Serum albumin promotes ATP-binding cassette transporter-dependent sterol uptake in yeast

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Abstract

Sterol uptake in fungi is a multistep process that involves interaction between external sterols and the cell wall, incorporation of sterol molecules into the plasma membrane, and subsequent integration into intracellular membranes for turnover. ATP-binding cassette (ABC) transporters have been implicated in sterol uptake, but key features of their activity remain to be elucidated. Here, we apply fluorescent cholesterol (NBD-cholesterol) to monitor sterol uptake under anaerobic and aerobic conditions in two fungal species, Candida glabrata (Cg) and Saccharomyces cerevisiae (Sc). We found that in both fungal species, ABC transporter-dependent uptake of cholesterol under anaerobic conditions and in mutants lacking HEM1 gene is promoted in the presence of the serum protein albumin that is able to bind the sterol molecule. Furthermore, the C. glabrata ABC transporter CgAus1p expressed in S. cerevisiae requires the presence of serum or albumin for efficient cholesterol uptake. These results suggest that albumin can serve as sterol donor in ABC transporter-dependent sterol uptake, a process potentially important for growth of C. glabrata inside infected humans.

Introduction

Sterols constitute an essential lipid class in eukaryotic membranes that determine membrane characteristics important for permeability, transport, and sorting (Jacquier & Schneiter, 2012). While cholesterol constitutes the main sterol found in mammalian cells, ergosterol is the primary sterol in fungal cell membranes. As a result, ergosterol and its biosynthetic pathway are important molecular targets for a number of antifungal agents. Polylene antibiotics such as amphotericin B are toxic to fungi due to their interaction with plasma membrane ergosterol (Ellis, 2002; Gray et al., 2012; Anderson et al., 2014), while other drugs such as azoles target the biosynthesis of ergosterol (Maertens, 2004; Carrillo-Munoz et al., 2006).

Some pathogenic fungi, such as Candida glabrata (Cg) can utilize exogenous cholesterol as a structural analogue and surrogate for ergosterol, thereby diminishing the effect of the ergosterol-specific antifungals (Bard et al., 2005; Hazen et al., 2005; Nakayama et al., 2007; Nagi et al., 2013a, b). Thus, systemic infections caused by this pathogenic yeast are difficult to treat (Pfaller et al., 2006; Pappas et al., 2009). The nonpathogenic yeast Saccharomyces cerevisiae (Sc), which is closely related to C. glabrata (Barns et al., 1991), does not incorporate exogenous sterols for growth under aerobic conditions, a phenomenon known as ‘aerobic sterol exclusion’ (Jacquier & Schneiter, 2012). However, under anaerobic growth conditions, or in mutants with a defective heme biosynthesis (which mimics anaerobic conditions in the presence of oxygen), S. cerevisiae depends on sterol uptake from the environment. This sterol uptake involves two plasma membrane ATP-binding cassette (ABC) transporters, ScAus1p and ScPdr11p (Lorenz & Parks, 1991; Wilcox et al., 2002; Li & Prinz, 2004; Reiner et al., 2006). Loss of both proteins completely abolishes sterol uptake, resulting in an anaerobic lethal phenotype (Li & Prinz, 2004).
An in silico-based search for sterol transporter genes in *C. glabrata* identified CgAUS1 as a single orthologue of the *ScAUS1* and *ScPDR11* genes, and previous studies revealed an essential role for CgAus1p in cholesterol uptake (Nagi et al., 2013a, b). Notably, the CgAus1p-dependent cholesterol uptake required fetal calf serum (FCS) in the growth media. FCS is a complex mixture of proteins, lipids, and growth factors. However, the nature of the promoting serum compounds has yet to be identified. In this study, we employ fluorescently labeled cholesterol (NBD-cholesterol) to investigate the effect of serum proteins on ABC transporter-dependent sterol uptake in *C. glabrata* and *S. cerevisiae*.

