Saccharomyces cerevisiae Npc2p Is a Functionally Conserved Homologue of the Human Niemann-Pick Disease Type C 2 Protein, hNPC2†

Adam C. Berger,1,2,3 Thomas H. Vanderford,4,5 Kim M. Gernert,6 J. Wylie Nichols,7 Victor Faundez,2 and Anita H. Corbett1*

Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia 30322; Department of Medicine, Emory University School of Medicine, Atlanta, Georgia 30322; BIMCORE (Biomolecular Computing Resource), Emory University, Atlanta, Georgia 30322; Department of Physiological Medicine, Emory University School of Medicine, Atlanta, Georgia 30322; Department of Cell Biology, Emory University School of Medicine, Atlanta, GA 30322; and Graduate Program in Biochemistry, Cell, and Developmental Biology† and Graduate Program in Population Biology, Ecology, and Evolution,* Emory University, Atlanta, Georgia 30322

Received 14 March 2005/Accepted 24 August 2005

Niemann-Pick Disease Type C (NP-C) is a fatal neurodegenerative disease, which is biochemically distinguished by the lysosomal accumulation of exogenously derived cholesterol. Mutation of either the hNPC1 or hNPC2 gene is causative for NP-C. We report the identification of the yeast homologue of human NPC2, Saccharomyces cerevisiae Npc2p. We demonstrate that scNpc2p is evolutionarily related to the mammalian NPC2 family of proteins. We also show, through colocalization, subcellular fractionation, and secretion analyses, that yeast Npc2p is treated similarly to human NPC2 when expressed in mammalian cells. Importantly, we show that yeast Npc2p can efficiently revert the unesterified cholesterol and GM1 accumulation seen in hNPC2−/− patient fibroblasts demonstrating that it is a functional homologue of human NPC2. The present study reveals that the fundamental process of NPC2-mediated lipid transport has been maintained throughout evolution.

Niemann-Pick Disease Type C (NP-C) is a fatal neurovisceral disorder that is characterized by the accumulation of low-density-lipoprotein (LDL)-derived cholesterol in endolysosomal compartments (37). NP-C is caused by mutation of either of two genes: hNPC1 (5, 30) or hNPC2 (34). Mutations in hNPC1 account for 95% of all patients (6, 37). Recently, it was demonstrated that the yeast homologue of hNPC1, scNcr1p, can functionally complement the loss of hNPC1 from mutant CHO cells (31). This finding suggests that there is an evolutionary conservation of function between species.

Human NPC2 (hNPC2), which is mutated in approximately 5% of NP-C disease (37), encodes a conserved 151-amino-acid secreted glycoprotein with an endoplasmic reticulum signal sequence (24). The NPC2 protein contains an MD-2-related lipid-recognition (ML) domain (21), which is predicted to mediate direct binding to lipids (21). This prediction is based in part on the finding that the MD-2 protein, the founding member of the ML domain family of proteins, binds directly to lipopolysaccharide (49). Structural analysis of an ML domain-contains protein, the dust mite allergen Der P 2, reveals that this protein folds into two β-strands with an internal cavity that is occupied by a hydrophobic ligand predicted to be a lipid (10). As seen for the other ML domain-containing proteins tested thus far (10, 49), the human, mouse, and porcine NPC2 orthologues all bind directly to lipid ligands, specifically cholesterol or cholesterol analogs (13, 26, 35). The recently solved crystal structure of bovine NPC2 (hNPC2) is consistent with a model wherein cholesterol binds within a loosely packed hydrophobic protein core (13). Taken together, these binding and structural studies suggest that NPC2 plays a direct role in sterol transport.

Here we report the identification of the yeast homologue of hNPC2 that we have termed Npc2p (scNpc2p). Our analysis of Saccharomyces cerevisiae Npc2p through phylogenetic and homology modeling studies suggests that this protein has been conserved from yeast to mammals. Most importantly, we show that yeast Npc2p can functionally replace human NPC2 in hNPC2−/− patient fibroblasts by reestablishing the transport of cholesterol and the ganglioside GM1. These findings demonstrate that yeast Npc2p is a functional homologue of hNPC2 and reveal that the yeast protein, like hNPC2 (34), can facilitate intracellular lipid trafficking.

