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Saccharomyces cerevisiae Na^+/H^+ Antiporter Nha1p Associates with Lipid Rafts and Requires Sphingolipid for Stable Localization to the Plasma Membrane

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The plasma membrane-type Na^+/H^+ antiporter Nha1p from budding yeast plays an important role in intracellular Na^+ and pH homeostasis by mediating the exchange of Na^+ for H^+ across the plasma membrane. However, the mechanism of intracellular targeting of Nha1p to the plasma membrane remains unknown. Here, we found that Nha1p exists predominantly in detergent-resistant membrane fractions (DRMs) following density gradient centrifugation. When ergosterol was extracted from membranes, Nha1p was transferred to a detergent-soluble fraction, suggesting that Nha1p associates with ergosterol-containing DRMs, also known as lipid rafts. Density gradient centrifugation of cell extracts of yeast mutants that were defective in different stages of the secretory pathway revealed that, unlike previously identified raft proteins, the association of Nha1p with DRMs occurs mainly at the plasma membrane. In *lcb1-100* cells, which are temperature-sensitive for sphingolipid synthesis, newly synthesized Nha1p failed to localize to the plasma membrane at the non-permissive temperature. Rather, Nha1p was distributed in an intracellular punctate pattern. The addition of phytosphingosine or the inhibition of endocytosis in *lcb1-100* cells restored the targeting of Nha1p to the plasma membrane. The results of the current study suggest that sphingolipids are required for the stable localization of Nha1p to the plasma membrane.

Key words: lipid rafts, membrane traffic, Na^+/H^+ antiporter, *Saccharomyces cerevisiae*, sphingolipid.

Abbreviations: Nha1p, Na^+/H^+ antiporter 1 protein; SDS–PAGE, SDS polyacrylamide gel electrophoresis; DRM, detergent-resistant membrane; MβCD, methyl β-cyclodextrin; ER, endoplasmic reticulum; TCA, trichloroacetic acid; PHS, phytosphigosine.

Intracellular ion homeostasis is important for the physiological well-being of all living cells (*1–3*). Na^+/H^+ antiporters are ubiquitous membrane proteins that play a major role in intracellular pH and Na^+ homeostasis (*4–7*). The budding yeast *Sacchromyces cerevisiae* has two Na^+/H^+ antiporters, Nha1 and Nhx1, that localize to the plasma and endosomal membrane, respectively (*8–10*). Both proteins remove excess Na^+ from the cytoplasm using a proton gradient generated by H^+ pumps (*9*, *11*). Accordingly, these antiporters confer salt resistance to yeast when cells are grown in high sodium medium (*12*, *13*). The structure of plasma membrane-type Nha1p consists of two domains, an integral membrane domain and a long hydrophilic tail domain (*14*). Overall, this molecular structure is similar to that of the mammalian Na^+/H^+ antiporters NHE1-9, although the primary sequence of these proteins is not well conserved between yeast and mammals (*14–22*). However, because of the similarities in molecular structure and characteristics, plasma membrane-type Nha1p is an important model of mammalian NHE function and the molecular mechanism of Na^+/H^+ antiport. To date, the mechanism of substrate (e.g. Na^+, K^+ and H^+) transport and the localization of Na^+/H^+ antiporters remains poorly understood.

Understanding the mechanism of targeting of Nha1p to the plasma membrane is an important aspect of the structure-function relationship of yeast Nha1p. Nha1p is transported from the endoplasmic reticulum (ER) through the Golgi complex to the plasma membrane, which is its site of function (*23*). In general, membrane protein transport is mediated by specific transport vesicles. The integration of membrane proteins into transport vesicles is often mediated by a cytoplasmic sorting signal that is present in the membrane protein, and which is recognized by vesicle coat or adaptor proteins. Some membrane proteins contain a sorting signal in their membrane-spanning regions (*24*). The sorting signal of Nha1p and the mechanism of Nha1p loading into specific transport vesicles are as-yet unknown. Previously, we showed that a variant of Nha1p that lacks most of the cytoplasmic tail domain localizes to the plasma membrane, similar to wild-type Nha1p (*23*). These results suggest that Nha1p contains a sorting determinant within its membrane-spanning

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regions. Since lipids interact with membrane-spanning regions of proteins, it is plausible that lipid composition affects the localization of membrane proteins.

Lipid rafts are formed by the lateral association of sphingolipids and cholesterol, and were first conceived in mammalian MDCK cells as a platform for polarized lipid and protein sorting (*25*, *26*). Comprehensive work has established a role of lipid rafts in membrane trafficking in different cell types (*27–30*). Furthermore, a role of lipid rafts as a signaling platform has been proposed as a fundamental element of numerous signal transduction cascades, and is thought to be crucial for immune system activation (*31*, *32*). The plasma membrane of *S. cerevisiae* contains sphingolipid- and ergosterol-rich micro-membrane domains called lipid rafts, which are insoluble in detergents such as TritonX-100 (*33*). It is believed that most yeast plasma membrane proteins associate with lipid rafts. The sphingolipids in *S. cerevisiae* are inositol-phosphoceramide (IPC), mannose-inositol-phosphoceramide (MIPC), and mannose-(inositol phosphate)$_2$-ceramide [M(IP)$_2$C] (*34–37*). During sphingolipid synthesis, ceramide is formed in the ER, and is then transported to the Golgi where it undergoes additional modifications (*34–37*). Sphingolipids are then transported to the plasma membrane, where they are concentrated. Sphingolipid synthesis is important in the cell surface targeting of the H$^+$-ATPase Pma1p, and the lack of sphingolipid causes the mis-sorting of Pma1p in the late Golgi and degradation in vacuoles (*38*, *39*). Moreover, Pma1p sorting in the late Golgi does not require the production of mannosylated sphingolipids (*40*). These observations suggest that yeast plasma membrane proteins, such as Pma1p, associate with sphingolipid-containing membranes (lipid rafts) in the early stages of the secretory pathway. Here, we investigated whether Nha1p interacts with lipid rafts, and whether lipid raft association was required for the localization of Nha1p to the plasma membrane. We found that Nha1p localizes predominantly to detergent-insoluble and ergosterol-rich membrane fractions. Moreover, Nha1p interacted with lipid rafts primarily at the plasma membrane, and not in the ER or Golgi apparatus. Blocking sphingolipid synthesis resulted in the targeting of newly synthesized Nha1p to intracellular structures.