**Materials and methods**

**Materials**

Yeast and bacteria growth media reagents were supplied from Difco. Phusion high-fidelity DNA polymerase and restriction enzymes were purchased from New England Biolabs (NEB). Prestained SDS-PAGE molecular markers were obtained from Thermo Scientific. Oligonucleotides were custom made by Integrated DNA Technologies (IDT). Anti-FLAG mouse M2 monoclonal antibody was purchased from Sigma-Aldrich (Denmark A/S). Alkaline phosphatase-conjugated polyclonal rabbit anti-mouse secondary antibody was obtained from Dako A/S (Glostrup, Denmark). NBD-cholesterol (25-[N-[7-nitro-2-1,3-benzoxadiazol-4-yl]methyl]amino]-27-norcholesterol) was purchased from Avanti Polar Lipids (Alabaster, AL). Tetramethylrhodamine-conjugated bovine serum albumin (BSA) and BCECF-AM were purchased from Life Technologies (Molecular Probes). All other reagents including heat-inactivated FCS, normal and essentially fatty acid-free BSA (fraction V) were reagent grade or better and heat-inactivated FCS, normal and essentially fatty acid-free BSA (fraction V) were reagent grade or better and were purchased from Sigma-Aldrich (Denmark, Brøndby) unless otherwise indicated.

**Strains and growth media**

For all plasmid amplifications, the *Escherichia coli* SURE strain was used and grown at 37 °C in either Luria–Bertani (LB) broth (Pappas et al.) or on LB agar plates. When appropriate, media were supplemented with ampicillin at 50 µg mL⁻¹ for plasmid selection. All yeast strains used in this study are listed in Table 1. Yeast strains were cultured at 30 °C in standard synthetic dextrose (SD) or galactose (SG) medium and when necessary supplemented with 20 µg mL⁻¹ 3-aminolevulinic acid (ALA). Medium supplemented with sterols contained 0.1% Tween 80 and 20 µg mL⁻¹ sterols. Solid media were added 2% (w/v) agar. Growth was undertaken under aerobic conditions unless stated otherwise. Anaerobic conditions were generated using an Anaerocult A system (Millipore). For drop dilution assays, cells grown overnight in liquid ALA-supplemented SD medium were washed twice with sterile water, adjusted to 0.2 OD₆₀₀ nm and subjected to consecutive fivefold dilution steps. Drops of 3 µL were spotted on standard synthetic galactose plates containing the indicated supplements, and plates were incubated for 4 days at 30 °C.

**Plasmid construction and yeast transformation**

PCR fragments containing the CgAUS1 gene was amplified from genomic DNA of *C. glabrata* (strain KUE200) using primers that contained homologous sequences for recombination with the EcoRI-digested vector pESC-URA (Agilent Technologies). *In vivo* recombination in yeast (strain W303-1a) between the PCR fragment and the EcoRI-digested vector led to formation of the pESC-URA-CgAUS1 plasmid in the cells. For generating the CgAus1p C-terminal fusion with GFP, a PCR fragment containing the GFP tag was amplified from plasmid pYM 27 (Euroscarf) using primers that contained homologous sequences for recombination with the NotI-digested vector pESC-URA-CgAUS1; *in vivo* recombination in yeast yielded plasmid pESC-URA-CgAUS1-GFP. Plasmids were recovered from yeast grown in liquid uracil dropout medium using QIAprep Miniprep.

Plasmid p122-ERG6-RFP plasmid in which ERG6-mRFP fusion gene is placed under the control of GalS promoter was constructed by first PCR-amplifying ERG6 gene from the genomic DNA of *S. cerevisiae* (strain W303-1a). The PCR product was then cotransformed along with XbaI-digested p122 vector (kind gift from Dr M. Surma, MPI-CBG, Dresden, Germany) into *S. cerevisiae* (strain W303-1a). Correct recombination between the PCR fragment and vector was confirmed by DNA sequencing. Site directed mutagenesis of CgAUS1 K793M was performed with the Quick Change XL Gold Mutagenesis Kit (Stratagene, La Jolla, CA) according to manufacturer conditions. Correct vector construction was confirmed by DNA sequencing. Yeast cells were transformed by the lithium acetate method (Ito et al., 1983). All oligonucleotide primers used in the study are listed in Table 2.