MATERIALS AND METHODS

Strains, plasmids, and cell culture. All DNA manipulations were performed according to standard protocols (42), and all yeast media were prepared by standard methods (1). All yeast strains, cell lines, plasmids, and viruses are described in Table 1. Chemicals were obtained from Fisher Scientific (Pittsburgh, PA), Sigma Chemical Co. (St. Louis, MO), Stratagene (La Jolla, CA), or US Biological (Swampscott, MA) unless otherwise noted. A complete deletion of scNPC2 was created as previously described by using a PCR-based strategy (27). Briefly, the diploid wild-type strain ACY247 was used as a starting strain to create a heterozygous diploid which was sporulated and subjected to tetrad dissection. Spores were backcrossed twice to the wild-type haploid strain...
ACY193 generating two separate isogenic isolates, ACY783 and ACY1260. Gene-specific deletion was confirmed by PCR analysis with one oligonucleotide ACY193 generating two separate isogenic isolates, ACY783 and ACY1260.

**Table 1. Yeast strains, cell lines, plasmids, and viruses**

<table>
<thead>
<tr>
<th>Strain, cell line, plasmid, or virus</th>
<th>Genotype and/or description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACY193</td>
<td>MATa ura3-52 leu2Δ1 his3Δ200</td>
<td>Research Genetics, Invitrogen Corp., Carlsbad, CA</td>
</tr>
<tr>
<td>ACY247</td>
<td>MATa/w ura3-52 ura3-52 leu2Δ1 his3Δ200/hisΔ200 ade2ADE2 ade3ADE3 lys2/LYS2 trp1/TRP1</td>
<td>This study</td>
</tr>
<tr>
<td>ACY402 (BY4741)</td>
<td>MATa his3 leu2 met15 ura3</td>
<td>MATa his3 leu2 met15 ura3</td>
</tr>
<tr>
<td>ACY783</td>
<td>MATa NPC2::HIS3 ura3 lys2 leu2</td>
<td>This study</td>
</tr>
<tr>
<td>ACY1037</td>
<td>MATa ARP1::KanMX4 his3 leu2 met15 ura3</td>
<td>This study</td>
</tr>
<tr>
<td>ACY1260</td>
<td>MATa NPC2::HIS3 ura3 lys2 leu2</td>
<td>This study</td>
</tr>
<tr>
<td>HEK293</td>
<td>E1Δ2Δ</td>
<td>V. Faundez</td>
</tr>
<tr>
<td>Wild-type mouse fibroblasts</td>
<td>Wild type</td>
<td>38</td>
</tr>
<tr>
<td>Wild-type human skin fibroblasts</td>
<td>Wild type</td>
<td>14</td>
</tr>
<tr>
<td>Human NPC2Δ skin fibroblasts</td>
<td>hNPC2Δ</td>
<td>14</td>
</tr>
<tr>
<td>pAC3 (pRS315)</td>
<td>LEU2 CEN</td>
<td>45</td>
</tr>
<tr>
<td>pAC45</td>
<td>pNUF2-NUF2-GFP URA3 2μ</td>
<td>scNPC2 was amplified by PCR from genomic DNA and cloned into pAC3 cut with PstI and XhoI</td>
</tr>
<tr>
<td>pAC1181</td>
<td>pNPC2-scNPC2-GFP LEU2 CEN</td>
<td>scNPC2 was amplified by PCR from genomic DNA and cloned into pAC45 cut with PstI and XhoI</td>
</tr>
<tr>
<td>pAC1185</td>
<td>pNPC2-scNPC2-GFP URA3 2μ</td>
<td>Clontech</td>
</tr>
<tr>
<td>pAC488</td>
<td>pEGFP-N3</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pAC1611</td>
<td>pShuttle-CMV</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pAC1612</td>
<td>pAdEasy-1</td>
<td>This study</td>
</tr>
<tr>
<td>scpNPC2-GFP adenovirus</td>
<td>pCMV-scNPC2-GFP</td>
<td>This study</td>
</tr>
<tr>
<td>hNPC2-GFP adenovirus</td>
<td>pCMV-hNPC2-GFP</td>
<td>This study</td>
</tr>
<tr>
<td>GFP adenovirus</td>
<td>pCMV-GFP</td>
<td>V. Faundez</td>
</tr>
</tbody>
</table>

