Targeting of NPC1 to Late Endosomes Involves Multiple Signals, Including One Residing within the Putative Sterol-sensing Domain*

Received for publication, June 1, 2004, and in revised form, August 6, 2004
Published, JBC Papers in Press, September 3, 2004, DOI 10.1074/jbc.M406090200

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The NPC1 protein is a multipass transmembrane protein whose deficiency causes the autosomal recessive lipid storage disorder Niemann-Pick type C1. NPC1 localizes predominantly to late endosomes and has a dileucine motif located within a small cytoplasmic tail thought to target the protein to this location. Our data have suggested previously that the protein can reach its correct location in the absence of its cytoplasmic tail, suggesting that other signals contribute to NPC1 targeting. By using various FLAG-tagged and CD32-NPC1 chimeric fusion constructs, we show that multiple signals are responsible for the trafficking of NPC1 to the endosomal compartment, including the dileucine motif and a previously unidentified signal residing within the putative sterol-sensing domain transmembrane domain 3. Neither region alone was capable of directing heterologous CD32 fusions to late endosomes exclusively via the trans-Golgi network to the late endosome route taken by wild-type NPC1; transmembrane domain 3 was unable to maintain CD32 in late endosomes, indicating that two or more signals work in concert to target and retain NPC1 in this compartment. In addition we confirm that the tail dileucine motif is not essential for NPC1 targeting to late endosomes, and we discuss the implications of this finding along with the previously unappreciated role for transmembrane domain 3 in NPC1 localization and function.

Following their biosynthesis, integral membrane proteins utilize sorting signals within their cytoplasmic domains to direct them from the trans-Golgi network (TGN),1 via clathrin-coated vesicles, to their proper intracellular destination (1–3). For transport of transmembrane proteins to endosomes and lysosomes, the most common signals are tyrosine and dileucine-based motifs (4–6). The tyrosine-based signals are more flexible, because a substitution of one leucine by isoleucine, methionine, or valine does not result in a loss of signal efficiency (7–9). Sorting is thought to occur by interaction of these signals with clathrin-coated vesicle adapter proteins (for a review see Ref. 10).

Niemann-Pick type C1 (NPC1) is a severe autosomal recessive lipidosis characterized at the cellular level by the accumulation of unesterified cholesterol in the endosomal/lysosomal (E/L) system (11). The disease-causing gene, NPC1, encodes a multitransmembrane protein that resides primarily in late endosomes and transiently associates with lysosomes and the TGN (12). Studies have shown that NPC1-containing vesicles undergo rapid outward movement to the cell periphery, a process that is inhibited when NPC1 is absent (13). NPC1 contains a putative dileucine motif at the C terminus of a small cytoplasmic tail (14). A 4-amino acid truncation of the cytoplasmic tail removing the dileucine motif abolishes NPC1 protein function; therefore, it was presumed that this motif is responsible for sorting NPC1 to the E/L pathway (15). A more recent study, however, has demonstrated that the NPC1 protein is capable of correcting the NPC1 phenotype even when devoid of the 27-amino acid cytoplasmic tail (14). These conflicting reports raise questions about the mechanism underlying the targeting of NPC1 to its correct subcellular location.

In this study, a series of FLAG-tagged NPC1 proteins with progressive truncations or specific mutations in their cytoplasmic tail were used to examine the role of the cytoplasmic tail in NPC1 trafficking to late endosomes. In addition, fusions of various NPC1 domains to a CD32 reporter were utilized to confirm their role in the targeting of the NPC1 protein. The data presented here demonstrate that multiple signals are involved in correctly directing NPC1 to late endosomes. We show that a C-terminal acidic sequence is required for efficient exit of NPC1 from the endoplasmic reticulum (ER), and we investigate the possibility that the tail dileucine is a plasma membrane retrieval signal involved in recovering mislocalized NPC1 rather than a late endosome targeting signal. Our studies also reveal the presence of important targeting information within the NPC1 sterol-sensing domain, transmembrane domain 3.