**Protein expression analysis**

Yeast transformants containing the expression plasmids were grown for 16 h in SG medium, and crude cellular membranes were isolated via glass bead lysis (Marek et al., 2011), separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (PVDF, Millipore).
Table 1. Yeast strains used in this study

<table>
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<th>Strain name</th>
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<td>Roland Lill, Philpps-Universität Marburg</td>
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<td>aus1Δ pdr11Δ</td>
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<td>hem1Δ</td>
<td>W303 pdr11Δ:loxP aus1Δ:loxP-HIS5Sp-loxP</td>
<td>Marek et al. (2011)</td>
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<tr>
<td>hem1Δ aus1Δ pdr11Δ</td>
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<td>Marek et al., 2011;</td>
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<td>Marek et al. (2011)</td>
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<td>This study</td>
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<tr>
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<td>This study</td>
</tr>
<tr>
<td>hem1Δ aus1Δ pdr11Δ + CgAUS1&lt;sup&gt;K793M&lt;/sup&gt;</td>
<td>W303 hem1Δ:LEU2 pdr11Δ:loxP aus1Δ:loxP-HIS5Sp-loxP &lt;pESC-URA-CgAUS1&lt;sup&gt;K793M&lt;/sup&gt;&gt;</td>
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<td>C. glabrata KUE200</td>
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<td>KUE200 aus1Δ</td>
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<td>Martin Zavrel, University of Missouri, Kansas City</td>
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Table 2. Templates and primers used in PCR amplifications for plasmid construction

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<th>Gene</th>
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<th>Primer</th>
<th>Oligonucleotide sequence</th>
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Uptake assays

Uptake of NBD-cholesterol was analyzed essentially as described (Marek et al., 2011), with minor modifications. Briefly, cells were cultured for 22 h in minimal medium containing 0.1% Tween 80 and 5 μg mL<sup>-1</sup> NBD-cholesterol. When appropriate, media were supplemented with 25% (v/v) FCS or 25% (v/v) fatty acid-free BSA if not stated otherwise. Before analysis by flow cytometry or confocal microscopy, cells were washed twice with ice-cold PBS containing 0.5% (v/v) Nonidet P-40, once with PBS and finally resuspended in PBS. Uptake of [<sup>14</sup>C]cholesterol was analyzed in cells cultured for 22 h in minimal medium containing 0.1% Tween 80, 20 μg mL<sup>-1</sup> NBD-cholesterol and 0.05 μCi mL<sup>-1</sup> [<sup>14</sup>C]cholesterol (Hartmann Analytic GmbH, Braunschweig, Germany). Equal optical density (OD) units of cells were collected, washed twice with ice-cold PBS containing 0.5% (v/v) Nonidet P-40 and finally washed twice with H<sub>2</sub>O. Cells were disrupted with glass beads in the presence of chloroform/
methanol (1 : 1), and lipids were extracted into the organic phase according to Bligh & Dyer (1959) and measured for radioactivity by scintillation counting. For BSA uptake studies, cells were incubated for 22 h with tetramethylrhodamine-conjugated albumin or free tetramethylrhodamine (both 100 µg mL⁻¹). For vacuolar labeling, cells were incubated with 50 µM BCECF-AM (Invitrogen) at 30 °C for 30 min before harvesting.

**Sterol analysis**

Total lipid extracts were separated by thin-layer chromatography (TLC) on silica gel 60 (Merck, Darmstadt, Germany) using hexane/diethyl ether/formic acid (80 : 80 : 20, v/v/v). Sterol standards were chromatographed on the same plate. Fluorescent and ¹⁴C-containing radiolabeled lipid spots were visualized using a Typhoon Trio variable-mode imager (GE Healthcare).