Antibodies. Primary antibodies used in the present study included anti-GM130 used at 1:250 and anti-Ap-1 γ used at 1:500 (BD Transduction Laboratories), anti-mouse LAMP1 or LAMPI used at 1:500 and anti-human LAMPI used at 1:100 (Developmental Studies Hybridoma Bank, University of Iowa), anti-cathepsin D used at 1:500 (Upstate, Waltham, MA), anti-green fluorescent protein (GFP) used at 1:5,000 (Synaptic Systems, Göttingen, Germany), and anti-tubulin used at 1:1,000 (kindly provided by Harish C. Joshi, Emory University, Atlanta, GA). Secondary antibodies used were: Alexa-conjugated goat-antimouse and goat-anti-rabbit 568 used at 1:1,000 dilution (Molecular Probes/InVitrogen Detection Technologies, Carlsbad, CA) and goat-anti-rabbit horseshod peroxidase (HRP) and goat-anti-mouse HRP used at 1:7,000 (Zymed Laboratories/Invitrogen Immunodetection, Carlsbad, CA).
BX60 epifluorescence microscope equipped with a GFP optimized barrier filter and a Photometrics Quantix digital camera. 

Phosphatidylcholine internalization in yeast. Cells were grown overnight in synthetic complete media containing 2% glucose and diluted the following morning to early log phase at 25°C. NBD-PC (1-meristeryl-2-h-[7-nitro-2-1,3-benz-oxadiazol-4-yl]aminooxyaniloxyl)-sn-glycero-3-phosphocholine) (Avanti-Polar Lipids, Alabaster, AL), dissolved in chloroform, was dried under a stream of nitrogen, resuspended in dimethyl sulfoxide, and then added to the culture at a final concentration of 5 μM. Cultures were then incubated with shaking for 30 min at 30°C. The cell suspension was washed twice with synthetic complete media containing 2% glucose, followed by three washes with SC-NaNa3, synthetic complete media lacking glucose but containing 2% sorbitol and 20 mM sodium azide, and cells were analyzed by direct fluorescence microscopy as described above.

Filipin assays. Yeast filipin assays were performed as follows. Cells were grown overnight at 30°C in synthetic complete media followed by dilution of cultures to an optical density of 0.1 and grown for 3 h. Filipin (Sigma) was dissolved in dimethyl sulfoxide and added to cultures at a final concentration of 5 μg/ml for 5 min at 30°C. Live cells were visualized by direct fluorescence microscopy as described above. Filipin assays performed on human fibroblasts were carried out as follows. scNPC2-GFP, hNPC2-GFP, or a GFP control adenovirus were used to infect normal human fibroblasts, and the cultures were then measured by using a Bio-Rad SmartSpec3000, and cells were grown in 800 g clearance as described for filipin assays. Prior to fixation, cells were washed twice with Ham’s F-12 media supplemented with 25 mM HEPES (pH 7.4) and 0.01% bovine serum albumin. Cells were incubated with 20 mM Alexa 550-conjugated CTxB (Molecular Probes/Invitrogen Detection Technologies, Carlsbad, CA) for 1 h at 37°C and then processed for fixation and microscopy as described above. Cells were then fixed (3%). Cells were fixed with 4% paraformaldehyde in PBS for 20 min. After fixation, paraformaldehyde was quenched by washing twice with PBS containing 25 mM glycine, fixed with a single wash with PBS. Cells were incubated with 50 μg of filipin/ml in PBS for 2 h at 37°C, followed by two changes of PBS. Slides were mounted in Gelvatol, and cells were visualized by confocal/two-photon microscopy using a Zeiss Axiovert 100 M microscope coupled to HeNe1, argon ion, and Verdi-pumped titanium:sapphire lasers. Filipin was excited with the Titanium:sapphire laser, and GFP was excited by using the argon ion laser. For fluorescence microscopy analysis, multiple GFP-positive cells were imaged for filipin fluorescence. Quantification of the microscopic analysis was performed by systematically analyzing ~150 different GFP-positive cells from fibroblasts individually infected with viruses encoding scNPC2-GFP, hNPC2-GFP, or GFP alone. These individual GFP-positive cells were then qualitatively scored for loss of filipin fluorescence intensity. Quantified cells were analyzed from two independent experiments. All images were viewed and acquired using a Plan Apochromat 63×/1.4 oil differential interference contrast (DIC) objective and Zeiss LSM 510 software.