EXPERIMENTAL PROCEDURES

Materials—All reagents were purchased from Sigma unless stated otherwise. Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from Mediatech (Herndon, VA) and HyClone (Logan, UT), respectively. LipofectAMINE, l-glutamine, gentamicin, trypsin/EDTA, and colcemid were obtained from Invitrogen. Filipin was from Polysciences, Inc. (Warrington, PA). VectaShield was purchased from Vector Laboratories (Burlingame, CA). Restriction endonucleases, polymersases, and ligases were from New England Biolabs (Beverly, MA). The FITC-conjugated fluorescent antibodies, FuGENE 6 transfection reagent and protein G-agarose beads, were from Roche Applied Science and Jackson ImmunoResearch (West Grove, PA). The Rhodamine Red-X-conjugated antibodies were from Jackson ImmunoResearch. The Rhodamine Red-X antibody labeling kit and Alexa fluorophore-conjugated antibodies were obtained from Molecular Probes (Eugene, OR). The anti-EEA1 monoclonal antibody was from Transduction Laboratories. The monoclonal antibody to human CD32 was produced from the

* This work was supported by National Institutes of Health Grants RO1 DK065793 and RO1 DK54736 and by a grant from the March of Dimes Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1724 solely to indicate this fact.
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1 The abbreviations used are: TGN, trans-Golgi network; TM, transmembrane; FITC, fluorescein isothiocyanate; NPC, Niemann-Pick type C1; ER, endoplasmic reticulum; Wt, wild type; E/L, endosomal/lysosomal.
hybridoma cell line IV.3 as described previously (16). Oligonucleotides were synthesized using phosphoramidite chemistry on a 380 B DNA Synthesizer (Applied Biosystems, Foster City, CA). Radioactive isotope-labeled PCR products were purified by PerkinElmer Life Sciences.

**Plasmid Construction—**All molecular biological manipulations were performed according to standard procedures (17). Vector constructs were confirmed by restriction digestion, PCR, andideoxyribonucleotide sequencing using an automated sequencer (Applied Biosystems, Foster City, CA).

**NPC1-FLAG—**An NPC1 cDNA construct was engineered to contain a FLAG sequence (DYKDDDK) at the unique Clal restriction endonuclease site of the NPC1 cDNA (14). This pGEM-NPC1-claF construct was digested with BglIII followed by partial digestion with Msel and then ligated with the mutated NPC1 tails. The NPC1-ClAf-tail cDNAs were removed from pGEM3zf (+) by digestion with EcoRI and ligated into the mammalian expression vector, pAsc9 (12). Plasmids were confirmed by digestion, and the mutations were reconfirmed by sequencing.

**CD32-Lamp2—**A chimeric cDNA, CD32-LAMPP2, was generated by PCR using the extracellular and transmembrane domains of the human IgG Feγ receptor II (12), using sense and antisense primers (5'-GCCAATTCCTTGCTGATGAGACC-3' and 5'-GCGAATTCATATTGCTCAGCAGATGGCTTGAGATGACAGGC-CC-3'). The product was fused to the 12-amino acid cytoplasmic tail of Lamp2. The CD32-LAMP2 fusion cDNA was inserted into pAsc9 (12) and confirmed by sequencing.

**CD32-NPC1 Chimera—**A chimeric cDNA, CD32-NPC1-Wt, was generated by overlap PCR using PCR-generated fragments of the extracellular domain of CD32 and the NPC1 transmembrane domain 13 (TM13) and cytoplasmic tail. The extracellular domain of CD32 was PCR-amplified using sense and antisense primers (5'-GCCAAATTCCTTGCTGATGAGACC-3' and 5'-GCCAATTCATATTGCTCAGCAGATGGCTTGAGATGACAGGCGCC-CC-3'). The product was mutated with the 12-amino acid cytoplasmic tail of Lamp2. The CD32-LAMP2 fusion cDNA was inserted into pAsc9 (12) and confirmed by sequencing.

**CD32-NPC1-TM3—**A chimeric cDNA, CD32-NPC1-TM3, was created by amplification of transmembrane domains 12 and 13 from pGEM-NPC1-ClaF followed by ligation of this product into CD32-NPC1-TM3 cDNA was created by amplification of transmembrane domains 12 and 13 from pGEM-NPC1-ClaF followed by ligation of this product into CD32-NPC1-TM3. Equimolar amounts of NPC1-Wt fragment and cloned into pAsc9.

