THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 35, Issue of August 27, pp. 36390–36396, 2004
Printed in U.S.A.

HOR7, a Multicopy Suppressor of the Ca^{2+}-induced Growth Defect in Sphingolipid Mannosyltransferase-deficient Yeast*

Received for publication, June 3, 2004
Published, JBC Papers in Press, June 18, 2004, DOI 10.1074/jbc.M406197200

Quirine Lisman‡§¶, Dorothy Urli-Stam§, and Joost C. M. Holthuis‡‖

From the ‡Department of Membrane Enzymology, Faculty of Chemistry, Utrecht University, 3584 CH Utrecht, The Netherlands and the §Department of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands

Yeast mutants defective in sphingolipid mannosylation accumulate inositol phosphorylceramide C (IPC-C), which renders cells Ca^{2+}- sensitive. A screen for loss of function suppressors of the Ca^{2+}-sensitive phenotype previously led to the identification of numerous genes involved in IPC-C synthesis. To better understand the molecular basis of the Ca^{2+}-induced growth defect in IPC-C-overaccumulating cells, we searched for genes whose overexpression restored Ca^{2+} tolerance in a mutant lacking the IPC mannosyltransferases Csg1p and Csh1p. Here we report the isolation of *HOR7* as a multicopy suppressor of the Ca^{2+}-sensitive phenotype of Δ*csg1*Δ*csh1* cells. *HOR7* belongs to a group of hyperosmolarity-responsive genes and encodes a small (59-residue) type I membrane protein that localizes at the plasma membrane. Hor7p is not required for high Ca^{2+} or Na^{+} tolerance. Instead, we find that Hor7p-overproducing cells display an increased resistance to high salt, sensitivity to low pH, and a reduced uptake of methylammonium, an indicator of the plasma membrane potential. These phenotypes are induced through a mechanism independent of the plasma membrane H^{+}-ATPase, Pma1p. Our findings suggest that induction of Hor7p causes a depolarization of the plasma membrane that may counteract a Ca^{2+}-induced influx of toxic cations in IPC-C-overaccumulating cells.

Sphingolipids are abundant components of eukaryotic plasma membranes with important functions in bilayer stability, stress adaptation, signaling, and possibly the formation of lipid microdomains (1–3). They consist of a ceramide linked through either a glucosyl or phosphodiester bond to a polar head group. Ceramides are comprised of a sphingoid base joined in amide linkage to a fatty acid and can be classified according to the level of hydroxylation of the sphingoid and fatty acid moieties. The yeast *Saccharomyces cerevisiae* produces phytoceramide based on C4-hydroxylated phytosphingosine and a C_{26} fatty acid that is usually hydroxylated on C2 (4, 5). Aur1p is required for transferring phosphoinositol from phosphatidylinositol onto the C1 hydroxyl group of phytoceramide, yielding inositol phosphorylceramide (IPC[1]; Ref. 6). IPC is mannosylated to form mannosyl-IPC (MIPC), which in turn can receive a second phosphoinositol group from phosphatidylinositol to generate the final and most abundant yeast sphingolipid, $M(IP)_2C$ (7). MIPC production requires the IPC mannosyltransferases Csg1p and Csh1p (8–10) as well as an EF-Ca^{2+}-binding domain-containing membrane protein, Csg2p (11). Csg2p interacts with Csg1p and Csh1p (9), but its precise role in MIPC production is unclear. Although Csg1p and Csh1p contain a region of homology with the yeast α-1,6-mannosyltransferase Och1p, Csg2p does not share this sequence and is not absolutely required for MIPC synthesis, suggesting that Csg2p serves a regulatory rather than a catalytic function.

Csg1p and Csg2p play an important role in Ca^{2+} tolerance because *csg1* and *csg2* mutants have been identified as Ca^{2+}-sensitive, Sr^{2+}-resistant mutants (12). The Ca^{2+}-sensitive phenotype appears to be due to the accumulation of IPC-C, which contains a phytosphingosine and a monohydroxylated C_{26} fatty acid. Indeed, the Ca^{2+}-sensitivity of *csg2* mutant cells is suppressed by mutations causing either a reduction in IPC-C levels or a change in its structure. Thus, suppressor mutations have been found in genes involved in sphingoid base synthesis (*LCB1, LCB2, TSC10*), palmitoyl-CoA synthesis (*FAS2*), fatty acid chain elongation (*TSC13*), conversion of dihydrosphingosine to phytosphingosine (*SUR2*), and hydroxylation of the C_{26} fatty acid (*SCS7*) (Fig. 1; Refs. 4, 8). Why IPC-C-overaccumulating cells become Ca^{2+}-sensitive is unknown. Several intermediates of sphingolipid metabolism have been reported to function as signaling molecules. For example, sphingoid base 1-phosphate is involved in heat stress resistance, diauxic shift, and Ca^{2+} mobilization (13–15), whereas accumulation of ceramide causes cell growth arrest (16, 17). Hence, one possibility is that Ca^{2+} activates hydrolysis of IPC-C, resulting in the formation of toxic levels of ceramide. Alternatively, IPC-C itself may act as a signaling molecule in a Ca^{2+}-signaling pathway. Suppressors of the Ca^{2+} sensitivity in the *csg2* mutant included two genes involved in signal transduction, namely the protein kinase *TOR2* and the phosphatidylinositol-4-phosphate 5-kinase *MSS4* (18). How the signaling pathways of Tor2p and Mss4p are connected to Ca^{2+}-induced cell death in *csg1* and *csg2* mutants remains to be established. Another model put forward to explain the Ca^{2+}-sensitive phenotype is that Ca^{2+} alters the permeability of *csg1* and *csg2* cells, causing an increased influx of toxic ions that leads to cell death. This idea is based on the observation that *csg1* and *csg2* mutants display an increased Ca^{2+} uptake and that the Ca^{2+}-sensitive phenotype can be reversed by addition of 0.8 M sorbitol to the growth

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¶ Supported by a grant from the Meelmeijer Foundation.
‖ Supported by a grant from the Royal Academy of Arts and Sciences.
To whom correspondence should be addressed: Dept. of Membrane Enzymology, Center for Biomembranes and Lipid Enzymology, H. R. Kruytgebouw N605, Padualaan 8, 3584 CH Utrecht, The Netherlands. Tel.: 31-30-253-6630; Fax: 31-30-252-2478; E-mail: j.c.holthuis@chem.uu.nl.