Yeast growth analysis. Yeast cells were grown overnight at 30°C in synthetic complete media supplemented with 2% glucose, diluted to early log phase the next morning, and grown for 3 h at the same temperature. The cell densities of the cultures were then measured by using a Bio-Rad SmartSpec3000, and cells were diluted to an optical density at 600 nm (OD600) of 0.1, followed by a further 40-fold dilution into synthetic media containing 2% glucose. A total of 100 μl of the diluted cell culture was added into an individual well from a 96-well plate. The plate was shaken continuously at 30°C for 30 h with 30 cell density readings taken every half hour at OD600 with a ELx808 automated microplate reader (Bio-Tek Instruments, Inc., Winooski, VT).

Secretion assay. Secretion of scNpc2p was assessed by infecting 2 × 105 wild-type mouse fibroblasts with adenovirus encoding scNpc2p-GFP, hNPC2-GFP, or GFP alone. After 7 h, the cells were washed with DMEM and then grown in 800 μl of fresh medium. After 72 h, the medium was collected, and cell lysates were prepared by washing cells twice with PBS, followed by lysis. Either 65 μl of medium or 30 μg of lysate was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis as described above for filipin culture fractionation.

CTxB assays. Cholera toxin B (CTxB) assays performed on human fibroblasts were carried out as follows. hNPC2−/− fibroblasts were grown and infected as described for filipin assays. Prior to fixation, cells were washed twice with Ham’s F-12 media supplemented with 25 mM HEPES (pH 7.4) and 0.01% bovine serum albumin. Cells were incubated with 20 mM Alexa 550-conjugated CTxB (Molecular Probes/Invitrogen Detection Technologies, Carlsbad, CA) for 1 h at 37°C and then processed for fixation and microscopy as described for filipin assays performed on human fibroblasts. Confocal microscopy was carried out by using a Zeiss Axiovert 100 M microscope coupled to HeNe1, argon ion, and Verdi-pumped titanium:sapphire lasers. CTxB was excited with the HeNe1 laser, and GFP was excited by using the argon ion laser. Quantification of the microscopic analysis was performed by analyzing GFP-positive cells from fibroblasts individually infected with viruses encoding hNPC2-GFP, scNPC2-GFP, or GFP alone. These individual GFP-positive cells were then qualitatively scored for loss of CTxB fluorescence intensity. Quantified cells were analyzed from two independent experiments. All images were viewed and acquired using a Plan Apochromat 63×/1.4 oil DIC objective and Zeiss LSM 510 software.

RESULTS

S. cerevisiae Homologue of hNPC2. The S. cerevisiae genome encodes an apparent homologue of the hNPC2 protein that we have termed scNpc2p (23% identical and 46% similar to hNPC2). scNPC2 corresponds to an uncharacterized open reading frame, YDL046w, which encodes a 173-amino-acid protein containing a single predicted N-terminal signal peptide (Fig. 1). Mammalian NPC2 proteins contain a conserved lipid binding domain, termed the ML domain, which is found in eukaryotes, plants, and fungi (21). Interestingly, scNpc2p is the only S. cerevisiae protein which contains this functional domain (21). Recent work shows that mammalian NPC2 binds to cholesterol (13, 26). Site-directed mutagenesis of the hNPC2 protein identified six residues that are critical for hNPC2 function as assessed by rescue of the cholesterol accumulation defect observed in hNPC2 mutant fibroblasts (see Table S2 in the supplemental material) (26). Protein sequence alignment shows that three of these six functionally important residues are conserved in scNpc2p (Fig. 1A).

To analyze the evolutionary relationship between yeast Npc2p and mammalian NPC2, 39 ML domain-containing proteins from animals and fungi were assessed for divergence from yeast Npc2p by using a maximum-likelihood phylogenetic tree built from aligned amino acid sequences and rooted on four ML-containing fungal proteins (Fig. 1B and Table S1 in the supplemental material). An analysis of phylogenetic distance from scNpc2p, averaged within the three major bootstrap supported clades (Fig. 1B, clades 1 to 3) and compared by analysis of variance, demonstrates that yeast Npc2p is more closely related to the animalia NPC2 proteins than it is to any of the MD proteins (clade 2 versus clade 3, Newman-Keuls
Thus, scNpc2p is more closely related to mammalian NPC2 than it is to other ML domain-containing proteins (e.g., the MD proteins), supporting the hypothesis that scNpc2p is the yeast orthologue of hNPC2.

