HOR7, a Multicopy Suppressor of the Ca2+-induced Growth Defect in Sphingolipid Mannosyltransferase-deficient Yeast*

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Yeast mutants defective in sphingolipid mannosyla-
tion accumulate inositol phosphorylceramide C (IPC-C), which renders cells Ca2+-sensitive. A screen for loss of function suppressors of the Ca2+-sensitive phenotype previously led to the identification of numerous genes involved in IPC-C synthesis. To better understand the molecular basis of the Ca2+-induced growth defect in IPC-C-overaccumulating cells, we searched for genes whose overexpression restored Ca2+ tolerance in a mutant lacking the IPC mannosyltransferases Csg1p and Csh1p. Here we report the isolation of HOR7 as a multicopy suppressor of the Ca2+-sensitive phenotype of Δcsg1Δcsh1Δ cells. HOR7 belongs to a group of hyperosmolarity-responsive genes and encodes a small (89-residue) type I membrane protein that localizes at the plasma membrane. Hor7p is not required for high Ca2+ 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 Ca2+-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 in amide linkage to a fatty acid and can be classified as a head group. Ceramides are comprised of a sphingoid base (4, 5). Aur1p is required for transferring phosphoinositol from phosphatidylinositol onto the C1 hydroxyl group of phytoceramide, yielding inositol phosphorylceramide (IPC; 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-Ca2+-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 α1,6-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 Ca2+-tolerance because csg1 mutants have been identified as Ca2+-sensitive, Sr2+-resistant mutants (12). The Ca2+-sensitive phenotype appears to be due to the accumulation of IPC-C, which contains a phytosphingosine and a monohydroxylated C26 fatty acid. Indeed, the Ca2+-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 sphingolipid base synthesis (LBC1, LBC2, TSC10), palmitoyl-CoA synthesis (FAS2), fatty acid chain elongation (TSC13), conversion of dihydrophosphi-
gosine to phytosphingosine (SUR2), and hydroxylation of the C26 fatty acid (SCS7) (Fig. 1; Refs. 4, 8). Why IPC-C-overaccumu-
lating cells become Ca2+-sensitive is unknown. Several inter-
mediates 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 Ca2+ mobilization (13–15), whereas accumulation of cer-
amide causes cell growth arrest (16, 17). Hence, one possibility is that Ca2+ 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 Ca2+-signaling pathway. Suppressors of the Ca2+-sensitivity in the csg2 mutant included two genes involved in signal transduction, namely the protein kinase TOR2 and the phosphatidylinositol-4-phosphate 5-kine-
rase GSS4 (18). How the signaling pathways of Tor2p and Gss4p are connected to Ca2+-induced cell death in csg1 and csg2 mutants remains to be established. Another model put forward to explain the Ca2+-sensitive phenotype is that Ca2+ 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 Ca2+ uptake and that the Ca2+-sensitive phenotype can be reversed by addition of 0.8 M sorbitol to the growth
Serine + Palmitoyl CoA

3-Ketosphingosine

Dihydro sphingosine

Phytosphingosine + C14:0-COA
Phytoceramide

OH-Phytoceramide

Inositolphosphorylceramide (IPC-C)

OH-IPC

Mannosyl-IPC

Mannosyl-di-IPC

myc-tagged HOR7 was ligated into multicopy vector pRS425 (2 μm, LEU2) and expressed in strain YJY021 (MATα ura3-52 his3-Δ200 leu2−3−112 trp1-Δ681 suc2−Δ9 lys2-Δ801) for localization studies.

Multicopy Suppressor Screen—A YEP13-based yeast genomic DNA library (AB320, ATCC 37323) was transformed into JHY090 (Δcsg1Δcsh1), and transformants were grown on SD plates supplemented with 20 μM CaCl2 at 28 °C. Ca2+-resistant colonies transformed with CSG1- or CSH1-containing plasmids were identified by colony PCR and discarded. Plasmids rescued from the remaining Ca2+-resistant colonies were isolated, retransformed in JHY090, and screened for their ability to complement the Ca2+-sensitive phenotype. This approach yielded three overlapping genomic sequences capable of restoring Ca2+ tolerance. Complementation experiments with restriction fragments and PCR-amplified gene products and the isolated genomic sequences led to the identification of HOR7 as a multicopy suppressor of the Ca2+-induced growth defect in JHY090.

Subcellular Membrane Fractionation—Cells were grown in 500 ml of SD medium, harvested, sonicated, and then lysed in a hypo-osmotic buffer as described (22). Subcellular membranes were collected at 100,000 × g (60 min, 4 °C) and loaded on top of a sucrose gradient prepared in buffer (10 mM Heps-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 (50 h, 4 °C), 20 × 0.6-ml fractions were collected from the top. Equal volumes per fraction were used in ATPase activity and for Western blot analysis (see below).