**Flow cytometry**

Flow cytometry was performed on a Becton Dickinson FACS Calibur (San Jose, CA) equipped with an argon laser using CELL QUEST software. One microliter of a 1 mg mL⁻¹ propidium iodide/formic acid solution was added to 1 × 10⁷ cells in 1 mL of PBS just before analysis. Twenty thousand cells were analyzed without gating during the acquisition. Live cells were selected based on forward-/side-scatter gating and propidium iodide exclusion. A histogram of the green fluorescence (GFP, NBD) of living cells was used to calculate the mean fluorescence intensity of total cells. Auto fluorescence of empty vector (e.v.)-transfected cells was detected as control. Data were analyzed with FLOWJO software (Tree Star).

**Fluorescence microscopy**

A confocal laser scanning microscope (Leica SP5-X) equipped with a 63x (numerical aperture 1.35) oil-immersion objective was used for all the microscopic studies. GFP and NBD-cholesterol were excited using a 488 nm laser and emission recorded between 500 and 560 nm; Tetramethylrhodamine was excited at 514 nm and emission recorded between 530 and 630 nm. For visualization of mRFP, fluorescence samples were excited at 536 nm, and emission was recorded between 574 and 700 nm. BCECF-AM was excited at 488 nm and emission between 500 and 580 nm was recorded.

**Sterol binding to BSA**

Binding of NBD-cholesterol to albumin was analyzed using a Fluoromax-4 spectrophotometer (Horiba, Edison, NJ) as previously described (Liu et al., 2009). NBD-cholesterol (from a methanol stock) was mixed with fatty acid-free BSA in a molar ratio of 1 : 34 or 1 : 400 and the emission intensity was recorded (excitation at 470 nm; emission at 520 nm).

**Statistical analysis**

Results are given as mean ± SD. Statistical analysis was carried out using Student’s t-test. A value of P < 0.05 was considered as statistically significant. All experiments were repeated at least three times (separate cell preparations) if not stated otherwise.

**Results**

**ABC transporter-dependent sterol uptake in the absence of serum**

We first assessed the uptake of NBD-cholesterol by *C. glabrata* and *S. cerevisiae* grown in the absence of FCS for 22 h under aerobic and anaerobic conditions. When examined by fluorescence microscopy, anaerobically grown cultures of both fungal species exhibited a strong staining of intracellular punctate structures (Fig. 1a, anaerobic). Colocalization studies with the lipid droplet marker protein Erg6–mRFP during NBD-cholesterol uptake in *S. cerevisiae hem1Δ* cells (which mimic anaerobic conditions in the presence of oxygen), identified the intracellular punctate structures as lipid droplets (Fig. 1b). Aerobically grown cultures of either fungal species did not show any intracellular fluorescence (Fig. 1a, aerobic), indicating that uptake of NBD-cholesterol was below the detection limit under these conditions. Consistent with this, analysis by flow cytometry revealed a substantial reduction in the level of cell-associated NBD fluorescence for aerobically vs. anaerobically grown cultures of both fungal species (Fig. 2a and b; note y-axis log scale). The anaerobic uptake of NBD-cholesterol was virtually abolished in the *S. cerevisiae aus1Δpdr11Δ* and *C. glabrata aus1Δ* strains (Fig. 2a and b), confirming the essential role of these ABC transporters for sterol influx in both yeast species (Wilcox et al., 2002; Li & Prinz, 2004; Nagi et al., 2013a, b).