To assess whether the primary sequence conservation of yeast and mammalian NPC2 proteins could translate to a conservation of secondary and tertiary protein structure as well, we created a structural model of yeast Npc2p using the solved crystal structures of bovine NPC2 (13) and the dust mite allergen Der P 2 (10). The yeast model, which was created by using the sequence alignment shown in Fig. 1A, encompassed only the amino acids aligned with the mature bNPC2 protein (amino acids 40 to 170). When folded onto the bNPC2 structure, the scNpc2p sequence fits well into seven beta strands (A to G) arranged into two opposing sheets. We find that the majority of amino acids that are mutated in the NP-C patient
FIG. 2. scNPC2 function in yeast. (A) Localization of scNpc2p in yeast. A plasmid encoding scNPC2-GFP (pAC1185) was transformed into wild-type yeast cells, and the GFP tagged protein was visualized by direct fluorescence microscopy. A corresponding DIC image is shown.

(B) Growth curve. Cells were grown in synthetic medium for 30 h with cell density readings (OD\textsubscript{600}) taken every 30 min. Cell density versus time is plotted for wild-type cells (black) and npc2Δ cells (gray). The average of three independent experiments is plotted. Standard deviations are indicated.

(C) NBD-PC uptake and trafficking is unaffected by deletion of scNPC2. Cells were incubated with 5 µM NBD-PC, washed with SC-NaN\textsubscript{3}, and assessed by direct fluorescence microscopy. Internalization and localization of NBD-PC is shown for npc2Δ (panel a), wild-type (panel c), and lem3Δ (panel e) cells. For the lem3Δ panel, the image was exposed 20-fold longer (10-s exposure time) than for npc2Δ and wild-type images (0.5-s exposure time) in order to visualize cells. Exposure of lem3Δ cells incubated with NBD-PC for 0.5 s results in panels devoid of any fluorescence (data not shown). The fluorescence seen in the presented lem3Δ panel is similar to that observed for cells not incubated with NBD-PC (data not shown). Corresponding DIC images are shown (panels b, d, and f).

(D) Ergosterol accumulates at the plasma membrane of npc2Δ cells. Cells were incubated with filipin, washed, and visualized by direct fluorescence microscopy. Filipin localization is shown for npc2Δ and wild-type cells. Corresponding DIC images are shown.

(E) npc2Δ cells are sensitive to nystatin. Cells were grown overnight, diluted to early log phase, grown for 3 h, and then serially diluted onto plates containing no drug (Control) or 5 µM nystatin. Growth is shown for two separate npc2Δ isolates, wild-type (nystatin-resistant control), and arv1Δ (nystatin-sensitive control) cells.
population are structurally conserved in the yeast model and are potentially important for either structural integrity or protein–protein interactions (Fig. S1 in the supplemental material). Thus, the modeling reveals that yeast Npc2p can assume the same three-dimensional structure as hNPC2.

Intracellular localization of scNpc2p. Our analyses suggest that a homologue of the hNPC2 protein exists in yeast. To examine whether the yeast and mammalian NPC2 proteins reside in functionally related compartments, we examined the localization of scNpc2p in yeast cells. The hNPC2 protein is found in lysosomal compartments (3, 34, 51). We therefore examined whether scNpc2p localizes to the equivalent structure in yeast, the vacuole. The subcellular localization of scNpc2p was assessed by creating the C-terminal GFP fusion protein, scNpc2p-GFP which is expressed from its endogenous yeast promoter. Immunoblot analysis shows that the scNpc2p-GFP protein migrates at approximately 51 kDa and, similar to human NPC2 (7), can be deglycosylated by Endo H treatment to run at its predicted size of ~47 kDa (data not shown). We find that scNpc2p-GFP is localized to the lumen of the vacuole in wild-type cells (Fig. 2A). These results are consistent with those obtained in the recent global localization study of yeast proteins (20). Thus, scNpc2p has a similar localization pattern to that found for hNPC2.