MATERIALS AND METHODS

Yeast Strains, Media and Growth Conditions—*S. cerevisiae* strains MKY0581 and MKY08101, carrying deletions of *NHA1* or *END3*, respectively, were derived from W303-1B (MATα *ade2-1 trp1-1 can1-100 leu2-3*/*112 his3-11*/*15 ura3-1*) (*41*). Strains MKY0591, MKY05101 and MKY05151, which are derivatives of MTsec6 (*sec6*), MTsec12 (*sec12*) and MTsec14 (*sec14*), respectively, were derived from YPH499 (*MAT***a** *ura3-52 lys2-80 ade2-10 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1*) and carried a deletion of *NHA1*. MTsec6, MTsec12 and MTsec14 were a kind gift from Dr Yoshinori Ohsumi (National Institute of Basic Biology, Okazaki, Japan). MKY08121 was derived from RH3804 (*MAT*α *lcb1-100 trp1 leu2 ura3 lys2 bar1*), a temperature-sensitive mutant of *LCB1* that was obtained from Dr Howard Riezman (Department of Biochemistry, Biozentrum of the University of Basel, Switzerland). For MKY0581, MKY0591, MKY05101, MKY05151, MKY08101 and MKY08121, disruption of the *NHA1* or *END3* locus was performed as follows. A *LEU2* or kanMX gene cassette was amplified by polymerase chain reaction (PCR) from pUG73 or pUG6 (*42*) using either primer #1 (5′-CTTAGCTAGATATTATGGCTATCTGGGAGCAACTA GAAGTCAGCTGAAGCTTCGTACGC-3′) and #2 (5′-TT GAGACCAAGCGTTTTTGATAGCGCCGACTTAACAGCA GGCATAGGCCACTAGTGGATCTG-3′), or primer #3 (5′-GTGGGTATTGGAAAGGCCGGTAAAGATAACAGGGAT CTCTGAAAACAGCTGAAGCTTCGTACGC-3′) and #4 (5′-ACAGTAAATATTACACATTCATGTACATAAAATTA ATTATCGGTGGCATAGGCCACTAGTGGATCTG-3′), respectively. Amplified DNA was introduced into W303-1B or RH3804 cells and the resulting Leu$^+$ or G418-resistant transformants were selected on medium lacking leucine or containing 200 μg/ml G418 disulfate, respectively. Insertion of the *LEU2* or kanMX gene was confirmed by PCR analysis of genomic DNA. Standard yeast media and genetic manipulations were performed as described by Sherman *et al*. (*43*), and yeast transformations were performed using the lithium acetate method (*43*). All yeast strains were routinely cultured at 30°C in YPD medium (1% yeast extract, 2% peptone and 2% glucose) or in SCD medium (0.17% yeast nitrogen base without ammonium sulfate or amino acids, 0.5% ammonium sulfate, 0.5% casamino acids and 2% glucose) supplemented with appropriate nucleotides and amino acids. A 5 mM phytosphingosine (PHS) stock solution was prepared by dissolving phytosphingosine hydrochloride (Sigma, St Louis, MO) in methanol. In the indicated experiments, the PHS stock solution was added to SCD medium at a final concentration of 5 μM. The control cells were treated with ethanol alone. *Escherichia coli* strains JM109 and BL21(DE3) were used to propagate plasmids and for the expression of recombinant proteins. *E. coli* was cultured in L broth supplemented with appropriate antibiotics for the selection of transformants, as described previously (*44*).

Plasmids—pRS314-NHA1-EGFP (*45*) is a derivative of the centromeric plasmid pRS314, and encodes a green fluorescent protein (GFP) fusion protein of Nha1p (Nha1p-GFP) under the control of the *NHA1* promoter (*46*). pRS314-HS4-NHA1-EGFP encodes Nha1p-GFP under the control of the *SSA4* promoter. The *SSA4* promoter region identified by Craig (*47*, *48*) was amplified from yeast genomic DNA by PCR using the oligonucleotide primers #5 (5′-ACGACTCACTATAGGG CGAATTGGAGCTCCACCGCGGTGGAGATTCGTTGTA TGTGCGGG-3′) and #6 (5′-TAGCTAAGTTCAGGATTT TTTTTTATAATGTACAAAAAAACTTGTTTTTGTTATTT ACTTTTTATTAGTGG-3′). The 5′-ends of primers #5 and #6 contained a 40 nucleotide sequence that was homologous to sequences upstream of the *NHA1* promoter and downstream of the *NHA1* ATG start codon, respectively, in pRS314-NHA1-EGFP. Amplified DNA was purified and transformed together with linearized pRS314-NHA1-EGFP into yeast. Transformants were selected on SD plates without tryptophan. pRS314-HS4-NHA1-EGFP was recovered from selected transformants and re-introduced into *E. coli*. Insertion of the *SSA4* promoter

in place of the *NHA1* promoter was verified by DNA sequencing.

Isolation of DRMs and Methyl β-cyclodextrin Extraction—DRM association analysis was performed as described previously (*33*). Briefly, yeast were grown to logarithmic phase, washed once in water and then collected. The cells were disrupted in TNE buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin) by vortexing with glass beads eight times for 30 s at 4°C. The lysate was clarified by centrifugation at $500 \times g$ for 5 min to remove unbroken cells and debris. The cleared lysate was incubated with TritonX-100 (1% final concentration) for 30 min on ice. After the extraction with TritonX-100, the lysate (1200 μl) was adjusted to a final concentration of 40% Optiprep with a solution of 60% Optiprep (Sigma, St Louis, MO), and then overlaid with 1920 μl of 30% Optiprep in TXNE (TNE plus 0.1% TritonX-100) and 320 μl of TXNE. The samples were subjected to centrifugation at 46,000 rpm for 3.5 h in a SW50.1 rotor (Beckman, Fullerton, CA), and nine fractions of equal volume were collected from the top. Fractions were precipitated by the addition of trichloroacetic acid (TCA) (10% final concentration) and then analysed by SDS–PAGE and immunoblot. All DRM association experiments were performed at least two times with essentially the same results. Methyl β-cyclodextrin extraction was performed as described previously (*33*). Crude membrane fractions were prepared from cleared lysate by ultracentrifugation at $100,000 \times g$ for 60 min. The membranes were treated with 100 mM methyl β-cyclodextrin (MβCD) (Sigma, St Louis, MO) for 15 min at 37°C and then precipitated again by centrifugation. The membrane pellet was resuspended in TNE buffer and then subjected to flotation analysis following TritonX-100 treatment.

SDS–PAGE, Immunoblot Analysis and Antibodies—Protein samples were resuspended in SDS sample buffer and subjected to electrophoresis on SDS polyacrylamide gels. The separated proteins were transferred to a GVHP filter (Millipore, Bedford, MA). The membrane was incubated with 10% skim milk in PBST (7.81 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 2.68 mM KCl, 0.1% Tween20), followed by incubation with the indicated primary antibody. After washing with PBST, the membrane was incubated with horseradish peroxidase-conjugated second antibody. Immunoreactive bands were visualized using the enhanced chemiluminescence method (Amersham Pharmacia Biotech, Piscataway, NJ). A rabbit polyclonal anti-Nha1p antibody (*49*) was prepared by immunizing a rabbit with bacterially expressed hydrophilic tail peptides of Nha1p (amino-acid residues 434–523), and was affinity purified before use. Goat anti-Pma1p antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-Vph1p and mouse anti-Pho8 antibodies were purchased from Molecular Probes (Eugene, OR). Rabbit anti-GFP serum was obtained form invitrogen (Carlsbad, CA). Horseradish peroxidase-conjugated secondary antibodies against rabbit, goat and mouse IgG were purchased from Vector Laboratories (Burlingame, CA).