**Effect of serum on sterol uptake**

We next analyzed NBD-cholesterol uptake in the presence of FCS. In both fungal species, FCS substantially increased the uptake of NBD-cholesterol under anaerobic conditions, while the effect was not observed under aerobic conditions or in the ABC transporter-deficient strains *S. cerevisiae* W303aus1Δpdr11Δ and *C. glabrata*.
KUE200aus1Δ (Fig. 2a and b). Likewise, the presence of serum increased accumulation of NBD-cholesterol in *S. cerevisiae* hem1Δ cells (Fig. 2c). Similarly to the anaerobic uptake experiments, this effect was not observed for *S. cerevisiae* hem1Δ cells lacking the ABC transporters ScAus1p and ScPdr11p or grown in media supplemented with ALA (the enzymatic product of the ScHem1p-catalyzed first step in heme biosynthesis) which restores the ergosterol biosynthesis pathway and leads to deactivation of the sterol uptake machinery. Although only a weak signal was detected, we noticed that aerobically grown *C. glabrata* aus1Δ cells exhibited lower NBD fluorescence levels than wild-type cells, albeit only statistically significant for cells grown in the presence of serum (Fig. 2a). Independent of serum supplementation, treatment with inhibitors of the ergosterol pathway such as fluconazole, amorolfine, terbinafine, and fenpropimorph did not stimulate the aerobic NBD-cholesterol uptake under the conditions used here (data not shown).

### Effect of albumin on sterol uptake

Serum contains high levels of albumin that acts as a carrier protein for steroids and fatty acids (Zhao & Marcel, 1996; Ha *et al.*, 2003). Hence, the effect of albumin on NBD-cholesterol uptake by *S. cerevisiae* hem1Δ cells was investigated. Medium supplementation with fatty acid-free albumin (FFA-BSA; A6003, Sigma-Aldrich) resulted in a dose-dependent increase in NBD-cholesterol uptake, which was maximal at an albumin concentration of 25% (Fig. 3a). Although to a lesser extent, this stimulating effect was also evident for normal Fraction V albumin (Fig. 3a, V-BSA; A4503, Sigma-Aldrich), for the uptake of [14C]cholesterol (Fig. 3b) as well as for the anaerobic NBD-cholesterol uptake by wild-type strains of *C. glabrata* and *S. cerevisiae* (Supporting Information, Fig. S1). Pretreatment of BSA by heat inactivation at 56 °C (which is a standard procedure during preparation of commercially available serum) did not abolish the promoting effect. In line with previous reports (Reiner *et al.*, 2006), TLC analysis of lipid extracts prepared from labeled cells confirmed uptake and esterification of intact NBD-cholesterol and [14C]cholesterol (Fig. 3a and b; insets).

To rule out the possibility that the increased NBD-cholesterol uptake in presence of BSA was caused by endocytosis, *S. cerevisiae* W303 hem1Δ cells were incubated with fluorescently labeled BSA (rhodamine-labeled albumin, TMR-albumin). BSA was not internalized during NBD-cholesterol uptake, while the free form of rhodamine efficiently accumulated in the vacuole, as revealed by...

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**Fig. 1.** NBD-cholesterol uptake by *Candida glabrata* and *Saccharomyces cerevisiae*. (a) *Candida glabrata* and *Saccharomyces cerevisiae* cells were incubated with NBD-cholesterol for 22 h under anaerobic or aerobic conditions before imaging by fluorescence (NBD) or differential interference contrast (DIC) microscopy. Scale bars, 3 μm. (b) *HEM1*-deficient *S. cerevisiae* mutant cells expressing the lipid droplet marker protein Erg6p–mRFP were incubated aerobically with NBD-cholesterol for 22 h. Subsequently, the colocalization of lipid droplets with NBD-cholesterol was examined by fluorescence (NBD, RFP) or DIC microscopy. Scale bars, 2 μm.
Fig. 2. Effect of serum on NBD-cholesterol uptake by Candida glabrata and Saccharomyces cerevisiae. Wild-type and mutant strains of C. glabrata (a) and S. cerevisiae (b) were incubated with NBD-cholesterol for 22 h in the absence (–) or presence (+) of FCS under anaerobic or aerobic conditions; (c) HEM1-deficient S. cerevisiae mutant was incubated aerobically with NBD-cholesterol for 22 h in the absence (–) or presence (+) of ALA. Subsequently, cells were washed and analyzed by flow cytometry. NBD-cholesterol uptake was expressed as percentage fluorescence intensity relative to control cells (–serum); 100% corresponds to 36 × 10^2 arbitrary units (C. glabrata), 23 × 10^2 arbitrary units (S. cerevisiae), and 51 × 10^2 arbitrary units (S. cerevisiae hem1Δ). Results represent the means ± SD of three independent experiments (note y-axis log scale). Single asterisks denote significant (P < 0.05) differences between wild-type and mutant strains; double asterisks denote a significant (P < 0.05) effect of serum supplementation. Growth conditions are indicated.