Analysis of npc2Δ yeast cells. A deficiency in hNPC2 leads to an accumulation of lipids in endocytic compartments, suggesting that human NPC2 plays a role in lipid trafficking. This hypothesis is supported by recent analyses demonstrating that hNPC2 binds directly to cholesterol (26). Furthermore, analysis of the bNPC2 structure reveals that the protein has the potential to accommodate cholesterol within a cavity in its hydrophobic core (13). Our homology modeling suggests that the hydrophobic interior core of scNpc2p may be able to accommodate an ergosterol molecule (Fig. S2 in the supplemental material), suggesting that it may play a role in yeast lipid metabolism. In order to examine the function that scNpc2p plays in yeast cells, we generated two isogenic haploids deficient for NPC2 (see Materials and Methods). scNPC2 is not essential for yeast viability and, like cells lacking the yeast hNPC1 homologue, scNcr1p (2, 31), npc2Δ cells show no readily discernible phenotype when grown at various temperatures (18, 25, 30, or 37°C) or on minimal or rich media (Fig. 2B and E and data not shown). This lack of differential growth is evidenced by growth curve analyses demonstrating that npc2Δ cells grow at a rate similar to that of wild-type cells (Fig. 2B).

We assessed lipid internalization and transport to the vacuole in npc2Δ cells by using a previously characterized fluorescently labeled phosphatidylcholine (NBD-PC) analog that is internalized across the plasma membrane by transmembrane transport and conveyed to the vacuole through the prevacuolar compartment (2, 15, 17, 19). As expected, wild-type cells accumulate NBD-PC in the vacuole (Fig. 2Ac), similar to wild-type cells, accumulate NBD-PC within the vacuole (Fig. 2Ca), suggesting that neither internalization nor intracellular trafficking of NBD-PC is greatly affected in these cells.

Since the proposed function of hNPC2 is in cholesterol transport (29), we assessed whether a deficiency in scNPC2 would have an effect on the equivalent yeast molecule, ergosterol. Similar to what has been previously demonstrated (31), ergosterol accumulates in the plasma membrane of wild-type yeast as detected by filipin fluorescence. Likewise, we saw no significant change in ergosterol localization in npc2Δ cells (Fig. 2D). However, we did find that npc2Δ cells are sensitive to the polyene antibiotic nystatin (Fig. 2E), which forms complexes with ergosterol (9). This sensitivity was observed for two isogenic npc2Δ deletion mutants. As controls, wild-type cells show no nystatin sensitivity and a known nystatin sensitive mutant, arv1Δ (47), shows significantly decreased growth. Since there is no apparent difference in growth rate between npc2Δ and wild-type cells (Fig. 2E, control plate, and see Fig. 2B), nystatin sensitivity suggests that npc2Δ cells have an ergosterol-dependent plasma membrane perturbation.

scNpc2p localization in cultured mammalian cells is similar to the localization of hNPC2. hNPC2 has previously been reported to colocalize with a subset of LAMPI (3), cathepsin D (51) and AP-1γ (3) positive structures, indicating that some fraction of the protein resides in lysosomal and Golgi compartments at steady state. To test the extent of conservation between species, we assessed the localization pattern of the yeast Npc2p protein expressed in mammalian cells. The subcellular localizations of yeast Npc2p and, as a control, hNPC2, were analyzed by creating C-terminal GFP fusion proteins. Mouse or human fibroblasts were infected with either scNpc2p-GFP or hNPC2-GFP adenovirus and the GFP fusion proteins were colocalized with LAMPI, LAMPII, and cathepsin D (markers for the late endosome and lysosome) as well as with GM130 (Golgi markers). Both hNPC2 and scNpc2p distribution and colocalization with Golgi and lysosomal organelles were indistinguishable in either mouse (Fig. 3) or human (Fig. 4) fibroblasts. The finding that hNPC2 and scNpc2p distribute similarly was confirmed by biochemical fractionation. The subcellular fractionation pattern of both proteins was examined by velocity sucrose gradient, and the results from this analysis demonstrate that hNPC2 and scNpc2p elute in identical fractions (Fig. S4), indicating that they reside in similarly sized vesicles and organelles.