[1] The abbreviations used are: IPC-C, inositol phosphorylceramide C; MIPC, mannosyl-IPC; MES, 4-morpholineethanesulfonic acid; SD, synthetic dextrose.

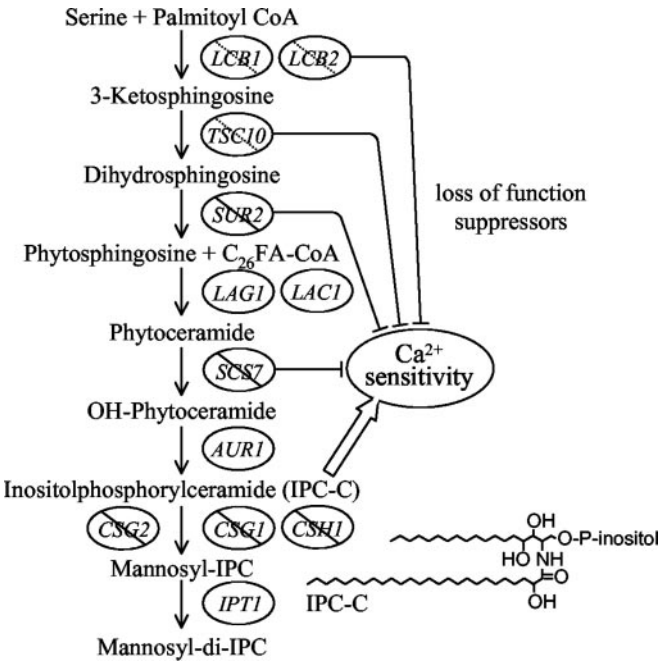

FIG. 1. **Defects in the yeast sphingolipid biosynthetic pathway affecting Ca^{2+} sensitivity.** Pathway intermediates and genes involved at each step are shown. Disruption of genes required for sphingolipid mannosylation (*CSG1, CSG2, CSH1*) causes accumulation of inositolphosphorylceramide C (*IPC-C*), which renders cells Ca^{2+}-sensitive. The Ca^{2+} sensitivity of IPC-C-overaccumulating cells is suppressed by mutations affecting IPC-C structure (*TSC10*, *SUR2*, *SCS7*) or production rate (*LCB1*, *LCB2*).

medium (8). Sorbitol reduces osmotic gradients and has been shown to prevent lysis of mutants with defective cell walls (19).

To gain further insight into the molecular basis of the Ca^{2+}-induced growth defect in IPC-C-accumulating cells, we searched for genes whose overexpression restores Ca^{2+} tolerance in a mutant strain lacking the IPC mannosyltransferases Csg1p and Csh1p. Here we report the isolation and characterization of *HOR7* as a multicopy suppressor of the Ca^{2+}-sensitive phenotype in Δ*csg1*Δ*csh1* cells. Our findings suggest that Hor7p protects Δ*csg1*Δ*csh1* cells from a Ca^{2+}-induced influx of toxic cations by reducing the plasma membrane electric potential.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—Unless indicated otherwise, yeast strains were grown at 28 °C to mid-logarithmic phase (0.5–1.0 A_{600}) in synthetic dextrose (SD) medium. Yeast transformations were carried out as described (20). Mutant strains Δ*csg1* (JHY075), Δ*csh1* (JHY088), Δ*csg1*Δ*csh1* (JHY090), Δ*ipt1* (JHY079) have been described previously (10). The *HIS3* marker in JHY090 was removed by excisive recombination using Cre recombinase, yielding strain JHY101. For deletion of the *HOR7* gene, 450–500 base pair fragments of the regions flanking the open reading frame were PCR-amplified from yeast genomic DNA and cloned into the NotI/EcoRI and SpeI/MluI sites located on either site of a *loxP-HIS3-loxP* cassette in a pBluescript KS^- vector (10). The gene deletion construct was linearized with NotI and MluI and transformed into EHY227 (*MATα sec6–4 TPI1*::*SUC2*::*TRP1 ura3–52 his3*-Δ*200 leu2–3 -112 trp1–1*) or JHY101 to generate Δ*hor7* (JHY161) and Δ*csg1*Δ*csh1*Δ*hor7* (JHY164) strains. Correct integration events were confirmed by PCR. Strains with Pma1p tagged at its N terminus with one copy of the HA epitope were generated using integration plasmid pRS305Δ51 (21). Promoter regions (650 bp) and open reading frames of *CSG1* and *CSH1* were PCR-amplified and ligated into single copy vector pRS416 (CEN, *URA3*). Promoter regions (650 bp) and open reading frames of *HOR7*, *DDR2,* and *YMR252C* were PCR-amplified and ligated into multicopy vector pRS426 (2 μm, *URA3*). To generate a *myc*-tagged version of *HOR7*, nine copies of the *myc* epitope were inserted in the open reading frame between residues 20 and 21 by PCR, using plasmid p9xMYCt-HIS5 (S. Munro, MRC-LMB, Cambridge, UK). *myc*-tagged *HOR7* was ligated into multicopy vector pRS425 (2 μm, *LEU2*) and expressed in strain SEY6210 (*MATα ura3–52 his3*-Δ*200 leu2–3 -112 trp1*-Δ*901 suc2*-Δ*9 lys2*-801) for localization studies.

Multicopy Suppressor Screen—A YEP13-based yeast genomic DNA library (AB320, ATCC 37323) was transformed into JHY090 (Δ*csg1*Δ*csh1*), and transformants were grown on SD plates supplemented with 20 mM $CaCl_2$ at 28 °C. Ca^{2+}-resistant colonies transformed with *CSG1*- or *CSH1*-containing plasmids were identified by colony PCR and discarded. Plasmids rescued from the remaining Ca^{2+}-resistant colonies were isolated, retransformed in JHY090, and screened for their ability to complement the Ca^{2+}-sensitive phenotype. This approach yielded three overlapping genomic sequences capable of restoring Ca^{2+} tolerance. Complementation experiments with restriction fragments and PCR-amplified open reading frames present in the isolated genomic sequences led to the identification of *HOR7* as a multicopy suppressor of the Ca^{2+}-induced growth defect in JHY090.