Fig. 3. Effect of albumin on sterol uptake by Saccharomyces cerevisiae and Candida glabrata. (a) Saccharomyces cerevisiae hem1Δ and hem1Δaus1Δpdr11Δ cells were incubated aerobically with NBD-cholesterol and the indicated concentrations of fatty acid-free (FFA) or normal Fraction V (V) BSA for 22 h; (b) S. cerevisiae hem1Δ and hem1Δaus1Δpdr11Δ cells were incubated aerobically with [14C]-cholesterol and 25% fatty acid-free BSA for 22 h. Subsequently, cells were washed and analyzed as described under Material and Methods. Results represent the means ± SD from two biological replicates and are expressed as percentage signal intensity relative to control cells (–BSA). #, not determined. Insets show representative TLC plates of lipid extracts prepared from cells incubated with fluorescent (NBD) or [14C]-labeled cholesterol (lane 1 and 3, hem1Δ; lane 2 and 4, hem1Δaus1Δpdr11Δ). The position of the respective free (FC), acetylated (STA), and esterified sterol (STE) is indicated to the right; O, origin. (c) Time course of NBD-cholesterol binding to fatty acid-free BSA (1 : 400 NBD-cholesterol/BSA molar ratio). Inset shows that fluorescence of NBD-cholesterol in PBS is relatively weak (blue squares) but increases substantially upon binding to albumin (red diamonds; 1 : 34 NBD-cholesterol/BSA molar ratio). PBS supplemented with BSA displayed comparable background signal as PBS alone. Results are the means ± SD from three independent experiments.
comparison with the vacuolar marker BCECF-AM (Plant et al., 1999) (Fig. S2). Moreover, fluorimetric analysis revealed binding of NBD-cholesterol to albumin (Fig. 3c). Taken together, these results suggest that albumin can bind sterol molecules and thereby makes them more accessible for import by the yeast ABC transporters.

Effect of serum on sterol uptake by heterologously expressed CgAus1p

We finally assessed the specific effect of bovine serum on ABC transporter-mediated NBD-cholesterol uptake by heterologously expressing CgAus1p in the S. cerevisiae strain hem1Δaus1Δpdr11Δ. The S. cerevisiae strain hem1Δaus1Δpdr11Δ grows normally on medium containing ALA but displays a defect in sterol uptake due to the lack of ScAus1p and ScPdr11p, and consequently does not grow on sterol-supplemented medium (Fig. S3). We first investigated the expression of the FLAG-tagged CgAus1p in this strain by immunoblotting the crude membrane extracts. Upon galactose induction, protein was readily detected within expected size range (Fig. 4a). Fluorescence microscopy revealed that CgAus1p-GFP localized to the plasma membrane and in internal structures (Fig. 4b), similar to what has been observed for its S. cerevisiae homologue (Li & Prinz, 2004; Sullivan et al., 2009; Marek et al., 2011). Analysis by flow cytometry showed that upon galactose induction about 30% of cells expressed CgAus1p-GFP at detectable level (Fig. 4c), similar to results obtained for other proteins expressed from 2-micron–based multicopy vectors (Xu et al., 2009; Albertsen et al., 2011; Marek et al., 2011; Jensen et al., 2014) and likely caused by fluctuating copy numbers of these plasmids within the cell population.