**Targeting of NPC1 to Late Endosomes**

**RESULTS**

NPC1 is a large polytopic glycoprotein that resides in late endosomes (12). It is composed of 13 transmembrane domains and a small cytoplasmic tail (14) that has been suggested to be essential for the transport of newly synthesized NPC1 to the late endosome (15). Examination of this cytoplasmic tail (numbered 1–27 from Gly1252) reveals several potential late endosome targeting regions, such as a dileucine motif at positions 24 and 25, a tyrosine at position 16, a cysteine at position 10, and a putative ER maturation signal (Fig. 1) (18). The 24 and 25 dileucine motif is of particular interest because its importance has been characterized in other transmembrane proteins targeted to the E/L system (19–21). Furthermore, the sequence surrounding this motif includes a threonine (Gln(22)) four residues away from the leucine pair (Fig. 1), which is consistent with observations that the targeting of dileucine motifs is often influenced by flanking upstream acidic residues (22, 23). The residues surrounding Tyr(16) do not constitute a typical tyrosine motif, which is usually YXXΦ (where X is any residue, and Φ is a hydrophobic amino acid), and has been shown to target proteins from the TGN to lysosomes both by a direct TGN to endosome route and indirectly via the plasma membrane (for a review see Ref. 24). Finally, the cysteine residue (Cys(10)) has the potential to be modified by lipidation and could alter the structure of the cytoplasmic tail, thus influencing trafficking as shown for the cation-dependent mannose 6-phosphate receptor (25).
FIG. 1. NPC1 mutant and chimeric proteins. The NPC1 cytoplasmic tail contains a cysteine, a tyrosine, and dileucine and acidic amino acid motifs that may function in directing the protein to late endosomes. FLAG-tagged NPC1 mutant constructs were generated in which the cysteine, tyrosine, or dileucine residues were replaced with alanine; the tail was truncated by 4, 8, or 16 amino acids; and the tail was removed completely (NPC1-TM13). These constructs were expressed in NPC1 fibroblasts followed by filipin staining to determine whether they were able (++) or unable (−−) to correct the NPC1 phenotype. Chimeric constructs were also generated by fusing the extracellular domain of CD32 to NPC1 TM13 and the various mutant tails as denoted.
NPC1 fibroblasts were grown in low density lipoprotein-depleted culture medium for 2 days followed by transfection of FLAG-tagged NPC1 mutant proteins. Cells were grown overnight in medium supplemented with 50 μg/ml low density lipoprotein and fixed and stained with anti-FLAG antibodies (Flag) to detect protein expression and filipin (Filipin) to detect free cholesterol. NPC1-Wt, NPC1-AA, NPC1-Y16A, NPC1-C10A, and NPC1-TR16 were all capable of clearing cholesterol from the E/L system (arrows). In contrast, NPC1-TR4 and NPC1-TR8 were unable to facilitate cholesterol egress from the E/L system indicating that these residues are also not critical for NPC1 localization to late endosomes (Fig. 2). The cysteine residue (Cys10), located ~10 amino acids from the lipid bilayer, is a potential site for acylation and could influence the conformation and interactions of the cytoplasmic tail by anchoring the juxtamembrane region to the bilayer. Labeling of the NPC1 protein with [3H]palmitate, however, indicated that Cys10 is not modified by palmitic acid (data not shown), providing further support to the notion that this residue is not required for NPC1 function or localization. Surprisingly, deletion of 16 amino acids (NPC1-TR16) or the complete NPC1 cytoplasmic tail (NPC1-TM13) did not hinder NPC1 localization and function. These mutants were able to correct the NPC1 phenotype (Fig. 2), implying that the cytoplasmic tail of the protein is not essential for its delivery to the endosomal system nor does it contribute to NPC1 function.

In contrast to the results obtained with the above NPC1 constructs, truncated constructs NPC1-TR4 and NPC1-TR8 were incapable of clearing the cholesterol accumulation in NPC1 fibroblasts (Fig. 2), in agreement with a previous report (15) where deletion of the C-terminal 4 amino acids of the cytoplasmic tail ablated NPC1 location and function. Inspection of the sequence surrounding the dileucine motif revealed that the C-terminal 8 amino acids of the tail contains a di-acidic sequence (ERE) conforming to the DExE motif proposed to elicit efficient COPII-mediated ER export of certain transmembrane proteins (18, 26–29). Inappropriate exposure of this motif in NPC1-TR4 and deletion of this motif in NPC1-TR8 most likely hinder movement of the proteins through the secretary pathway to such an extent that they are prevented from reaching late endosomes and thus are incapable of restoring NPC1 function in NPC1-deficient cells (see “Discussion”). In support of this hypothesis, more extensive immunofluorescence microscopy of expressed NPC1-TR4 and NPC1-TR8 constructs revealed a predominantly ER localization (data not shown). These results are summarized in Fig. 1.