Subcellular Membrane Fractionation—Cells were grown in 500 ml of SD medium, harvested, spheroplasted, and then lysed in a hypo-osmotic buffer as described (22). Subcellular membranes were collected at 100,000 × g_{av} (60 min, 4 °C) and loaded on top of a sucrose gradient prepared in gradient buffer (10 mM Hepes-KOH, pH 7.2, 1 mM EDTA, 0.8 M sorbitol) using the following steps: 0.5 ml 60%, 1 ml 40%, 1 ml 37%, 1.5 ml 34%, 2 ml 32%, 2 ml 29%, 1.5 ml 27%, and 1.5 ml 22% (w/w) sucrose. After centrifugation at 130,000 × g_{av} in a Beckman SW40Ti rotor (18 h, 4 °C), 20 × 0.6-ml fractions were collected from the top. Equal volumes per fraction were used to assay for ATPase activity and for Western blot analysis (see below).

Western Blot Analysis—Myc-tagged Hor7p was detected with anti-*myc* rabbit polyclonal (A14) or mouse monoclonal antibody (9E10) and HA-tagged Pma1p with anti-HA rabbit polyclonal antibodies (Santa Cruz Biotechnology). Other antibodies were directed against Gos1p (22), Dpm1p (Molecular Probes, Eugene, OR), and Sso2p (S. Keränen, Biotechnology and Food Research, Espoo, Finland). For immunoblotting, all antibody incubations were carried out in phosphate-buffered saline containing 5% dried milk and 0.5% Tween 20. After incubation with peroxidase-conjugated secondary antibodies (Bio-Rad), blots were developed using a chemiluminescent substrate kit (Pierce).

ATPase Assay—The protein content of pooled gradient fractions was measured using a micro BCA protein assay reagent kit (Pierce) with bovine serum albumin as a standard. ATPase assays were performed on 70 μg of protein at 30 °C in a volume of 25 μl (10 mM Hepes-KOH, pH 7.2, 0.8 M sorbitol, 2 mM ATP, 5 mM $MgCl_2$). Reactions were stopped after 30 min with 175 μl of 40 mM H_2SO_4. Then 50 μl of 6 M H_2SO_4 containing 0.001% malachite green was added. After a 30-min incubation at room temperature, the absorbance was measured at 595 nm. The specific ATPase activity was calculated from the amount of P_i released after 30 min and expressed as nmol P_i/min/mg of protein.

Immunofluorescence Microscopy—Exponentially grown cells were fixed and mounted on poly(L)lysine-coated glass slides as described previously (22). Antibody incubations were performed in phosphate-buffered saline supplemented with 2% dried milk and 0.1% saponin for 2 h at room temperature. Anti-Myc mouse monoclonal antibody 9E10 was used at a dilution of 1:100 and rabbit polyclonal antibody to Sso2p at a dilution of 1:200. Goat-anti-mouse fluorescein- and goat-anti-rabbit Cy3-conjugated secondary antibodies (Amersham Biosciences) were used at a dilution of 1:100. Fluorescence microscopy and image acquisition were carried out using a Leica DMRA microscope (Leitz, Wetzlar, Germany) equipped with a cooled CCD camera (KX85, Apogee Instruments Inc., Tucson, AZ) driven by Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).

Measurement of Methylammonium Uptake—Cells (5 A_{600}/ml) were incubated in 50 mM glucose, 10 mM MES, pH 6.0, at 2 °C. After a 5-min incubation, [^{14}C]methylamine hydrochloride (2 mM and 2.5 μCi/ml final concentration; Amersham Biosciences) was added. At the indicated time points, 100-μl aliquots were diluted into 10 ml of ice-cold 20 mM $MgCl_2$, filtered through a 0.45-μm nitrocellulose filter (Millipore HAWP), and washed twice with 10 ml of the same solution. Filters were transferred to a scintillation mixture and radioactivity measured using a liquid scintillation counter.

RESULTS

Loss of Csg1p and Csh1p Causes Hypersensitivity toward Calcium—S. cerevisiae cells have a high tolerance for Ca^{2+}. Thus, cells display normal growth in medium containing up to 100 mM $CaCl_2$ (data not shown). A screen for genes specifically involved in Ca^{2+} regulation in *S. cerevisiae* revealed *CSG1,* a gene with a critical function in high Ca^{2+} tolerance (Beeler

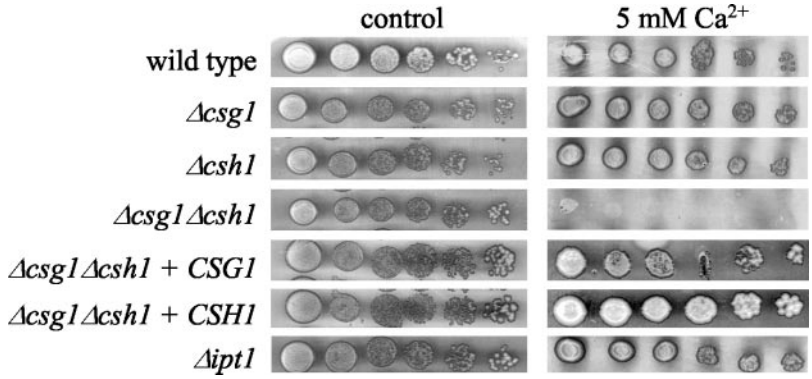

FIG. 2. **Loss of mannosyltransferases Csg1p and Csh1p causes hypersensitivity toward calcium.** Serial 3-fold dilutions of exponentially grown wild type, Δ*ipt1,* Δ*csg1*, Δ*csh1,* Δ*csg1*Δ*csh1*, or Δ*csg1*Δ*csh1* cells transformed with *CSG1* or *CSH1* on a single copy vector were spotted onto SD plates with or without 5 mM $CaCl_2$ as indicated. Plates were scanned after 3 days of incubation at 30 °C.

et al., Ref. 12). Subsequent studies showed that Csg1p is homologous to Csh1p and that the two proteins function as IPC mannosyltransferases (8–10). To study the effects of Δ*csg1* and Δ*csh1* mutations on Ca^{2+} sensitivity, cells were grown on SD plates containing 5 mM $CaCl_2$ at 30 °C. As shown in Fig. 2, Δ*csg1* and Δ*csh1* cells were resistant to exogenous Ca^{2+} and displayed normal growth. In contrast, the Δ*csg1*Δ*csh1* double mutant was highly sensitive to Ca^{2+} and did not grow on the medium. Transformation of Δ*csg1*Δ*csh1* cells with the *CSG1* or *CSH1* gene on a single copy vector fully suppressed the Ca^{2+}-induced growth defect. Deletion of *IPT1*, a gene required for $M(IP)_2C$ synthesis (23, 24) had no effect on Ca^{2+} tolerance. Thus, the Ca^{2+} sensitivity of yeast cells correlates with the level of MIPC synthesis rather than $M(IP)_2C$ synthesis.