To test for sterol transport activity, S. cerevisiae hem1Δaus1Δpdr11Δ cells expressing CgAus1p upon galactose induction were incubated with NBD-cholesterol and subsequently analyzed by flow cytometry. CgAus1p without GFP tag was employed here as NBD and GFP both excite at 488 nm and exhibit emission overlap. In the absence of serum supplementation, a small fraction (< 5%) of CgAus1p-expressing cells accumulated NBD-cholesterol, whereas the remaining cells showed only a very low level or no uptake (Fig. 5a). In presence of serum, the cell fraction with high levels of NBD-cholesterol uptake increased to about 32% (Fig. 5b). The fraction of cells displaying high levels of NBD-cholesterol uptake corresponded well to the fraction of cells that showed high expression of CgAus1p-GFP (cf. Figs 4c and 5b). Supplementation with FCS or BSA did neither affect the expression nor the localization of CgAus1p-GFP (data not shown), further supporting the conclusion that cholesterol bound to serum proteins is more accessible for CgAus1p-mediated uptake. Yet, CgAus1p–mediated uptake of cholesterol in the absence of serum proteins was sufficient to rescue the growth defect of the hem1Δaus1Δpdr11Δ mutant strain cultured on solid media supplemented with sterol (Fig. 6).

To verify that a catalytically active CgAus1p is required for complementation, a nonfunctional version of the transporter was generated by introducing a lysine to
methionine substitution in the Walker A region of nucleotide binding domain 2 (CgAus1pK793M), which is known to block the ATPase activity of ABC proteins (Gao et al., 2000; Marek et al., 2011). Upon galactose induction, the CgAus1pK793M mutant was expressed and reached the yeast plasma membrane (data not shown), but was unable to rescue the sterol uptake defect of the hem1Δaus1Δpdr11Δ mutant strain (Figs 5c and 6).

**Discussion**

In this study, we analyzed the effects of the serum protein albumin on sterol uptake in two fungal species, *C. glabrata* and *S. cerevisiae* by employing fluorescent NBD-cholesterol. This approach has the advantage over assays using radiolabeled sterols or high-performance liquid chromatography of allowing direct visualization of sterol uptake and the exclusion of dead cells (which readily absorb sterol) from the analysis. However, until now NBD-cholesterol has not been employed on other yeast species. In line with previous studies in *S. cerevisiae* (Reiner et al., 2005, 2006; Marek et al., 2011), we confirmed that the uptake of NBD-cholesterol was dependent on the two ABC transporters ScAus1p and ScPdr11p and only occurred efficiently under anaerobic conditions or in mutants lacking the HEM1 gene. Under these conditions, NBD-cholesterol rescued sterol auxotroph yeast, demonstrating that the fluorescent analogue can substitute natural cholesterol. Furthermore, we were able to show here that in *C. glabrata* uptake of NBD-cholesterol was dependent on the ABC transporter CgAus1p and occurred efficiently only under anaerobic conditions or in mutants lacking the HEM1 gene. Under these conditions, NBD-cholesterol rescued sterol auxotroph yeast, demonstrating that the fluorescent analogue can substitute natural cholesterol. Collectively, these results demonstrate that NBD-cholesterol is a sensitive biocompatible probe for monitoring sterol uptake in fungal species. While our flow cytometric analysis revealed evidence for CgAus1p-dependent aerobic cholesterol uptake, fluorescence levels

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**Fig. 5.** NBD-cholesterol uptake by CgAus1p expressed in *Saccharomyces cerevisiae*. Accumulation of NBD-cholesterol in *hem1Δaus1Δpdr11Δ* cells transformed with either e.v. or expression plasmids carrying CgAUS1 or CgAUS1K793M. Cells were incubated with NBD-cholesterol in the absence (a) and presence of serum (b and c) as indicated for 22 h and then analyzed by flow cytometry. Shown is one representative flow cytometric analysis. Numbers in the upper and middle panels represent percentages of cells with high NBD fluorescence.