Subcellular Localization of CD32-NPC1 Chimeras—The above results suggested that regions of NPC1 in addition to the tail must be involved in targeting the protein to the E/L system because deletion of the entire cytoplasmic tail did not ablate NPC1 targeting to late endosomes. To characterize further the elements involved in NPC1 targeting, portions of NPC1 were fused to the extracellular region of the plasma membrane protein CD32 (30). To confirm that CD32 could be redirected to the E/L system and thus serve as an appropriate marker for these studies, the cytoplasmic tail and transmembrane domain of CD32 were initially replaced with those of human Lamp2. NPC1 fibroblasts were then transfected with wild-type CD32 or the CD32-lamp2 chimera. Wild-type CD32 decorated the plasma membrane of transfection-positive cells as expected (Fig. 3). The chimeric CD32-lamp2 protein, however, displayed a vesicular pattern indicative of lysosomes (Fig. 3). Colocalization studies with the lysosomal marker α-galactosidase A confirmed that these organelles were lysosomes (data not shown).

Nine CD32-NPC1 chimeras were constructed as shown in Fig. 1. The CD32 extracellular domain was linked to transmembrane domain 13 and the cytoplasmic tail of NPC1 (CD32-Wt); to transmembrane domain 13 with mutated NPC1 tails as described above (Leu24 → Ala/Leu25 → Ala (CD32-AA), Cys10 → Ala (CD32-C10A); Tyr16 → Ala (CD32-Y16A)); to transmembrane domain 13 and the 4- and 8-amino acid truncated tails (CD32-TR4 and CD32-TR8); to transmembrane domain 13 alone (i.e., without the cytoplasmic tail) (CD32-TM13); to transmembrane domains 3, 12, and 13 (CD32-TM3/12/13); and to transmembrane domain 3 alone (CD32-TM3). Transmembrane domain 3 was selected based on previous results (14) suggesting the presence of a targeting motif within this region. The
CD32-TM3/12/13 fusion was constructed to contain both TM3 and TM13, with TM12 included to maintain these domains in their correct topology (14).

COS7 cells were transfected with each CD32 chimera and then immunostained for CD32 to determine the subcellular distribution of the expressed proteins. Similar to the results using the full-length FLAG-tagged NPC1 protein (NPC1-Wt), wild-type NPC1 transmembrane domain 13 and tail (CD32-Wt) directed CD32 to vesicular structures, most likely components of the E/L system (Fig. 4). Vesicular targeting appeared to be diminished but not completely abolished when the dileucine motif was mutated to alanines (CD32-AA) (Fig. 4), correlating with previous findings that NPC1-AA can correct the cholesterol accumulation in the E/L system of NPC1 fibroblasts. The CD32-Y16A and CD32-C10A mutations did not prevent CD32 from reaching vesicular structures, consistent with the capability of NPC1-Y16A and NPC1-C10A mutants to correct the NPC1 cellular phenotype. Truncation of 4 or 8 amino acids from the tail of chimeric constructs (CD32-TR4 and CD32-TR8) resulted in retention of the chimeric proteins in the ER (Fig. 4), reinforcing the assertion that this region of the tail contains a potential ER maturation or exit signal (see “Discussion”). The observed vesicular distribution of the CD32-NPC1 chimeric proteins indicated that they localized correctly to the E/L system. To determine the identity of these vesicles, COS7 cells were transfected with the CD32-NPC1 constructs and analyzed by double immunofluorescence microscopy with E/L marker proteins as follows: early endosomal antigen-1 (EEA1) for early endosomes, MLN64 for late endosomes, and β-galactosidase for lysosomes. The results are summarized in Table I. Both CD32-Wt and CD32-AA were directed to MLN64-positive late endosomes (Fig. 5 and Table I), indicating that the tail dileucine motif is not essential for targeting to this compartment. However, it was observed that CD32-AA trafficked less efficiently to late endosomes, as many cells exhibited increased ER staining compared with CD32-Wt. It was also noted that despite its dispensability, the NPC1 cytoplasmic tail could influence CD32 trafficking as in its absence (CD32-TM13) less chimeric protein was localized to late endosomes (Fig. 5 and Table I). The CD32-TM3/12/13 chimera was mostly found trapped in the ER; however, the fraction of this chimera that could overcome this retention was spread among all three vesicular compartments and appeared qualitatively to favor late endosomes (Fig. 5 and Table I). CD32-TM3 had a vesicular location and localized predominantly to β-galactosidase-positive lysosomes (Fig. 5 and Table I). The lysosomal distribution of CD32-TM3 suggests that this transmembrane domain contains sufficient vesicle targeting information to direct CD32 to vesicles of the E/L system but not to retain the chimera in late endosomes.