Isolation of HOR7 as a Multicopy Suppressor of the Ca^{2+}-induced Growth Defect in Δ*csg1*Δ*csh1 Cells*—In the absence of sphingolipid mannosylation, yeast cells accumulate IPC-C. Suppressor mutations that reverse the Ca^{2+} sensitivity of MIPC-deficient cells have been found to either decrease the synthesis of IPC-C or to alter its structure (Fig. 1). None of the suppressor mutants synthesized MIPC, indicating that it is the accumulation of IPC-C, rather than the absence of mannosylated sphingolipids, that makes cells Ca^{2+}-sensitive. To gain further insight into the molecular basis of the Ca^{2+}-induced growth defect in IPC-C-overaccumulating cells, we performed a multicopy suppressor screen aimed at the identification of genes whose overexpression restores Ca^{2+} tolerance in the Δ*csg1*Δ*csh1* mutant. To this end, Δ*csg1*Δ*csh1* cells were transformed with a yeast genomic DNA library on a multicopy vector, and transformants that regained tolerance to 20 mM Ca^{2+} were selected. Of the 64,000 transformants screened (6 times the amount required to cover the entire library), 168 grew in the presence of Ca^{2+}. Sixty Ca^{2+}-resistant colonies were picked for further analysis. Colonies transformed with *CSG1*- or *CSH1*-containing plasmids were identified by colony PCR and discarded (49 of 60). Plasmids rescued from the 11 remaining Ca^{2+}-resistant colonies were retransformed in Δ*csg1*Δ*csh1* cells and screened for their ability to restore Ca^{2+} tolerance. The three plasmids for which this was the case were sequenced. All three had a 25-kb insert derived from the same region in the right arm of chromosome XIII (765,000–790,000 bp), harboring the complete open reading frames of six different genes (Fig. 3*A*). Complementation studies with restriction fragments of the genomic sequence were used to eliminate four of the six genes as possible suppressors of the Ca^{2+}-induced growth defect in Δ*csg1*Δ*csh1* cells (Fig. 3*B*). Open reading frames of the remaining two genes were PCR-amplified from yeast genomic DNA, ligated into a multicopy expression vector, and then screened for their ability to restore Ca^{2+} tolerance. This led to the identification of a gene named *HOR7* whose overexpression suppressed the Ca^{2+}-induced growth defect in Δ*csg1*Δ*csh1* cells (Fig. 3*B*).

HOR7 Encodes a Small Plasma Membrane-associated Protein—HOR7 was originally identified as one of seven hyperosmolarity-responsive genes co-induced by shifting cells to 1 M NaCl (25). The gene codes for a 59-amino acid protein of unknown function with a predicted N-terminal signal peptide and C-terminal membrane span (Fig. 4*A*). Searching the Protein Data Bank for homologous proteins revealed Ddr2p, a 61-amino acid yeast protein sharing 46% amino acid sequence identity with Hor7p. No additional homologues were found. Ddr2p is encoded by a DNA damage-responsive gene (26). Its expression is also induced by osmotic shock, heat shock, and oxidative stress (27). The function of Ddr2p is unknown. Despite being highly related to Hor7p, overexpression of Ddr2p did not restore Ca^{2+} tolerance in Δ*csg1*Δ*csh1* cells (Fig. 4*B*). A recent global analysis of protein localization in yeast revealed that Ddr2p is associated with the vacuole (28). To investigate the localization of Hor7p, the protein was tagged with nine copies of the *myc* epitope that were inserted immediately behind the putative signal peptide cleavage site (between residues 20 and 21). Epitope-tagging, whether internally or at the C terminus, abolished the ability of Hor7p to restore Ca^{2+} tolerance in Δ*csg1*Δ*csh1* cells (data not shown). *myc*-tagged Hor7p expressed in wild type cells migrated as a 37-kDa protein on polyacrylamide gels. Following high speed centrifugation of a cell lysate, Hor7p-Myc was found exclusively in the membrane pellet (Fig. 5*A*). Immunofluorescence microscopy revealed a peripheral staining pattern and extensive co-localization with the plasma membrane-associated syntaxin, Sso2p (Fig. 5*B*). When cellular membranes were fractionated on an equilibrium sucrose density gradient, the bulk of Hor7p-Myc co-migrated with Sso2p and was separated from markers for the endoplasmic reticulum (*Dpm1p*), Golgi (*Gos1p*), endosomes (Pep12p), and vacuoles (Vam3p) (Fig. 5*C* and data not shown). Taken together, these results indicate that Hor7p resides at the yeast plasma membrane.

Hor7p contains a putative N-terminal signal sequence and C-terminal membrane span that predict a type I membrane topology where the N terminus is situated in the lumen/extracellular environment (Fig. 5*D*). To test this prediction, cells expressing Hor7p-Myc were spheroplasted and then immunostained with anti-Myc and anti-Sso2p antibodies either before (prestained) or after fixation and permeabilization (poststained). As expected, post-stained cells were labeled with both antibodies (Fig. 5*E, upper panel*). In contrast, prestained cells were positive for anti-Myc antibodies but negative for anti-Sso2p antibodies (Fig. 5*E, upper panel*). These findings indicate that the N terminus of Hor7p-Myc is exposed to the extracellular environment, hence consistent with the topology depicted in Fig. 5*D*.