hNPC2 is a soluble, secreted protein (34). To test whether scNpc2p can also be secreted from mammalian cells, we infected wild-type mouse fibroblasts with adenoviruses encoding scNpc2p-GFP, hNPC2-GFP, or GFP alone. Cells were grown for 72 h, followed by collection of the media to evaluate the levels of secreted protein. Lysates were also prepared from the infected cells as a control for cellular NPC2 protein level in each sample. Our immunoblot analysis reveals that both

FIG. 3. Subcellular distribution of hNPC2 and scNPC2p in wild-type mouse fibroblasts. Cells were infected with an adenovirus encoding either hNPC2-GFP or scNPC2p-GFP. The GFP-tagged proteins (green) were assessed for their codistribution with LAMPI and LAMPII, markers of late endosomes/lysosomes, and AP-1, a Golgi marker, (red) by confocal microscopy. Colocalization is indicated in yellow in the merged images. Enlarged images are magnified 200%. Bars, 10 μm.
scNpc2p-GFP and hNPC2-GFP are detectable in the culture media (Fig. 5B). These results confirm that like hNPC2, scNpc2p can be secreted into the media.

**Yeast Npc2p expression functionally complements loss of hNPC2.** Human NPC2 fibroblasts accumulate unesterified cholesterol and the ganglioside GM1 in late endosomes and lysosomes (34). We tested whether yeast Npc2p could reverse the hNPC2−/− lipid accumulation defects. Human NPC2−/− fibroblasts (Fig. 6), as well as wild-type control cells (data not shown), were infected with adenovirus encoding scNpc2p-GFP, hNPC2-GFP, or GFP alone. Cholesterol accumulation was assessed by filipin staining and direct fluorescence two-photon confocal microscopy. GM1 accumulation was assessed by CTxB staining and direct fluorescence confocal microscopy. To quantify the degree of complementation by scNpc2p, ~150 GFP-positive cells from two independent experiments were counted and scored for reversion of cholesterol and GM1 accumulation defects. The results indicate that, as expected, hNPC2-GFP efficiently restores the transport of cholesterol and GM1 in hNPC2−/− cells (Fig. 6A and C), whereas GFP alone does not (Fig. 6A and C). Importantly, expression of yeast Npc2p-GFP also restores cholesterol and GM1 transport in hNPC2−/− cells (Fig. 6A and C). When the ability of hNPC2 and scNpc2p to rescue the lipid accumulation defects is quantified, we find that scNpc2p is able to rescue to the same extent as hNPC2 (Fig. 6B and D). These findings in human cells indicate that yeast Npc2p is able to functionally complement the loss of hNPC2 and demonstrate that the yeast Npc2 protein is a functional homologue of hNPC2.

**DISCUSSION**

In the present study we have identified a functional homologue of the Niemann-Pick disease type C protein, hNPC2, in...
FIG. 6. Yeast Npc2p is a functional homologue of hNPC2. hNPC2<sup>+/−</sup> patient fibroblasts were infected with an adenovirus encoding hNPC2- GFP as a positive control, scNpc2p-GFP, or GFP alone as a negative control. (A) After infection, cells were fixed and incubated with filipin to detect unesterified cholesterol. Fluorescence was viewed by two-photon/confocal microscopy. Bar, 10 μm. (B) Quantification of rescue of cholesterol accumulation. The percentage of GFP-positive cells scored filipin negative (% GFP<sup>+</sup> filipin-negative cells) is shown for hNPC2, scNpc2p, and GFP alone (empty vector). For each sample, n = 152. (C) Cells were incubated with 20 nM CTxB for 1 h at 37°C prior to fixation to detect GM1. Fluorescence was viewed by confocal microscopy. Bar, 10 μm. (D) Quantification of rescue of GM1 accumulation. The percentage of GFP-positive cells scored CTxB negative (% GFP<sup>+</sup> CTxB-negative cells) is shown for hNPC2, scNpc2p, and GFP alone (empty vector). For each sample, n = 150.
yeast. The hNPC2 protein is hypothesized to play a critical role in cellular cholesterol homeostasis, specifically the retrograde movement of cholesterol from late endosomes (29), with mutations in this protein leading to the fatal neurological disorder, NP-C (37). This protein is a member of a specialized class of lipid-binding proteins (the ML domain-containing family [21]), and as such, binds directly to (26) and facilitates the movement of cholesterol (34).