Fluorescence Microscopy—Yeast cells that expressed Nha1p-EGFP were grown in SD medium and observed under a fluorescence microscope (BX51, Olympus, Tokyo, Japan) equipped with a NIBA filter. Images were recorded using an ORCA-ER1394 digital camera (Hamamatsu Photonics, Hamamatsu City, Japan).

Other Procedures and Materials—DNA manipulations were performed according to published procedures (*50*). Protein concentration was measured as described previously (*51*). Restriction enzymes, KOD DNA polymerase and T4 DNA ligase were purchased from Toyobo (Osaka, Japan) and Takara (Otsu, Japan). The oligonucleotides used in this study were synthesized by Invitrogen (Carlsbad, CA). Other materials were of the highest commercially available grade.

RESULTS

Nha1p Associates with DRMs—To determine whether Nha1p interacted with DRMs, yeast cell lysate was treated with 1% TritonX-100 for 30 min at 4°C and then subjected to Optiprep density gradient centrifugation (Fig. 1A). After floatation in a density gradient, the distribution of Nha1p was analysed by immunoblot using an anti-Nha1p antibody. DRMs floated to the interface of 0 and 30% Optiprep (Fig. 1A, fraction 2, arrowhead), whereas detergent-soluble proteins remained in the bottom fractions (fractions 6–9). In the absence of detergent, membrane proteins localized to fraction 2 (Fig. 1B, left). After treatment with detergent, a portion of Pma1p, a H^+-ATPase that is a known raft-associated protein, localized to fraction 2, whereas the 100 kDa subunit of the vacuolar H^+-ATPase Vph1p and the vacuolar alkaline phosphatase Pho8p, which are non-raft proteins, were detected in the bottom fractions, as reported previously (*33*) (Fig. 1B, right). Nha1p was detected in faction 2 at a level that was nearly equal to that of Pma1p (Fig. 1C). These results suggested that Nha1p associates with DRMs. Unexpectedly, after treatment with the detergent, a small portion of Nha1p and Pma1p was detectable not only in fraction 2 but also in the bottom fractions (Fig. 1B and C). This suggests that Nha1p or Pma1p in the bottom fractions was partially solubilized, since these proteins were present in only fraction 2 in the absence of detergent. This result also suggests that a portion of Nha1p or Pma1p in yeast cells does not interact with DRMs. These unbound proteins may be present in intermediate-membranes during Nha1p biogenesis or degradation.

Nha1p-Containing DRMs are Enriched in Ergosterols—To determine whether Nha1p-containing DRMs were lipid rafts, we investigated DRM integrity in the presence and absence of ergosterol. Previously, it was shown that the association of GPI-anchored protein Gas1p and Pma1p with DRMs is reduced in ergosterol-depleted (*erg1*Δ cells, reflecting the fact that ergosterol is one of the major components of lipid rafts, and may be required for raft formation (*33*). We incubated total crude membranes with a buffer containing MβCD for 15 min at 37°C to extract ergosterol from the membranes. Since MβCD is less efficient in extracting ergosterol than cholesterol, we performed the extraction using a higher