HOR7 Overexpression Causes a Growth Defect at Low pH without Affecting Plasma Membrane H^+-ATPase Activity—To determine the physiological function of Hor7p, the chromo-

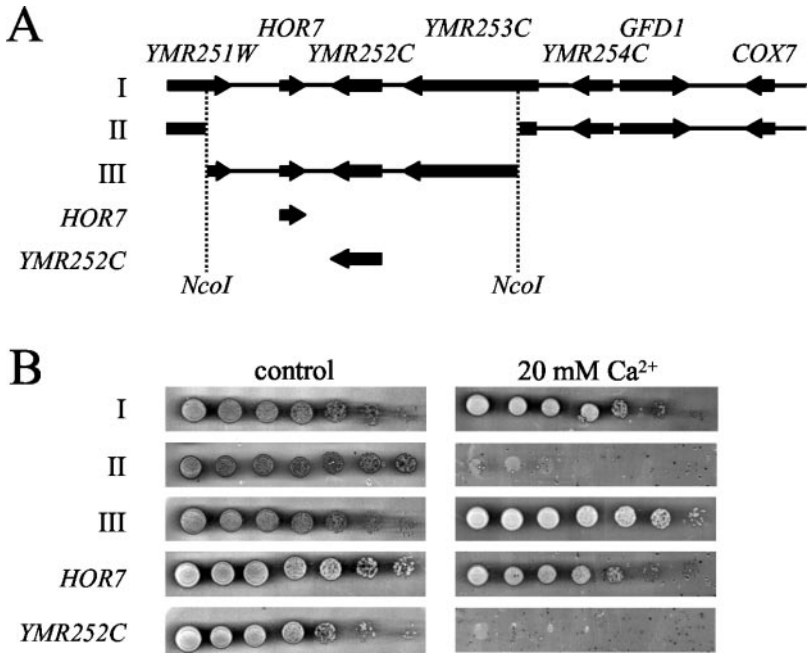

FIG. 3. **Identification of *HOR7* as a multicopy suppressor of the Ca^{2+}-induced growth defect in Δ*csg1*Δ*csh1* cells.** *A*, a screen for multicopy suppressors of the Ca^{2+}-induced growth defect in Δ*csg1*Δ*csh1* cells yielded a genomic DNA sequence harboring the complete open reading frames of six different genes (*I*). *HOR7* was identified by testing restriction fragments (*II*, *III*) and PCR-amplified open reading frames for their ability to complement the Δ*csg1*Δ*csh1* Ca^{2+}-sensitive phenotype. *B*, serial 3-fold dilutions of exponentially grown Δ*csg1*Δ*csh1* cells transformed with genomic DNA fragments (*I-III*) or PCR-amplified open reading frames (*HOR7*, *YMR252C*) on a multicopy vector were spotted onto SD plates with or without 20 mM $CaCl_2$ as indicated. Plates were scanned after 3 days of incubation at 30 °C.

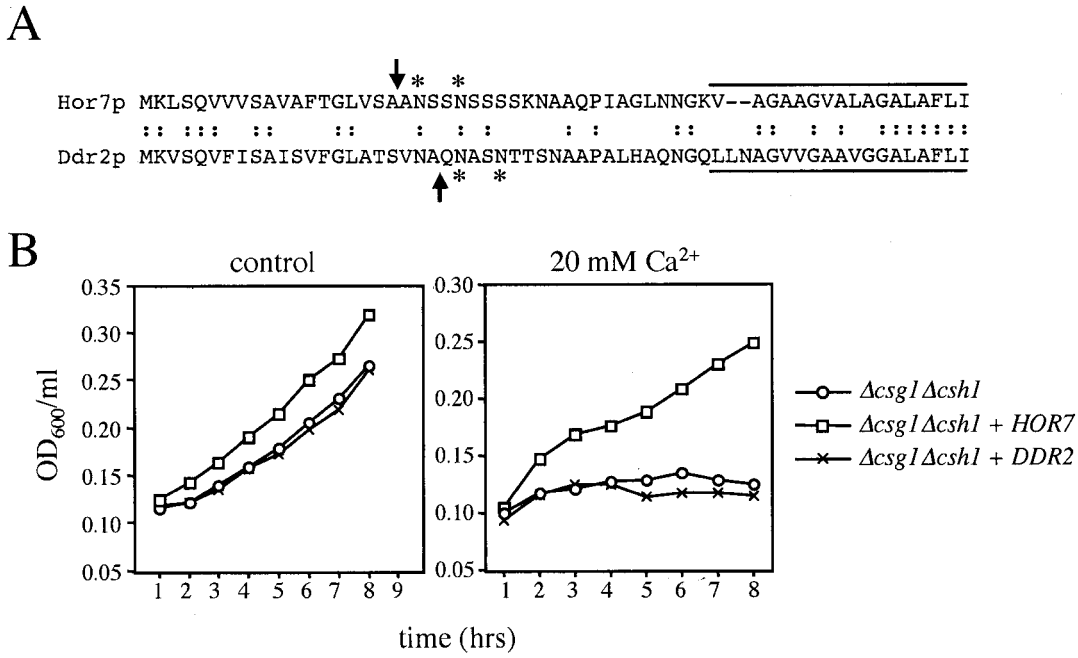

FIG. 4. **Overexpression of *HOR7*, but not of the homologous gene *DDR2*, suppresses the Ca^{2+}-induced growth defect in Δ*csg1*Δ*csh1* cells.** *A*, alignment of the amino acid sequences of Hor7p and Ddr2p. Putative signal peptide cleavage sites are indicated by *arrows*. Potential transmembrane domains are *underlined*. Putative *N*-linked glycosylation sites are marked by *asterisks*. *B*, *csg1*Δ*csh1* cells transformed with *HOR7* or *DDR2* on a multicopy vector were grown in SD medium with or without 20 mM $CaCl_2$. At the indicated times, 1-ml aliquots were withdrawn for measurement of the A_{600}.