On the basis of phylogenetic and molecular modeling analyses, we suggest that the yeast genome encodes a homologue of the mammalian NPC2 protein. Specifically, we find the evolutionary distance between scNpc2p and the mammalian NPC2 proteins is smaller than the distance between scNpc2p and the majority of ML domain-containing proteins examined (Fig. 1). Indeed, this phylogenetic analysis suggests that the yeast protein may be a progenitor of the mammalian NPC2 proteins. Interestingly, scNpc2p is very closely related to both Aspergillus oryzae and Neurospora crassa phospholipid transfer proteins (39) (Fig. 1). Based upon this relationship as well as the relationship to the cholesterol binding NPC2 proteins (13, 26, 35), we suggest that yeast Npc2p may also bind directly to a lipid cargo.

Our in vivo analyses provide further evidence to support the conclusion that scNpc2p is a functional homologue of hNPC2. Specifically, we find that yeast cells lacking scNPC2 are sensitive to an ergosterol interacting drug, nystatin (Fig. 2E). This finding is similar to what has been observed for the yeast homologue of NPC1, scNcr1p, where a perturbation of this protein also causes cells to become sensitive to this drug (31). This finding uncovers a functional link between the two yeast NP-C proteins.

When scNpc2p was expressed in mammalian cells, we found that the yeast protein has a similar localization and identical subcellular fractionation pattern to that observed for hNPC2 (Fig. 3, 4, and 5A). We found that scNpc2p-GFP and hNPC2-GFP partially colocalize with the late endosomal and lysosomal markers LAMP1, LAMPII, and Cathepsin D (Fig. 3 and 4), as well as with the Golgi markers GM130 and AP-1 γ (Fig. 3 and 4), suggesting that adenoviral expressed hNPC2-GFP and scNpc2p-GFP have a steady-state localization in both late endosomal/lysosomal structures and Golgi. Although these proteins are expressed from adenoviruses, these results are consistent with previous localization studies of endogenous hNPC2 (3, 51). Overall, these results suggest that both hNPC2 and scNpc2p reach the same intracellular compartments.

As a definitive test of the evolutionary conservation of hNPC2, we analyzed the function of scNpc2p in mammalian cells. We found that expression of yeast Npc2p in hNPC2−/− fibroblasts is able to correct both the cholesterol and GM1 accumulation defects observed in these cells (Fig. 6A and C). These results demonstrate that scNpc2p is able to facilitate the movement of both cholesterol and GM1. Since hNPC2 is hypothesized to mediate cholesterol transport through direct binding to this lipid (26), the ability of the yeast protein to induce cholesterol and GM1 transport provides supportive evidence for our suggestion that yeast Npc2p can bind directly to a lipid cargo. Thus, yeast Npc2p is homologous to mammalian NPC2, not only in sequence and structure but also in function. These results highlight the mechanistic conservation of lipid recognition and transport, particularly that of sterols, between species.

The present study, in combination with previous work, demonstrates that the yeast genome encodes functional homologues of both hNPC1 (2, 31) and hNPC2. The finding that both NP-C proteins are expressed in a single-celled eukaryote coupled with their ubiquitous expression in human cells (28, 37), suggests that they play a role in basic cellular metabolism, likely in lipid or cholesterol/ergosterol homeostasis. The demonstration that both scNcr1p and scNpc2p are able to efficiently clear cholesterol from NP-C patient cells suggests that the mechanism of recognizing and transporting cholesterol by hNPC1 and hNPC2 has also been conserved in these yeast proteins. Therefore, studies in simple genetic model systems, such as yeast, may be helpful in defining the normal cellular function of these proteins, thereby providing insight into the molecular mechanisms that underlie NP-C disease.

ACKNOWLEDGMENTS

We thank Harish C. Joshi, Daniel S. Ory, and Silvija I. Staprans for supplying reagents. We thank Murray Stewart for critical reading of the manuscript and John Logsdon for helpful suggestions on our phylogenetic analysis.

This study was supported by a predoctoral NRSA fellowship from the National Institute of Neurological Disorders and Stroke, National Institutes of Health, to A.C.B. (NS047473), a grant from the National Institutes of Health to V.F. (NS42599), and a grant from the National Niemann-Pick Disease Foundation to A.H.C.

REFERENCES