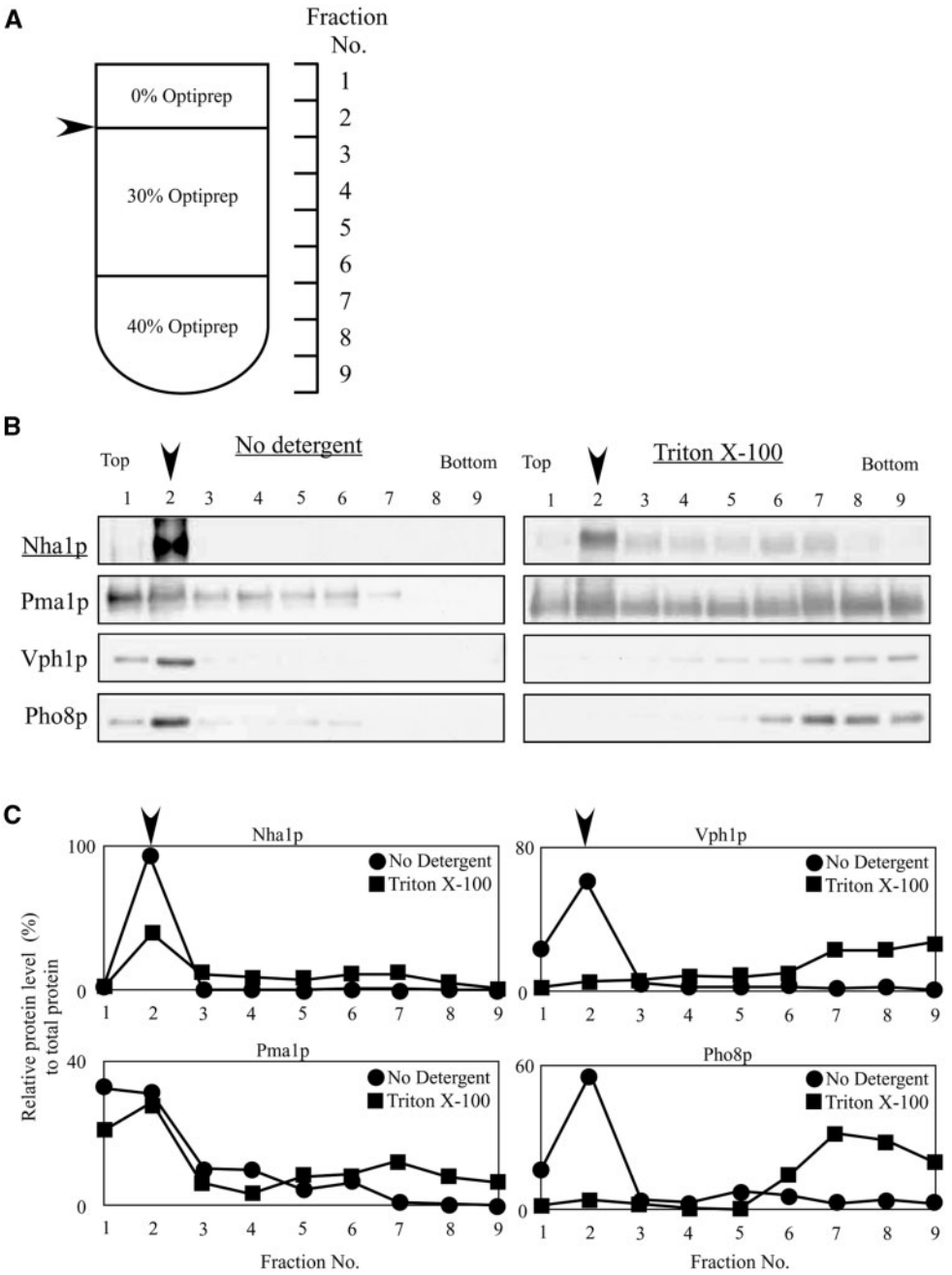

Fig. 1. **Nha1p associates with DRMs.** (A) Schematic representation of the floatation analysis of the TritonX-100 solubility of membrane proteins. Intact membranes and TritonX-100-insoluble membranes float to the interface (arrowhead) between 0% and 30% Optiprep, which corresponds to fraction 2. (B) Intact membranes from W303-1B (wild-type) cells were incubated with TNE buffer or TritonX-100 and then subjected to floatation analysis, as described in MATERIALS AND METHODS section. Proteins in each fraction were precipitated with 10% TCA, separated by SDS–PAGE and then analysed by immunoblot using anti-Nha1p and -Pma1p antibodies, or anti-Vph1p and -Pho8p antibodies. (C) The data shown in (B) was quantified based on the protein band intensity in each fraction, and protein levels in each fraction were expressed as a percentage of total protein.

concentration of MβCD than that used in previous experiments (*33*, and MATERIALS AND METHODS section). MβCD was removed by centrifugation, and the membranes were resuspended in a buffer and subjected to floatation analysis after incubation with TritonX-100. Following MβCD treatment, the level of Pma1p in DRMs was markedly reduced, from 45 to 8%, whereas the distribution of the vacuolar proteins Vph1p and Pho8p was unchanged (Fig. 2A and B). The level of Nha1p in DRMs was also reduced, from 50% to 20%, following incubation with MβCD (Fig. 2A and B). These results suggested that Nha1p localizes to ergosterol-enriched DRMs, or lipid rafts.

Nha1p-GFP Exhibits a Regular Punctate Distribution Pattern in the Plasma Membrane—In *S. cerevisiae*, the distribution of most DRM-associated proteins, such as

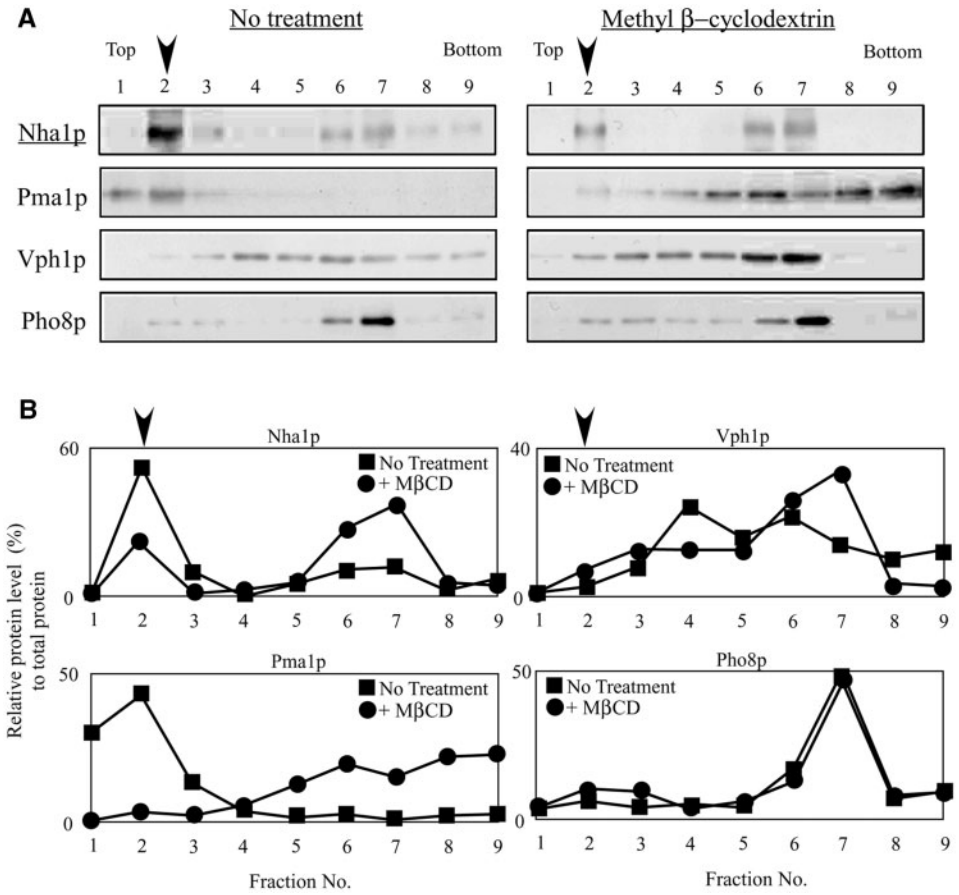

Fig. 2. **Nha1p-containing DRMs are enriched in ergosterol.** (A) Intact membranes from W303-1B (wild-type) cells were treated with TNE buffer or TNE buffer containing MβCD. Membranes were recovered by ultracentrifugation, incubated with TritonX-100, and then subjected to floatation analysis. Proteins in each fraction were precipitated with 10% TCA, separated by SDS-PAGE and then analysed by immunoblot using anti-Nha1p and -Pma1p antibodies, or anti-Vph1p and -Pho8p antibodies. (B) The data shown in (A) was quantified based on the protein band intensity in each fraction, and protein levels in each fraction were expressed as a percentage of total protein.

Pma1p, arginine/H^+ symporter Can1p and uracil permease Fur4p, is non-homogenous within the plasma membrane, as observed by confocal microscopy (*52*, *53*). In contrast, hexose permease Hxt1p exhibits a homogenous distribution pattern within the cell membrane, independent of its association with DRMs (*52*, *54*). To visualize Nha1p *in vivo*, *nha1*Δcells were transformed with pRS314-NHA1-EGFP (*23*), which encoded Nhap1 fused at its C-terminus to GFP. Transformed *nha1*Δcells expressing Nha1p-GFP were highly resistant to high salinity growth conditions (*23*, *45*), which indicated that Nha1p-GFP was fully functional. When we analysed the solubility of Nha1p-GFP after TritonX-100 treatment on Optiprep density gradients, the majority of Nha1p-GFP was present in the DRM fraction (data not shown), similar to endogenous wild-type Nha1p (see Figs 1 and 2). These results indicated that the fusion of GFP to the C-terminus of Nha1p does not affect the ion exchange activity and detergent-solubility of Nha1p, and that Nha1p-GFP can be used to analyse the localization and function of Nha1p. When we examined transformed cells by fluorescence microscopy, we observed a regular punctate distribution pattern of Nha1p-GFP throughout the cell membrane (Fig. 3), similar to Pma1p, Can1p and Fur4p (*52*, *54*). These results indicated that the localization of Nha1p-GFP is not homogenous within the plasma membrane, and that the distribution pattern of Nha1p is similar to the raft proteins Pma1p, Can1p and Fur4p.