All CD32-NPC1 chimeras localized to β-galactosidase-positive lysosomes to a varying degree, and none of them appeared to localize significantly with EEA1-positive early endosomes (Fig. 5 and Table I). Of note, attempts to colocalize endogenous

**Fig. 3.** *Redirection of the plasma membrane marker CD32 to lysosomes by the LAMP2 cytoplasmic tail.* COS7 cells were transfected with wild-type CD32 or CD32 extracellular domain fused to the transmembrane and cytoplasmic domains of human LAMP2. Cells were cultured overnight and fixed and stained with anti-CD32 antibodies. Wt CD32 decorates the plasma membrane (A), whereas fusion to the LAMP2 tail redirects CD32 to vesicular structures (B).

**Fig. 4.** *Intracellular distribution of CD32-NPC1 fusion proteins.* COS7 cells were transfected with each CD32-NPC1 chimera (see Fig. 1) and fixed and stained with anti-CD32 antibodies to determine the intracellular distribution of the fusion proteins. CD32-Wt (Wt), CD32-C10A (C10A), CD32-AA (AA), and CD32-Y16A (Y16A) all localize to vesicular structures, although CD32-AA to a lesser extent than the other three chimeras, consistent with the ability of the analogous NPC1 FLAG-tagged mutants to correct the NPC1 phenotype. CD32-TR4 (TR4) and CD32-TR8 (TR8) exhibited a strong ER distribution, suggesting that these mutants are unable to efficiently exit the ER.
Targeting of NPC1 to Late Endosomes

**Table I**

Summary of data obtained for the subcellular localization of CD32-NPC1 chimeras

| Chimeras       | Early endosomes | Late endosomes | Lysosomes | Plasma membrane |
|----------------|-----------------|----------------|-----------|-----------------|
| CD32-Wt        |                | ++ + + +       | ++        | Low             |
| CD32-AA        |                | ++ + +         | ++ + + +  | Intermediate    |
| CD32-TM3       |                | ++ + +         | ++ + + +  | High            |
| CD32-TM3/12/13 | +              | ++             | +         | High            |
| CD32-TR4       | -a             | -a             | -a        | Low             |
| CD32-TR8       | -a             | -a             | -a        | High            |
| CD32-C10A      | ND             | ND             | ND        | ND              |
| CD32-Y16A      | ND             | ND             | ND        | ND              |

*Similar to full-length NPC1 mutants, these constructs appear to be retained in the ER.

Fig. 5. Colocalization of CD32-NPC1 fusion proteins with E/L markers. CD32-NPC1 chimeras were expressed in COS7 cells and costained with anti-CD32 antibodies (green) and markers of the E/L system (red). Markers were for early endosomes (EEA1), late endosomes (MLN64), and lysosomes (α-GalA). CD32-Wt and CD32-AA were most abundant in late endosomes (arrows), whereas CD32-TM3 was found predominantly in lysosomes (arrows). Results shown are representative of at least five random images per chimera.

Summary of data obtained for the subcellular localization of CD32-NPC1 chimeras

Images from immunofluorescence colocalization with marker proteins for early endosomes, late endosomes, or lysosomes (Fig. 5) were assigned an arbitrary score reflecting the extent of colocalization. Results are based on comparison of a minimum of five random images per chimera. Relative plasma membrane localization was determined by densitometry as outlined in Fig. 6. Plasma membrane levels of protein are as follows: low (10–15% of total expressed protein), intermediate (~25%), high (30–35%). ND, not determined.