Western Blot Analysis—Myc-tagged Hor7p was detected with antimyc 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 Cos1p (22), Dsm1p (Molecular Probes, Eugene, OR), and Sso2p (S. Kerrin, 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 specific 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 Heps-KOH, pH 7.2, 0.8 M sorbitol, 2 mM ATP, 5 mM MgCl2). Reactions were stopped after 30 min with 175 μl of 40 mM H2SO4. Then 50 μl of 6 M H2SO4 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 Pi released after 30 min and expressed as nmol Pi/min/mg of protein.

Immunofluorescence Microscopy—Exponentially growing cells were fixed and mounted on polylysine-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 Cy5-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 (KX55, Apoege Instruments Inc., Tucson, AZ) driven by Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).

Measurement of Methylmangium Uptake—Cells (5 A600/ml) were incubated in 50 mM glucose, 10 mM MES, pH 6.0, at 32 °C. After a 5-min incubation, [14C]methylmangium hydrochloride (2 mM and 2.5 μM/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 MgCl2, filtered through a 0.45-μm nitrocellulose filter (Millipore Corp., Bedford, MA), and washed twice with 1 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 Ca2+.

Thus, cells display normal growth in medium containing up to 100 μM CaCl2 (data not shown). A screen for genes specifically involved in Ca2+ regulation in S. cerevisiae revealed CSG1, a gene with a critical function in high Ca2+ tolerance (Beeler...
et al., Ref. 12). Subsequent studies showed that Csg1p is homologous to Chs1p and that the two proteins function as IPC mannosyltransferases (8–10). To study the effects of Δcsg1 and Δcsh1 mutations on Ca sensitivity, cells were grown on SD plates containing 5 mM CaCl₂ at 30 °C. As shown in Fig. 2, Δcsg1 and Δcsh1 cells were resistant to exogenous Ca⁺⁺ and displayed normal growth. In contrast, the Δcsg1Δcsh1 double mutant was highly sensitive to Ca⁺⁺ and did not grow on the medium. Transformation of Δcsg1Δcsh1 cells with the CSG1 or CSH1 gene on a single copy vector fully suppressed the Ca⁺⁺-induced growth defect. Deletion of IFT1, a gene required for MIP₃/C synthesis (23, 24) had no effect on Ca⁺⁺ tolerance. Thus, the Ca⁺⁺ sensitivity of yeast cells correlates with the level of MIPC synthesis rather than MIP₃/C synthesis.

Isolation of HOR7 as a Multicopy Suppressor of the Ca⁺⁺-induced Growth Defect in Δcsg1Δcsh1 Cells—In the absence of sphingolipid mannosylation, yeast cells accumulate IPC-C. Suppressor mutations that reverse the Ca⁺⁺ 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⁺⁺-sensitive. To gain further insight into the molecular basis of the Ca⁺⁺-induced growth defect in IPC-C-overaccumulating cells, we performed a multicopy suppressor screen aimed at the identification of genes whose overexpression restores Ca⁺⁺ tolerance in the Δcsg1Δcsh1 mutant. To this end, Δcsg1Δcsh1 cells were transformed with a yeast genomic DNA library on a multicopy vector, and transformants that regained tolerance to 20 mM CaCl₂ 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⁺⁺. Sixty Ca⁺⁺-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⁺⁺-resistant colonies were retransformed in Δcsg1Δcsh1 cells and screened for their ability to restore Ca⁺⁺ 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. 3A). Complementation studies with restriction fragments of the genomic sequence were used to eliminate four of the six genes as possible suppressors of the Ca⁺⁺-induced growth defect in Δcsg1Δcsh1 cells (Fig. 3B). 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⁺⁺ tolerance. This led to the identification of a gene named HOR7 whose overexpression suppressed the Ca⁺⁺-induced growth defect in Δcsg1Δcsh1 cells (Fig. 3B).

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. 4A). 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⁺⁺ tolerance in Δcsg1Δcsh1 cells (Fig. 4B). 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⁺⁺ tolerance in Δcsg1Δcsh1 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. 5A). Immunofluorescence microscopy revealed a peripheral staining pattern and extensive co-localization with the plasma membrane-associated syntaxin, Sso2p (Fig. 5B). 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. 5C 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. 5D). 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. 5E, upper panel). In contrast, prestained cells were positive for anti-Myc antibodies but negative for anti-Sso2p antibodies (Fig. 5E, 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. 5D.