The Association of Nha1p With DRMs Occurs Mainly at the Plasma Membrane—Newly synthesized Nha1p in the ER is transported to the plasma membrane via the secretory pathway, as evidenced by the fact that Nha1p biogenesis is blocked in temperature-sensitive mutants of successive steps in the secretory pathway (*23*), including *SEC12* (ER to COPII vesicles), *SEC14* (Golgi complex to secretory vesicles) and *SEC6* (secretory vesicles to plasma membrane) (*55*, *56*) (Fig. 4A). First, we performed flotation analysis of cell lysates obtained from *sec* mutants after incubation with TritonX-100. However, it was impossible to determine the solubility of endogenous Nha1p as the amount of newly synthesized Nha1p after the temperature-shift to the non-permissive temperature was too small to be distinguished from the Nha1p synthesized prior to the temperature-shift by immunoblotting with the anti-Nha1p antibody. This probably

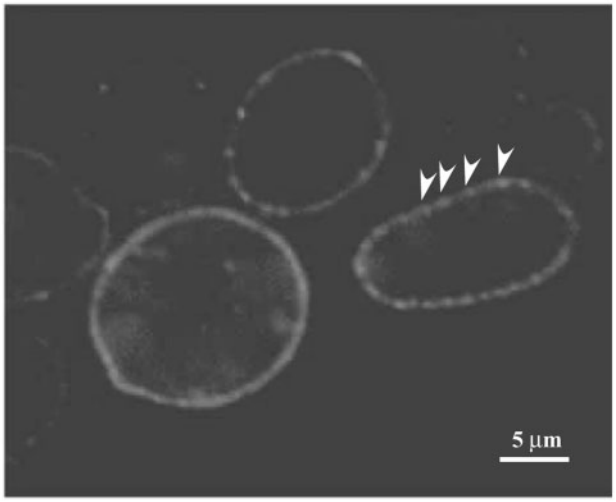

Fig. 3. **Nha1p-GFP exhibits a punctate distribution pattern within the plasma membrane.** Fluorescence pattern of Nha1p-GFP in MKY0581 (*nha1*Δ) cells. Cells were transformed with pRS314-NHA1-EGFP and cultured at 30°C until logarithmic growth phase, at which point they were analysed by fluorescence microscopy. Arrowheads indicate the location of Nha1p-GFP within the plasma membrane. Bar: 5 μm.

reflects the low expression level of Nha1p under these conditions. Therefore, we were unable to detect changes in Nha1p solubility in *sec* mutants grown at the non-permissive temperature. To determine the effect of inhibiting specific stages of the secretory pathway on newly synthesized Nha1p-GFP, we transformed *sec* mutants with pRS314-HS4-NHA1-EGFP, which encoded Nha1p-GFP under the control of a heat shock promoter derived from *SSA4*, a member of the *HSP70* family of heat shock proteins (Fig. 4B). Transformed cells were maintained at 24°C, and then shifted to 37°C for 2 h to induce the expression of Nha1p-GFP. The expression of Nha1p-GFP was clearly evident in all *sec* mutants after incubation at 37°C, whereas little Nha1p-GFP was detected in cells grown at 24°C (Fig. 4B). As expected, Nha1p-GFP localized to secretory vesicles, ER membranes and the Golgi complex in *sec6*, *sec12* and *sec14* mutants, respectively (data not shown). We then subjected cell lysates of the transformed *sec* mutants to floatation analysis after incubation with TritonX-100 to determine the solubility of Nha1p in intermediate-membranes (ER membrane, Golgi complex and secretory vesicle) during Nha1p biogenesis (Fig. 4C and D). There was a major peak of Nha1p-GFP in wild-type (*SEC*$^+$) cells in the DRM fraction (48% of the total Nha1p), which

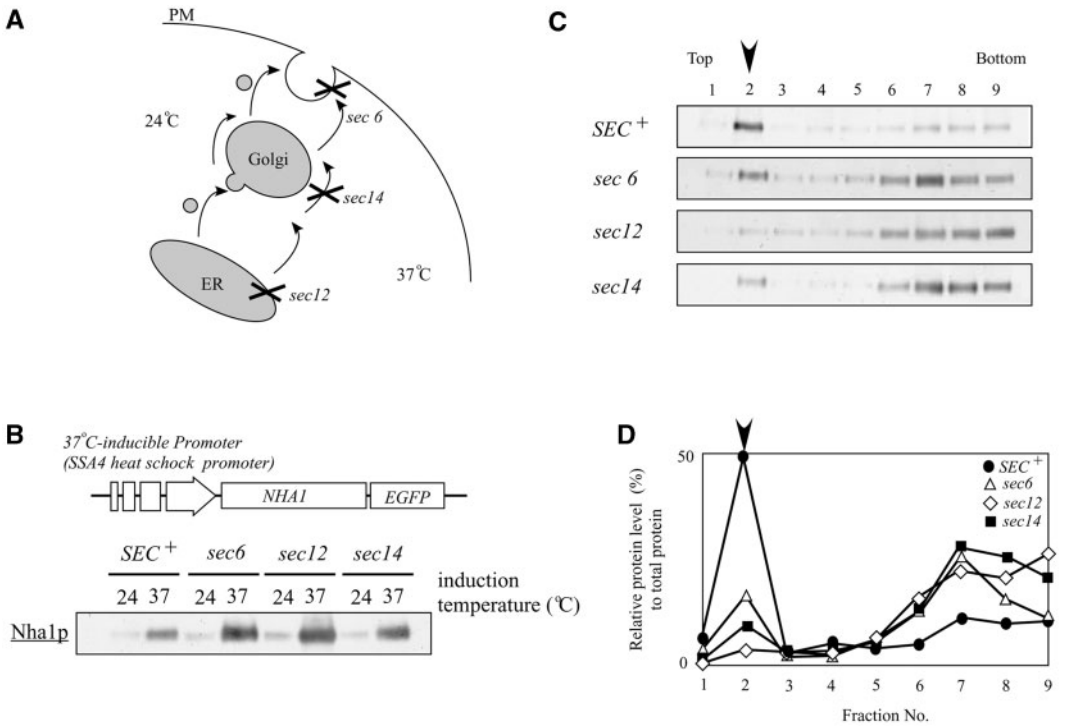

Fig. 4. **Nha1p associates with DRMs at the plasma membrane.** (A) Schematic illustration of the transport of newly synthesized Nha1p from the ER to the plasma membrane through the secretory pathway. Nha1p biogenesis is blocked by temperature-sensitive mutations in genes that govern successive steps of the pathway: *SEC12* (ER to COPII vesicles), *SEC14* (Golgi complex to secretory vesicles) and *SEC6* (secretory vesicles to plasma membrane). (B) MKY0581 (*nha1*Δ), MKY0591 (*sec6 nha1*Δ), MKY05101 (*sec12 nha1*Δ and MKY05151 (*sec14 nha1*Δ) cells were transformed with pRS314-HS4-NHA1-EGFP, grown to logarithmic phase at 24°C, and then shifted to 37°C for 2 h. Membranes were isolated, and then analysed by immunoblot using an anti-Nha1p antibody. (C) Yeast cell lysates were subjected to flotation analysis, as described in MATERIALS AND METHODS section. Proteins in each fraction were precipitated with 10% TCA, resolved by SDS–PAGE, and then analysed by immunoblot using an anti-Nha1p antibody. (D) The data in panel (C) was quantified based on the protein band intensity of Nha1p in each fraction, and Nha1p levels in each fraction were expressed as a percentage of total Nha1p. *SEC*$^+$, closed circle; *sec6*, open triangle; *sec12*, open diamond; *sec14*, closed square.

