substrate recognition and binding of the fungal permease in more detail. *myo*-inositol is the most abundant of nine naturally occurring inositol isomers. Most of these isomers occur predominantly in plant materials as secondary metabolites, but *myo*-, *D*-chiro- and *L*-chiro-*inositol are also found in human tissue and secreted in the urine (Ostlund et al., 1993). We used eight of these isomers as competitors with radiolabelled *myo*-inositol and found that *neo*-inositol was the only other inositol isomer recognized by the *Candida* *myo*-inositol transport system, suggesting that the C-5 hydroxyl is not important for substrate recognition. Neither scylo-, *D*-chiro- nor *epi*-inositol affected *myo*-[2-$^3$H]*inositol uptake even at 100-fold excess, and our studies showed that C-2, C-3 and C-4 hydroxyls are critical for substrate recognition in *C. albicans* *myo*-inositol transport. Furthermore, this finding allowed an explanation for the high substrate specificity of the fungal transport system: the critical C-2 hydroxyl in *myo*-inositol is missing in all pyranose sugars where the ring oxygen is occupying the C-2 position of *myo*-inositol (Fig. 6). This finding for the *Candida* transporter is unique compared to the other characterized inositol transporters which all recognize scylo-inositol as a substrate [human SMI and SMI2 (Hager et al., 1995; Coady et al., 2002), human HMIT (Uldry et al., 2001), *Leishmania* donovani MIT (Drew et al., 1995), *Klebsiella* aerogenes inositol transporter (Deshusses & Reber, 1977)]. Furthermore, in the human host, SMI2 (Coady et al., 2002) and HMIT (Uldry et al., 2001) also recognize *D*-chiro*-inositol with high affinity.

The specific kinetics and substrate selectivity that characterize and distinguish the *C. albicans* *myo*-inositol transporter from the transport systems in the human host are further complemented by the different pharmacology between the fungal and human systems. *C. albicans*...
myo-inositol transport was not affected by phlorizin concentrations five times higher than the \( K_v \) value for human SMIT. One class of drugs against Candida includes fluoro-nucleoside analogues such as 5-fluorocytosine (Georgopapadakou & Walsh, 1996). Moreover, fluorinated myo-inositol analogues as 3-fluoro-3-deoxy-myoinositol have been reported to possess anti-proliferative activity in cancer cells through inhibition of phosphatidylinositol signalling (Powis et al., 1991; Offer et al., 1993; Johnson et al., 1993). Hence, our finding that 3-fluoro-3-deoxy-myoinositol is recognized by the C. albicans myo-inositol transporter with similar affinity as myo-inositol is particularly encouraging to further probe the Candida myo-inositol transporter as an attractive target for rational drug design and delivery of cytotoxic substrate analogues.

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by irreversible inactivation of permease and

incorporation into phospholipid in permeabilized cells.

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