somal copy of *HOR7* was disrupted by homologous recombination. Haploid Δ*hor7* and Δ*csg1*Δ*csh1*Δ*hor7* strains were obtained, indicating that Hor7p is not essential under standard growth conditions, *i.e.* in YEPD or SD medium at 30 °C. Hor7p is not required for high salt, high Ca^{2+}, or low pH tolerance, because Δ*hor7* cells grew as well as wild type on 1 M NaCl, 50 mM $CaCl_2$ or at pH 2.5 (Fig. 6*A*). However, we noticed that cells overproducing Hor7p from a multicopy vector grew better on 1 M NaCl and were more sensitive to low pH than wild type or Δ*hor7* cells. Although Hor7p-overproducing cells grew better than Δ*hor7* cells at pH 6.5, the situation was reversed at pH 4.5 or below (Fig. 6 and data not shown). The plasma membrane H^+-ATPase, Pma1p, plays a key role in the regulation of intracellular pH and yeast cells carrying mutations that reduce Pma1p activity, are sensitive to low pH, and become resistant to Na^+ (29–31). Pma1p is associated with two small (38-residue) and highly hydrophobic isoproteins, Pmp1 and Pmp2, that are required for maximal ATPase activity (32, 33). Pma1p is one of the most abundant proteins in the yeast plasma membrane and has been estimated to consume as much as one quarter of cellular ATP (34). To investigate whether Hor7p acts as a negative regulator of Pma1p, we determined the rates of MgATP hydrolysis in plasma membranes derived from Δ*hor7* cells transformed with *HOR7* on a multicopy plasmid (*HOR7–2 μm*) or with empty vector. To this end, cells were lysed and the plasma membranes separated from intracellular organelles by

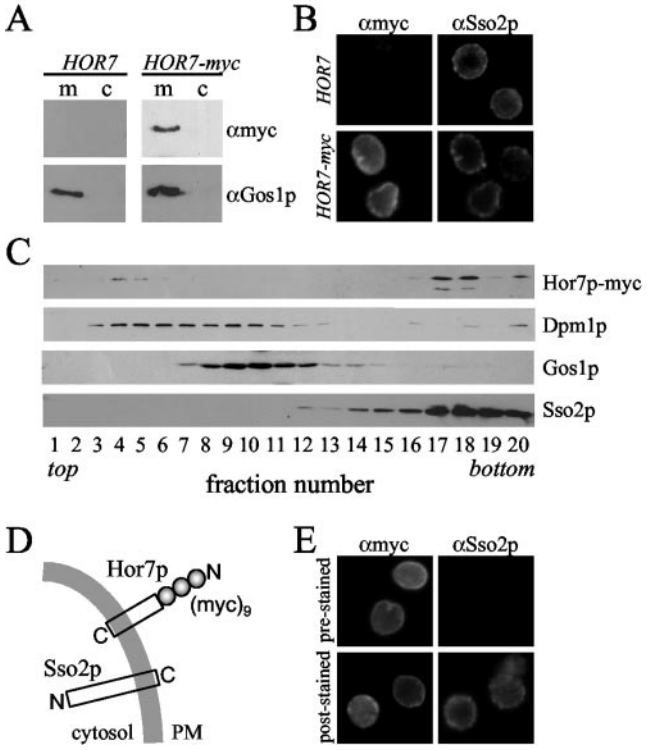

FIG. 5. **Hor7p localizes at the plasma membrane.** *A*, Hor7p is membrane-associated. High speed membrane pellets (100,000 × *g*) were prepared from yeast cells expressing untagged (*HOR7*) or *myc*-tagged *HOR7* (*HOR7-myc*) from a multicopy vector. Membranes (*m*) and cytosol (*c*) were analyzed by immunoblotting using anti-*myc* monoclonal and anti-Gosp1 polyclonal antibodies. *B*, immunofluorescence micrographs of cells expressing untagged or *myc*-tagged *HOR7* (*HOR7-myc*) from a multicopy vector were co-stained with anti-*myc* monoclonal and anti-Sso2p polyclonal antibodies as indicated. *C*, subcellular fractionation of Hor7p. A high-speed membrane pellet (100,000 × *g*) prepared from cells expressing *myc*-tagged *HOR7* was fractionated on a sucrose step gradient. Fractions were analyzed by immunoblotting using antibodies against the *myc* epitope, the endoplasmic reticulum marker Dpm1p, the Golgi marker Gos1p, and the plasma membrane marker Sso2p. *D*, schematic view of the (predicted) membrane topologies of *myc*-tagged Hor7p and Sso2p. *E*, immunofluorescence micrographs of cells expressing *myc*-tagged *HOR7* from a multicopy vector and co-stained with mouse monoclonal anti-*myc* and rabbit polyclonal anti-Sso2p antibodies either before (prestained) or after fixation and permeabilization (post-stained).

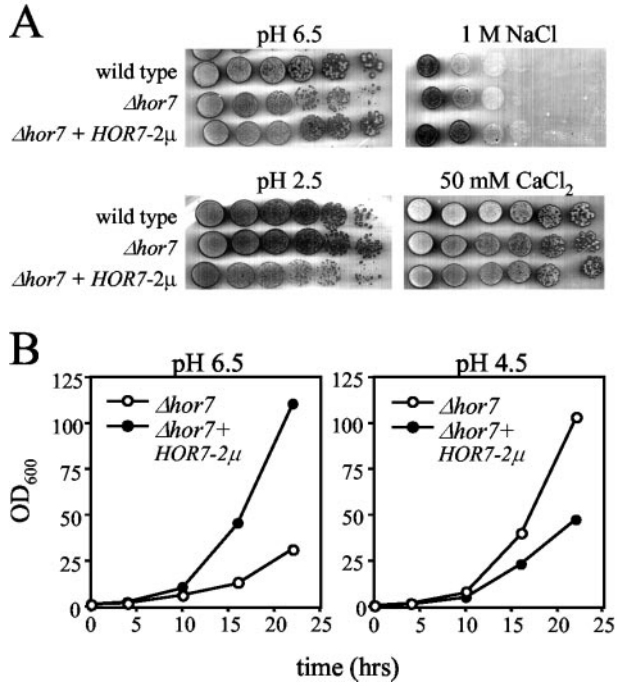

FIG. 6. ***HOR7* overexpression causes a growth defect at low pH.** *A*, serial 3-fold dilutions of exponentially grown wild type, Δ*hor7*, and Δ*hor7* cells transformed with *HOR7* on a multicopy vector (*HOR7–2 μm*) were spotted onto SD plates supplemented with the indicated concentrations of H^+, NaCl, or $CaCl_2$. *B*, Δ*hor7* and Δ*hor7* cells transformed with *HOR7–2 μm* were grown in SD medium supplemented with the indicated concentrations of H^+. The cultures were kept in log phase by regular dilution in fresh medium. At the indicated times, 1-ml aliquots were withdrawn for measurement of the A_{600}. Total A_{600} values for each culture are given. The experiment was repeated twice with similar results.