**Fig. 6.** CgAus1p expressed in *Saccharomyces cerevisiae* rescues the growth defect of the *hem1Δaus1Δpdr11Δ* mutant. Serial fivefold dilutions of sterol uptake-competent (hem1Δ) and sterol uptake-deficient (hem1Δaus1Δpdr11Δ) mutant strains transformed with e.v. or expression plasmids carrying FLAG-tagged CgAUS1 or CgAus1K793M were spotted onto standard synthetic galactose plates supplemented with either ALA, 20 μg mL⁻¹ or cholesterol (20 μg mL⁻¹) and Tween 80 (0.1%). Plates were incubated at 30 °C for 4 days.
were too low to allow for microscopic visualization, indicating that aerobic cholesterol uptake reported for this fungus is very limited.

In both fungi, ABC transporter-dependent uptake of NBD-cholesterol was substantially increased in media supplemented with FCS or BSA. This promoting effect was not limited to NBD-cholesterol, as it was also observed for 14C-cholesterol. Furthermore, efficient NBD-cholesterol uptake by heterologously expressed CgAus1p required the presence of FCS or BSA. While the influence of the remaining components of FCS on sterol uptake remains to be investigated, our data indicate that the sterol can be complexed to serum proteins such as albumin and thereby become more accessible for ABC transporter-mediated uptake in fungi. Structurally, albumin is a globular protein composed of three similar domains (I, II and III), each containing two subdomains (A and B). Sterols were found to bind within a hydrophobic pocket in subdomains IIA and IIB (Sudhamalla et al., 2010; Cheng, 2012); while fatty acids bind to sites localized in subdomains IIA, IIB, IIIA, and IVB, and on the subdomain interfaces (Sugio et al., 1999). Studies on mammalian cells showed that albumin can bind and mediate transfer of cholesterol between cells and extracellular lipoproteins (Fielding & Moser, 1982; Zhao & Marcel, 1996; Sankaranarayanan et al., 2013). Conceivably, albumin might thus serve as a sterol donor facilitating the loading of sterol molecules onto fungal cell wall proteins that have been proposed to serve as sterol-binding proteins (Kohut et al., 2011). Atomic force microscopy observation suggests that S. cerevisiae cell walls have pores of around 200 nm that could increase to 400 nm in stress conditions (de Souza Pereira & Geibel, 1999). Such putative pores remain to be characterized but would allow BSA to penetrate the cell wall and bring sterol molecules directly to the plasma membrane – the location of Aus1p and Pdr11p. This mechanism would resemble uptake of nutrients in prokaryotes, where substrate-binding proteins are important for the action of ABC transporter-dependent substrate import (Davidson & Chen, 2004). The precise mechanism of sterol uptake and involvement of ABC transporters, namely whether these proteins are directly facilitating sterol import, requires further functional studies, like their reconstitution into model membranes. Moreover, in C. glabrata, alternative sterol uptake transporters/pathways may exist (Zavrel et al., 2013). Using S. cerevisiae strain hem1Δaus1Δpdr11Δ as a heterologous expression system, we here consolidate the function of CgAus1p in sterol uptake. The availability of a heterologous expression system for CgAus1p may prove useful for the expression, purification, and reconstitution of CgAus1p to enable detailed molecular analysis of its direct role in sterol uptake. Given that CgAus1p is a potential target for antifungal drug treatment, our results also provide a foundation for the development of high throughput screens aimed at identifying novel antagonists of CgAus1p that might help in the treatment of candidiasis and related infections in humans.

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References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Effect of albumin on sterol uptake by wild-type strains of *Candida glabrata* (KUE200) and *Saccharomyces cerevisiae* (W303).

**Fig. S2.** Albumin is not internalized during NBD-cholesterol uptake.

**Fig. S3.** *Saccharomyces cerevisiae* strain *hem1-Δaus1Δpdr11Δ* displays a growth defect on sterol-supplemented medium.