was consistent with previous data (Figs 1C and 2B). These results suggested that the DRM association of Nha1p-GFP induced in response to heat shock is similar to endogenous Nha1p. A relatively small fraction of Nha1p localized with DRMs in *sec6*, *sec12* and *sec14* mutants (14%, 3% and 8% of the total Nha1p, respectively). These results suggested that the association of Nha1p with DRMs takes place mainly after Nha1p exits the Golgi complex, possibly at the plasma membrane, but not in the ER membrane and Golgi complex.

Synthesis of Sphingolipids is Required for the Stable Localization of Nha1p to the Plasma Membrane—To determine whether the association of Nha1p with DRMs was required for plasma membrane localization, we examined the localization of Nha1p-GFP in sphingolipid-deficient *lcb1-100* cells. The *lcb1-100* mutation (*33*) is a temperature-sensitive mutation in a subunit of serine palmitoyltransferase that blocks the first step of the sphingolipid biosynthetic pathway (Fig. 5A). We examined the localization of newly synthesized Nha1p-GFP, expressed under the control of the *SSA4* heat shock promoter, in wild-type (*LCB1*$^+$) and *lcb1-100* cells (Fig. 5B and C). When wild-type cells were shifted to 37°C for 2 h, Nha1p-GFP was detected at the plasma membrane, whereas Nha1p-GFP was undetectable in cells grown at 24°C (Fig. 5B). In *lcb1-100* cells, Nha1p-GFP was distributed in a punctate pattern throughout the cytoplasm, and was not detected at the plasma membrane (Fig. 5C, upper panel). To determine whether this mis-localization of Nha1p to intracellular compartments was due to a disruption in the association of Nha1p-GFP with DRMs, we examined the association of newly synthesized Nha1p-GFP with DRMs in *lcb1-100* cells that were grown at the non-permissive temperature. However, Nha1p-GFP was undetectable by immunoblot under these conditions, since Nha1p-GFP was unstable in sphingolipid-depleted condition (Fig. 6B, lane 2). We then examined the localization of Nha1p-GFP in *lcb1-100* cells grown in the presence of phytoshpingosine (PHS), a sphingolipid biosynthetic intermediate. The mis-localization of Nha1p-GFP was restored by exogenous PHS (Fig. 5C, lower panels). These results suggested that the loss of DRMs results in the mis-localization of Nha1p-GFP, and that sphingolipids are required for the proper plasma membrane localization of Nha1p.

In Fig. 5, Nha1p-GFP expression from the heat shock promoter was higher than that from its own promoter. Moreover, it has been reported that sphingolipid synthesis is affected by heat shock (*57*). Therefore, there was a possibility that mis-localization of Nha1p-GFP was due rather to Nha1p-GFP over-expression or changes in sphingolipid synthesis than to the *lcb1-100* mutation. To exclude these possibilities, we examined the intracellular localization of Nha1p-GFP in yeast cells depleted of sphingolipids by treating them with myriocin (inhibitor of serin palmitoyltransferase Lcb1p) in the absence of heat shock. When Nha1p-GFP was expressed from its own promoter, fluorescent signals of Nha1p-GFP were observed in intracellular compartments (possibly the lumen of vacuoles), in addition to the plasma membrane (data not shown). This result suggests that mis-localization of newly synthesized Nha1p-GFP in