fractionation on equilibrium sucrose density gradients (Fig. 7*A*). Of the 20 fractions collected/gradient, the last 4 high density fractions contained the bulk of plasma membrane (*Pma1p*, *Sso2p*) and were devoid of significant amounts of endoplasmic reticulum (*Dpm1p*), Golgi (*Gos1p*), vacuoles (Vam3p), and endosomes (Pep12p) (Fig. 7*A* and data not shown). Plasma membrane-enriched fractions for each gradient were pooled and then normalized for Pma1p levels by Western blot analysis. As shown in Fig. 7*B*, there was no significant difference in plasma membrane-associated ATPase activity between Hor7p-deficient and -overproducing cells. Hence, it appears unlikely that Hor7p renders cells sensitive to low pH by reducing the activity of plasma membrane H^+-ATPase.

HOR7 Overexpression Causes a Depolarization of the Plasma Membrane—The plasma membrane electric potential maintained by H^+-ATPase Pma1p has been reported to be a major determinant of toxic cation tolerance (29, 31). Therefore, it is feasible that Hor7p overexpression alters the membrane potential, which might reduce the rate of cation uptake into the cell and, consequently, the sensitivity of the cell to toxic cations. To investigate this possibility, we measured [^{14}C]methylammonium uptake as an indicator of membrane potential (35, 36). As expected, wild type cells preincubated with protonophore carbonyl cyanide *p*-chlorophenylhydrazone displayed a significant reduction in methylammonium uptake compared with untreated cells (Fig. 8). Hor7p-overproducing cells displayed a similar decrease in methylammonium uptake when compared with wild type or Hor7p-deficient cells. Collectively, our results suggest that Hor7p overexpression causes a depolarization of the plasma membrane through a mechanism independent of Pma1p function.

DISCUSSION

Yeast mutants defective in IPC mannosylation accumulate IPC-C, which renders cells Ca^{2+}-sensitive. These mutants provide a positive selection for IPC-C synthesis mutants because the latter are suppressors of the Ca^{2+}-sensitive phenotype. How IPC-C induces Ca^{2+} sensitivity is not well understood. Here we have identified *HOR7* as a multicopy suppressor of the Ca^{2+}-induced growth defect in cells lacking the IPC mannosyltransferases Csg1p and Csh1p. Our findings suggest that Hor7p causes a depolarization of the plasma membrane that may counteract a Ca^{2+}-induced influx of toxic cations in IPC-C-overaccumulating cells.

Our multicopy suppressor screen on 60,000 transformed Δ*csg1*Δ*csh1* colonies yielded *HOR7* as the only non-IPC mannosyltransferase-encoding gene capable of restoring high Ca^{2+} tolerance. *HOR7* was originally identified as one of seven hyperosmolarity-responsive genes whose transcript levels are increased over 10-fold by 1 M NaCl or 1.5 M sorbitol (25). *HOR7* encodes a 59-amino acid protein with a predicted signal sequence and C-terminal membrane span. Membrane fractionation analysis and immunofluorescence microscopy of cells ex-

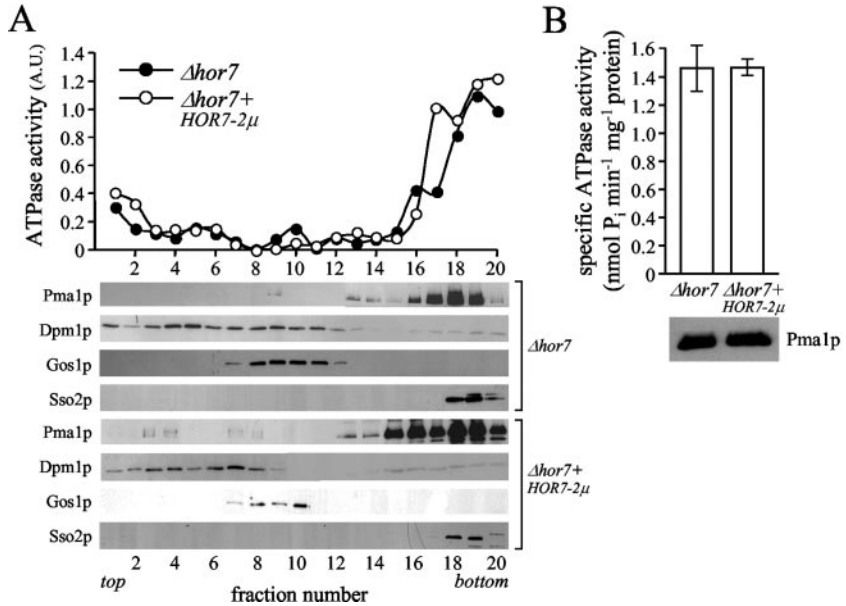

FIG. 7. **Overexpression or deletion of *HOR7* has no effect on plasma membrane H^+-ATPase activity.** *A*, subcellular fractionation of plasma membrane H^+-ATPase Pma1p from cells lacking or overexpressing *HOR7*. High speed membrane pellets (100,000 × *g*) prepared from Δ*hor7* cells expressing HA-tagged Pma1p and transformed with empty vector (Δ*hor7*) or *HOR7* on a multicopy vector (Δ*hor7* + *HOR7–2* μ*m*) were fractionated on sucrose step gradients. Fractions were collected from the top and analyzed for ATPase activity and by immunoblotting. ATPase activity is expressed in arbitrary units based upon the absorbance measured at 595 nm as described under "Experimental Procedures." Immunoblots were stained with anti-HA antibodies to detect HA-tagged Pma1p and with antibodies against Gos1p, Sso2p, and Dpm1p. *B*, ATPase activity in plasma membrane-enriched fractions derived from Hor7p-deficient (Δ*hor7*) and Hor7p-overproducing cells (Δ*hor7* + *HOR7–2* μ*m*). Fractions 16–20 from gradients shown in *panel A* were pooled, normalized, and assayed (70 μg of protein/measurement) for ATPase activity as described under "Experimental Procedures." Each *bar* represents the mean ± S.D. of three independent measurements. Immunoblotting with anti-HA antibodies on pooled fractions revealed that both strains contained similar amounts of Pma1p.