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-
somal copy of \textit{HOR7} was disrupted by homologous recombination. Haploid \textit{H9004 hor7} and \textit{H9004 csg1\textbackslash{}csh1 hor7} strains were obtained, indicating that \textit{Hor7p} is not essential under standard growth conditions, \textit{i.e.} in YEPD or SD medium at 30°C. \textit{Hor7p} is not required for high salt, high Ca\textsuperscript{2+}, or low pH tolerance, because \textit{H9004 hor7} cells grew as well as wild type on 1 M NaCl, 50 mM CaCl\textsubscript{2} or at pH 2.5 (Fig. 6A). However, we noticed that cells overproducing \textit{Hor7p} from a multicopy vector grew better on 1 M NaCl and were more sensitive to low pH than wild type or \textit{Hor7} cells. Although \textit{Hor7p}-overproducing cells grew better than \textit{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 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\textsuperscript{+} (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 \textit{Hor7p} acts as a negative regulator of Pma1p, we determined the rates of MgATP hydrolysis in plasma membranes derived from \textit{H9004 hor7} cells transformed with \textit{HOR7} on a multicopy plasmid (\textit{HOR7}\textendash{}2\textendash{}m) or with empty vector. To this end, cells were lysed and the plasma membranes separated from intracellular organelles by

**Fig. 3.** Identification of \textit{HOR7} as a multicopy suppressor of the Ca\textsuperscript{2+}-induced growth defect in \textit{Δcsg\textbackslash{}Δcsh1} cells. \textbf{A}, a screen for multicopy suppressors of the Ca\textsuperscript{2+}-induced growth defect in \textit{Δcsg\textbackslash{}Δcsh1} cells yielded a genomic DNA sequence harboring the complete open reading frames of six different genes (I). \textit{HOR7} was identified by testing restriction fragments (II, III) and PCR-amplified open reading frames for their ability to complement the \textit{Δcsg\textbackslash{}Δcsh1 Ca\textsuperscript{2+}}-sensitive phenotype. \textbf{B}, serial 3-fold dilutions of exponentially grown \textit{Δcsg\textbackslash{}Δcsh1} cells transformed with genomic DNA fragments (I-III) or PCR-amplified open reading frames (\textit{HOR7}, \textit{YMR252C}) on a multicopy vector were spotted onto SD plates with or without 20 mM CaCl\textsubscript{2} as indicated. Plates were scanned after 3 days of incubation at 30°C.

**Fig. 4.** Overexpression of \textit{HOR7}, but not of the homologous gene \textit{DDR2}, suppresses the Ca\textsuperscript{2+}-induced growth defect in \textit{Δcsg\textbackslash{}Δcsh1} cells. \textbf{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. \textbf{B}, \textit{csg\textbackslash{}csh1} cells transformed with \textit{HOR7} or \textit{DDR2} on a multicopy vector were grown in SD medium with or without 20 mM CaCl\textsubscript{2}. At the indicated times, 1-ml aliquots were withdrawn for measurement of the A\textsubscript{600}.

![Diagram](image-url)
fractionation on equilibrium sucrose density gradients (Fig. 7A). 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. 7A 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. 7B, 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 the plasma membrane-associated ATPase. There was no significant difference in plasma membrane-associated ATPase activity between Hor7 deficient and -overproducing cells.

**DISCUSSION**

**Yeast mutants defective in IPC mannosylation accumulate IPC-C**
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 \( \Delta csg1 \Delta csh1 \) 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 hyperosmosality-responsive genes whose transcript levels are increased over 10-fold by 1 M NaCl or 1.5 M sorbitol (25). HOR7 encodes a 59-aa protein with a predicted signal sequence and C-terminal membrane span. Membrane fractionation analysis and immunofluorescence microscopy of cells expressing untagged (HOR7) or myc-tagged HOR7 (HOR7-myc) from a multicopy vector. Membranes (m) and cytosol (c) were prepared from yeast cells expressing untagged (myc) or -HOR7 low pH by reducing the activity of plasma membrane H^+-ATPase. Hence, it appears unlikely that Hor7p renders cells sensitive to the plasma membrane-associated ATPase activity between Hor7p-deficient and -overproducing cells.

**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.

**FIG. 5.** Hor7p localizes at the plasma membrane. A, Hor7p is membrane-associated. High speed membrane pellets (100,000 \( \times \) 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-Gos1p 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 \( \times \) 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 topology 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, \( \Delta \)hor7, and \( \Delta \)hor7 cells transformed with HOR7 on a multicopy vector (HOR7-2 \( \mu \)m) were spotted onto SD plates supplemented with the indicated concentrations of H^+, NaCl, or CaCl_2. B, \( \Delta \)hor7 and \( \Delta \)hor7 cells transformed with HOR7-2 \( \mu \)m 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.
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 Δseg1/Δcsh1 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 Δseg1/Δcsh1 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.

Ca²⁺ has previously been proposed to induce an irreversible alteration in the plasma membrane of csg1 and csg2 yeast mutants that increases the influx of Ca²⁺ and perhaps other toxic cations, resulting in cell death (8). Our data suggest that Hor7p restores high Ca²⁺ tolerance in IPC mannosyltransferase-deficient yeast by counteracting the Ca²⁺-induced influx of toxic cations through a depolarization of the plasma membrane. Whether Ca²⁺ 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 Ca²⁺ target that initiates Ca²⁺-induced cell death in IPC-C-overaccumulating yeast.

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