NPC1 with vesicle marker proteins in these cells were unsuccessful, whereas expression of FLAG-tagged wild-type NPC1 produced giant vesicle structures that altered marker protein distribution.

**Quantitation of the Plasma Membrane Localization of CD32-NPC1 Chimeras—**Many lysosomal targeting signals have been shown to direct plasma membrane proteins into clathrin-coated pits for internalization at the plasma membrane (2). Thus, mislocalized NPC1 protein at the plasma membrane could be redirected to the E/L system via this transport pathway. Because the dileucine motif in the cytoplasmic tail does not appear to be essential for targeting NPC1 to the E/L system, we hypothesized that this motif may function in the retrieval of mislocalized NPC1 from the plasma membrane. To investigate this possibility, the relative level of each CD32-NPC1 chimera at the plasma membrane was determined by using a plasma membrane labeling assay.

As shown in Fig. 6 the CD32-NPC1 chimeras can be broadly classified into three groups as follows: those that localize to the plasma membrane at low levels (low, ~10–15% of total expressed protein); those that localize at an intermediate level (intermediate, ~20–25%); and those that localize at a high level (high, ~30–35%) (Table I). CD32-Wt, CD32-AA, and CD32-TR4 reached the plasma membrane in low levels suggesting that the targeting signals present in each of these chimeras could efficiently direct them to internal cellular compartments (late endosomes for CD32-Wt and CD32-AA, ER for CD32-TR4) with minimal protein reaching the plasma membrane en route. These data also suggested that the dileucine motif is not a strong plasma membrane retrieval signal for mislocalized NPC1 protein. Alternatively, diminished vesicle targeting of CD32-AA compared with CD32-Wt observed previously would suggest that mutation of the dileucine in CD32-AA leads to less efficient trafficking of the protein through the secretory pathway thus causing the apparent low levels of this chimera at the plasma membrane. CD32-TM3 was found to localize to the plasma membrane at an intermediate level supporting previous immunofluorescence data indicating that CD32-TM13 localized less efficiently to late endosomes than the CD32 chimeras with the cytoplasmic tail (i.e. CD32-Wt and CD32-AA). CD32-TM3, CD32-TR8, and CD32-TM3/12/13 showed the highest levels of protein at the plasma membrane indicating a lack of strong E/L targeting signals in these chimeras.

The CD32-NPC1 Chimeras Traffic by Both Direct and Indirect Routes—As shown above, the different CD32-NPC1 chimeras accumulated at the plasma membrane to varying degrees. All fusion proteins investigated, however, could also access the E/L system as shown by colocalization with specific E/L system markers (Fig. 5). By using a trafficking assay, the route by which the CD32-NPC1 chimeras reached their vesicular destination was analyzed, and after treating cells with cycloheximide to restrict expression of the chimERIC proteins, chimeras residing at the plasma membrane were labeled with an anti-CD32 monoclonal antibody as described under “Experimental Procedures.” Cells were subsequently fixed and stained to label the chimeras that bypassed the plasma membrane and trafficked directly from the TGN to endosomes.

The results from this study indicate that CD32-Wt reaches...
beled with [35S]methionine/cysteine for 2 h followed by a 1-h chase proteins. COS7 cells expressing the CD32-NPC1 chimeras were la-
expressed eight times longer than that PM
constructs were shown on the top panel. The photographic film showing plasma membrane (PM) expression was exposed eight times longer than that showing total construct expression levels. The bottom panel shows the relative levels of each chimera at the plasma membrane as determined by quantitative densitometry of photographic film. NPC1-CD32 chime-
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ras can be broadly classified into three groups as follows: low plasma membrane localization (10–15% of total expressed protein) (CD32-Wt, CD32-AA, and CD32-TR4), intermediate plasma membrane localization (~25%) (CD32-TM13), and high plasma membrane localization (30–35%) (CD32-TM3, CD32-TR8, and CD32-TM3/12/13). Results are an average of two independent experiments. See Table I for summary.