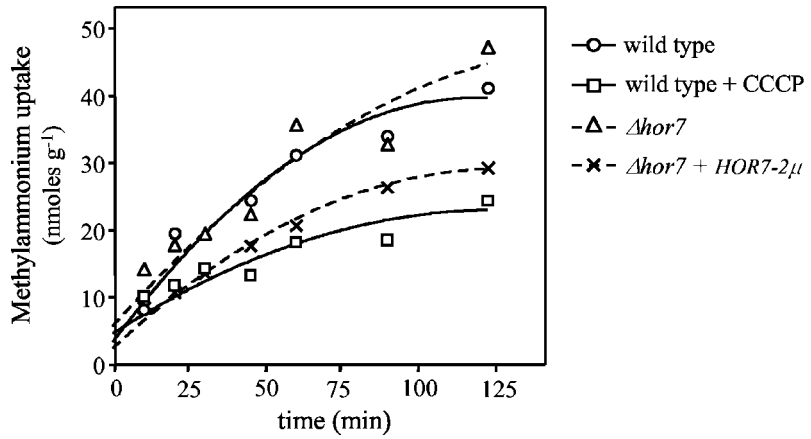

FIG. 8. ***HOR7* overexpression causes a reduction in methylammonium uptake.** Wild type cells incubated with or without 50 μM carbonyl cyanide *p*-chlorophenylhydrazone (*CCCP*) and cells lacking (Δ*hor7*) or overexpressing *HOR7* from a multicopy vector (Δ*hor7* + *HOR7–2* μ*m*) were analyzed for [^{14}C]methylammonium uptake. At the indicated times, 100-μl aliquots of cells incubated with [^{14}C]methylammonium were collected, filtered, and washed as described under "Experimental Procedures." Filters were transferred to a scintillation mixture and radioactivity was counted. The experiment was repeated twice with similar results.

pressing epitope-tagged Hor7p revealed that the protein resides in the plasma membrane with its N terminus facing the cell surface, hence consistent with a type I membrane topology. Hor7p shares 49% sequence identity with Ddr2p, a 61-amino acid yeast protein encoded by a DNA damage- and hyperosmolarity-responsive gene (37). However, unlike *HOR7*, *DDR2* was unable to serve as a multicopy suppressor of the Ca^{2+}-induced growth defect in Δ*csg1*Δ*csh1* cells. A global protein localization study revealed that Ddr2p is not associated with the plasma membrane but with the yeast vacuole (28). Hence, it is feasible that Hor7p and Ddr2p perform identical functions at different locations within the cell.

Even though Hor7p is induced in response to high salt and serves as a multicopy suppressor of the Ca^{2+}-induced growth defect in Δ*csg1*Δ*csh1* cells, disruption of the corresponding gene had no effect on high salt or high Ca^{2+} tolerance. Overexpression of Hor7p, on the other hand, conferred an increased resistance to Na^+ and sensitivity to low pH. Moreover, Hor7p-overproducing cells displayed a decreased uptake of methylammonium, an indicator of the plasma membrane potential (35, 36). The latter finding suggests that Hor7p overexpression causes a depolarization of the plasma membrane. Plasma membrane depolarization, low pH sensitivity, and an increased Na^+ resistance have previously been reported for yeast strains bearing mutations in the plasma membrane H^+-ATPase, Pma1p (29, 31). This activity is required for cytosolic pH homeostasis as well as for maintaining the electrochemical potential that drives transport of multiple nutrients and ions across the yeast plasma membrane (35, 38, 39). Pma1p function is dependent on Pmp1p and Pmp2p, two small (38-residue) and highly hydrophobic isoproteins whose removal causes a drastic reduction in the rate of plasma membrane-associated ATP hydrolysis (33). A role for Hor7p as negative regulator of Pma1p might explain the reduced methylammonium uptake, low pH sensitivity, and increased Na^+ tolerance of Hor7p-overproducing cells. However, this possibility is unlikely given our finding that cells

lacking or overexpressing Hor7p display similar rates of ATP hydrolysis in their plasma membranes. Hence, it appears that Hor7p causes a depolarization of the plasma membrane through a mechanism independent of Pma1p. It is possible that Hor7p induces a depolarizing proton leak or mediates the proton leak itself. This would be consistent with the low pH sensitivity of Hor7p-overproducing cells. A similar function has previously been attributed to Pmp3p, a small 55-residue hydrophobic polypeptide found in the yeast plasma membrane with high sequence similarity to a family of plant polypeptides that are induced by high salinity (40). Whereas removal of Pmp3p causes a hyperpolarization of the plasma membrane (40), loss of Hor7p had no obvious effect on the membrane potential (this study). Further analysis of the proton permeability of Hor7p-deficient and -overproducing cells will be required to understand the precise role of Hor7p in the regulation of the plasma membrane potential.

Ca^{2+} has previously been proposed to induce an irreversible alteration in the plasma membrane of *csg1* and *csg2* yeast mutants that increases the influx of Ca^{2+} and perhaps other toxic cations, resulting in cell death (8). Our data suggest that Hor7p restores high Ca^{2+} tolerance in IPC mannosyltransferase-deficient yeast by counteracting the Ca^{2+}-induced influx of toxic cations through a depolarization of the plasma membrane. Whether Ca^{2+} affects the ion permeability of the plasma membrane via an IPC-C-dependent signaling pathway or by directly interacting with cell surface-exposed IPC-C remains an open issue. Hence, future studies will be necessary to identify the Ca^{2+} target that initiates Ca^{2+}-induced cell death in IPC-C-overaccumulating yeast.

Acknowledgments—We thank Amy Chang, Sean Munro, and Sirkka Keränen for generously providing plasmids and antibodies and Maurice Jansen for technical assistance.

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