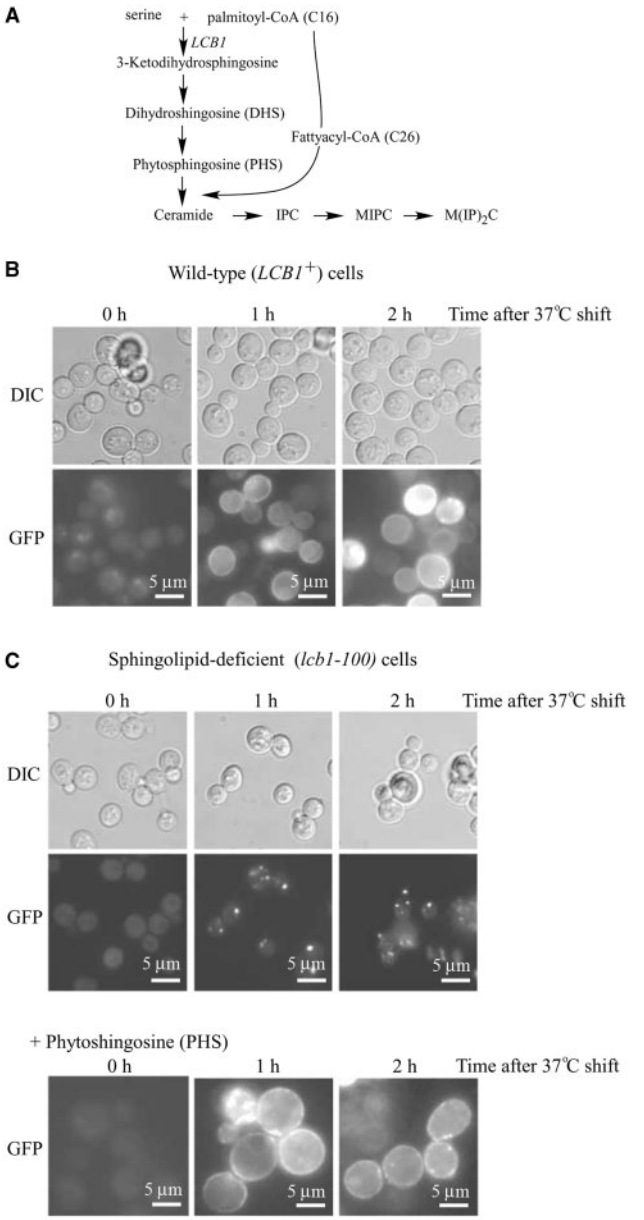

Fig. 5. **Sphinglipid is essential for the localization of Nha1p to the plasma membrane.** (A) Schematic illustration of the sphingolipid biosynthetic pathway, indicating the biosynthetic intermediates and genes of the current study. IPC, inositolphosphorylceramide; MIPC, mannosyl-inositolphosphorylceramide; $M(IP)_2C$, mannosyl-diinositolphosphorylceramide. (B) Cellular localization of Nha1p-GFP in W303-1B (*LCB1*$^+$) cells. W303-1B cells transformed with pRS314-HS4-NHA1-EGFP were grown at 24°C to logarithmic phase in SD medium, and then shifted to 37°C. After incubation for the indicated times, cells were observed by fluorescence microscopy. (C) Cellular localization of Nha1p-GFP in temperature-sensitive sphingolipid deficient RH3804 (*lcb1-100*) cells. *lcb1-100* cells transformed with pRS314-HS4-NHA1-EGFP were grown at 24°C to logarithmic phase in SD medium, and then shifted to 37°C. After incubation for the indicated times, cells were observed by fluorescence microscopy. The lower panel shows the localization of Nha1p-GFP in the presence of 5 μM phytosphingosine. Bar: 5 μm.

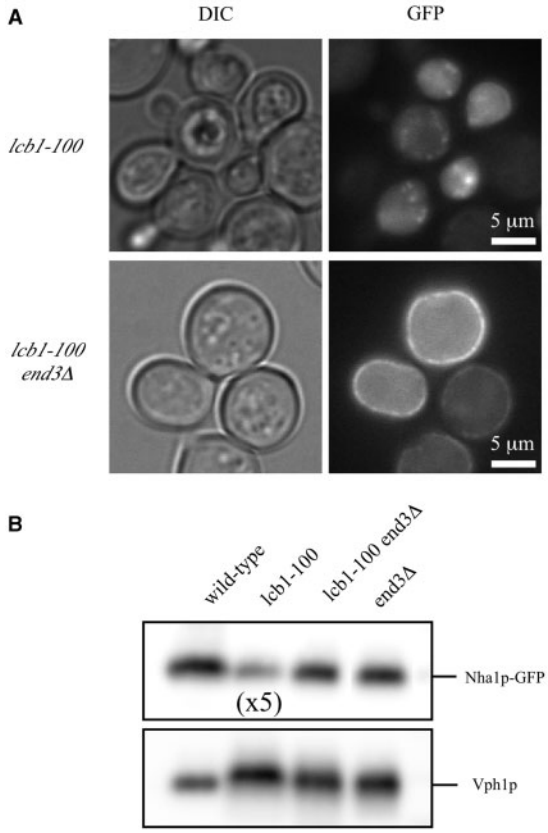

Fig. 6. **Nha1p is rapidly internalized from the plasma membrane in *lcb1-100* cells at the non-permissive temperature.** (A) Cellular localization of Nha1p-GFP in RH3804 (*lcb1-100*; upper panels) and MKY08121 (*lcb1-100 end3*Δ; lower panels) cells. RH3804 and MKY08121 cells transformed with pRS314-HS4-NHA1-EGFP were grown at 24°C to logarithmic phase, and then shifted to 37°C for 2 h. Cells were then observed by fluorescence microscopy. Bar: 5 μm. (B) W303-1B (wild-type), RH3804 (*lcb1-100*), MKY08121 (*lcb1-100 end3*Δ) and MKY08101 (*end3*Δ) cells transformed with pRS314-HS4-NHA1-EGFP were grown at 24°C to logarithmic phase, and then shifted to 37°C for 2 h. Total cell lysates (5 μg for wild-type, *lcb1-100 end3*Δand *end3*Δcells; 25 μg for *lcb1-100* cells) of these strains were prepared and subjected to immunoblot analysis using anti-GFP antibody (upper panel). As a control, total cell lysates (5 μg each) of these strains were also subjected to immunoblot analysis using anti-Vph1p antibody (lower panel).

lcb1-100 cells grown at the non-permissive temperature of 37°C was not due to Nha1p-GFP over-expression or to changes in sphingolipid synthesis under heat shock conditions.

In immunoblot analysis of total cell lysate, the levels of Nha1p-GFP in *lcb1-100* cells were markedly decreased as compared to wild-type cells (Fig. 6B), which suggested that Nha1p-GFP is unstable just in *lcb1-100* cells, and consequentially degraded in the vacuole. To investigate this hypothesis, we examined the localization of Nha1p-GFP in *lcb1-100* cells that lacked End3p (*58*), which is required for the internalization step of endocytosis, or Vps1p (*59*), which is required for the transport of proteins from the Golgi to the vacuole. Cells transformed with pRS314-HS4-NHA1-EGFP were grown at the permissive temperature of 24°C and then shifted to 37°C to induce the expression of Nha1p-GFP. After incubation for 2 h, the localization of Nha1p-GFP was analysed by fluorescence microscopy. Nha1p-GFP localized to the plasma membrane in *lcb1-100 end3*Δ cells (Fig. 6A), but not in *lcb1-100 vps1*Δ cells (data not shown). These results suggested that in the absence of sphingolipid, newly synthesized Nha1p in *lcb1-100* cells is initially transported to the plasma membrane, and subsequently localizes to intracellular structures. The deletion of *END3* restored the protein levels of Nha1p-GFP in *lcb1-100* cells to some extent (Fig. 6B), which suggested that newly synthesized Nha1p-GFP in *lcb1-100* cells is rapidly internalized from the plasma membrane via endocytosis and degraded in the vacuole. Thus, sphingolipids are required for the stable localization of Nha1p to the plasma membrane.

DISCUSSION

In the current study, we demonstrated that the Na^+/H^+ antiporter Nha1p associates with DRMs in the plasma membrane (Fig. 1). Nha1p-associated DRMs were identified as lipid rafts based on the following criteria: (i) Nha1p-associated DRMs were enriched in ergosterol (Fig. 2) and (ii) Nha1p was distributed in non-homogenous compartments within the plasma membrane (Fig. 3). In *S. cerevisiae*, plasma membrane proteins such as Gas1p (*33*), Pma1p (*33*), Fur4p (*60*), Can1p (*52*) and Hxt1p (*54*) also associate with DRMs. The association of Gas1p, Pma1p and Fur4p with DRMs takes place in the ER or Golgi apparatus (*33*, *39*, *53*), suggesting that the association of plasma membrane proteins with DRMs occurs at earlier steps (ER or Golgi) in the secretory pathway. We found that the association of Nha1p with DRMs occurs predominantly at the plasma membrane, the last stage of the secretory pathway, rather than in the ER or Golgi (Fig. 4). These results demonstrate for the first time that a plasma membrane protein associates with DRMs in the plasma membrane rather than the ER or Golgi.

Platelet-derived growth factor receptor (PDGFR) and influenza hemagglutinin are present in sphingolipid- and cholesterol-rich microdomains, termed caveolae, which are similar to lipid rafts, and the interaction of these proteins with sphingolipids and/or cholesterol is believed to be mediated through their transmembrane domains (*61–63*). Based on these findings, it has been proposed that the interaction of proteins with lipid rafts depends on the molecular compatibility of the protein and the lipids (sphingolipids and/or sterols) present in lipid rafts (*64*). Thus, differences in molecular compatibility might explain why Nha1p associates selectively with DRMs at the plasma membrane, which is a substantially different mechanism than that of previously-characterized raft proteins.

In *S. cerevisiae*, there are at least three types of lipid raft (*52*, *54*). Studies using GFP fusion proteins to analyse the localization of membrane proteins have shown that Pma1p, Can1p and Hxt1p are segregated into different lateral compartments, possibly with different types of lipid rafts, within the plasma membrane (*52*, *54*). Pma1p-RFP and Can1p-GFP are distributed in

distinct punctate patterns at the cell surface. In contrast, Hxt1p-GFP is distributed in a homogenous manner within the cell membrane, even though it is also present in DRMs. Moreover, these compartments have been shown to be composed of distinct chemical components. Ergosterol is abundant in the compartments that contain Can1p, whereas Pma1p is located within compartments that contain relatively lower levels of ergosterol (*65*). Therefore, it is possible that the association of Nha1p with DRMs at the plasma membrane reflects the presence of a novel type of DRM (or lipid raft), which is distinct from the three previously characterized lipid rafts in the yeast plasma membrane. It has been reported that the formation of lipid rafts is initiated in the ER, where sphingolipids are synthesized, and then the rafts are modified by mannosylation of sphingolipids in the Golgi (*35*, *37*). In addition, the concentration of ergosterol in membranes increases along the secretory pathway from the ER, where ergosterol is synthesized, to the plasma membrane (*66*). Within the plasma membrane, lipid rafts are concentrated and mature into micro-domains that contain mannosylated sphingolipids and a high concentration of ergosterol (*33*, *37*, *38*). Thus, the composition of lipid rafts formed in the ER and Golgi differs from those that are present in the plasma membrane. Another possibility, therefore, for the unique mechanism of localization of Nha1p is that Nha1p requires a mature form of lipid raft for successful association with the plasma membrane, while other raft proteins do not. This raises the question of whether Nha1p-containing DRMs are similar to other DRMs, such as those that contain Pma1p, Can1p or Hxt1p. While further studies are required to resolve this question, our results suggest that Nha1p does not co-localize with Hxt1p-containing lipid rafts (Fig. 3), since, unlike Hxt1p, Nha1p-GFP was distributed in a non-homogenous manner in the plasma membrane (*52*, *54*).

We also explored the physiological significance of the association of Nha1p with lipid rafts in yeast. In *lcb1-100* cells, which are temperature-sensitive for sphingolipid synthesis, at the non-permissive temperature, there was an accumulation of Nha1p in intracellular punctate structures (Fig. 5), indicating that lipid rafts play a crucial role in the localization of Nha1p specifically at the plasma membrane. The intracellular structures containing Nha1p-GFP that we observed in *lcb1-100* cells are most likely endocytic vesicles or fragmented vacuoles. Thus, our results suggest that in *lcb1-100* cells at the non-permissive temperature, Nha1p is initially transported to the plasma membrane, but is rapidly internalized and degraded in vacuoles (Fig. 6A and B). The secretion of invertase, a secretory enzyme, is normal in *lcb1-100* cells even under the non-permissive temperature, which indicates that sphingolipid synthesis is not a general requirement for the trafficking of proteins through the secretory pathway (*67*). Moreover, the lipid composition of the membrane is thought to be a structural determinant of intrinsic membrane proteins (*68*, *69*). Thus, the association of Nha1p with lipid rafts might be required for the structural stability of Nha1p and its stable localization in the plasma membrane, allowing the protein to escape rapid endocytosis and degradation. Pma1p in sphingolipid-depleted cells and a mutant of Pma1p that does not associate with lipid rafts (Pma1-7) are also mis-targeted to vacuoles. Both proteins are mis-sorted from the Golgi apparatus to vacuoles for degradation through the endosomal (prevacuolar) compartment (*70*). Moreover, it was reported that the general amino acid permease Gap1p is delivered to the plasma membrane but undergoes rapid internalization by endocytosis when synthesized in the absence of sphingolipid (*71*), as shown also for Nha1p in this study. This suggests that sphingolipids play a common role in the localization of these proteins to the plasma membrane. Furthermore, Pma1-7p and Gap1p synthesized in the absence of sphingolipids appear to be misfolded, presumably because they are more extensively ubiquitinated than their wild-type counterparts (*70*, *71*). These findings support our hypothesis that lipid rafts play a crucial role in maintaining the structure of integral membrane proteins. On the other hand, Nha1p associates with lipid rafts mainly at the plasma membrane, unlike other known raft proteins (e.g. Pma1p, Gap1p). The roles of different raft-association sites in the physiological functioning of lipid rafts are currently known and warrant further study.

Exogenous addition of PHS restored mis-localization of Nha1p in *lcb1-100* cells, indicating that PHS itself and metabolized phytoceramide derivatives, IPC, MIPC and $M(IP)_2C$, might be required for the proper localization of Nha1p to the plasma membrane as shown in Fig. 5. PHS is metabolized to ceramide in the ER and then further modified by the Golgi-localized mannotranferases Csh1p and Csg1p to produce MIPC or $M(IP)_2C$ (*72*). These findings may suggest that specific forms of MIPC and $M(IP)_2C$ are required for proper localization of Nha1p to the plasma membrane, since Nha1p associates with lipid rafts after its exit from the Golgi, possibly at the plasma membrane.

We also examined the intracellular distribution of Nha1p-GFP in ergosterol-deficient cells. These cells contain a deletion of *ERG6*, which encodes delta(*24*)-sterol C-methyltransferase, a component of the ergosterol biosynthetic pathway (*73*). The TritonX-100 solubility of Nha1p was significantly increased by the depletion of ergosterol from membranes (Fig. 2). Interestingly, the localization of Nha1-GFP to the plasma membrane in *erg6*Δ cells was similar to wild-type cells (data not shown). These results indicate that unlike sphingolipids, ergosterol is not essential for the proper localization of Nha1p-GFP to the plasma membrane. It is possible that ergosterol contributes to the efficient association of Nha1p with lipid rafts.

In summary, the association of Nha1p with lipid rafts occurs at the plasma membrane, which is a different mechanism of localization that that of previously identified raft proteins. The association of Nha1p with lipid rafts plays a crucial role in maintaining the structural integrity of Nha1p and stable localization in the plasma membrane. It remains to be determined whether lipid raft association affects the ion transport activity of Nha1p, and whether the interaction between Nha1p and lipid rafts is regulated by extracellular or other stimuli, such as salinity or osmotic shock.

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CONFLICT OF INTEREST

None declared.

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