the E/L system by both a direct TGN to E/L route and an indirect route via the plasma membrane (Fig. 7). Mutation of the dileucine motif in CD32-AA leads to increased protein trafficking to vesicles via the indirect plasma membrane route, indicated by the increased red plasma membrane and vesicle fluorescence and reduced green vesicle fluorescence compared with CD32-Wt (Fig. 7). This result provides further support for the notion that although the tail dileucine is not essential for delivering NPC1 to late endosomes, it does play a role in targeting NPC1 directly from the TGN to late endosomes. The increased plasma membrane staining apparent with CD32-AA compared with CD32-Wt indicates that the rate of CD32-AA internalization is slower than CD32-Wt, suggesting that the dileucine plays a role in the internalization process also but is not essential for this to occur. The CD32-TM13 chimera reached vesicles almost completely by the indirect route (Fig. 7) consistent with earlier data showing that CD32-TM13 has greater levels of protein at the plasma membrane compared with CD32-Wt and CD32-AA (Fig. 6). As noted previously, CD32-TM3 localized exclusively to vesicles. Most interestingly, this chimera reached its destination by using both the direct TGN to E/L and indirect plasma membrane routes equally, suggesting that both plasma membrane internalization and lysosomal targeting signals may be present within this domain (Fig. 7). The fact that none of the CD32-NPC1 chimeric proteins tested reached the E/L system exclusively via the TGN suggests that none of the chimeras analyzed contained all the signals necessary to mimic NPC1 protein transport to its cellular destination. In support of this hypothesis, native FLAG-tagged NPC1 protein was found to traffic to vesicles exclusively via the direct TGN to endosome route (Fig. 7), confirming that a combination of multiple signals is required to achieve proper NPC1 trafficking.

**DISCUSSION**

NPC1 is a multitransmembrane protein that localizes to late endosomes and may cycle between this compartment and the TGN (12). Analysis of the primary structure of NPC1 has revealed various regions with the potential for mediating late endosomal localization. Four dileucines and an acidic sequence

**Fig. 6. Plasma membrane localization of CD32-NPC1 fusion proteins.** COS7 cells expressing the CD32-NPC1 chimeras were labeled with [35S]methionine/cysteine for 2 h followed by a 1-h chase period. NPC1-CD32 protein at the plasma membrane was labeled with anti-CD32 antibodies before lysing, and antibody-bound protein was immunoprecipitated on protein G-agarose beads. Total NPC1-CD32 protein was isolated by incubating with anti-CD32 antibodies and immunoprecipitating after cell lysis. The relative plasma membrane levels of each NPC1-CD32 chimera were determined by densitometry. Samples were normalized to CD32, which for these purposes was taken to be 100% at the plasma membrane. The relative levels of each construct at the plasma membrane and the total amount expressed in cells are shown on the top panel. The photographic film showing plasma membrane (PM) expression was exposed eight times longer than that showing total construct expression levels. The bottom panel shows the relative levels of each chimera at the plasma membrane as determined by quantitative densitometry of photographic film. NPC1-CD32 chime-
ras can be broadly classified into three groups as follows: low plasma membrane localization (10–15% of total expressed protein) (CD32-Wt, CD32-AA, and CD32-TR4), intermediate plasma membrane localization (~25%) (CD32-TM13), and high plasma membrane localization (30–35%) (CD32-TM3, CD32-TR8, and CD32-TM3/12/13). Results are an average of two independent experiments. See Table I for summary.

**Fig. 7. Analysis of the route taken by CD32-NPC1 chimeras to reach their vesicular destination.** COS7 cells transfected with the CD32-NPC1 chimeras had their expression levels restricted by treatment with cycloheximide. CD32-NPC1 proteins at the plasma membrane were detected by labeling the cells for 15 min with rhodamine X-labeled anti-CD32 (PM) or anti-FLAG (to detect the NPC-FLAG and NPC-TM1/2 constructs) followed by a 1-h chase. Cells were fixed and stained with FITC-labeled anti-CD32 to detect CD32-NPC1 protein that reached vesicles directly from the TGN, bypassing the plasma membrane (Direct). CD32-Wt and CD32-TM3 trafficked to vesicles by both the indirect plasma membrane and direct TGN to endosome routes. CD32-AA and CD32-TM13 showed increased trafficking via the plasma membrane, suggesting a role for the tail in direct targeting of NPC1 to late endosomes. As a control, full-length FLAG-tagged NPC1 (NPC-FLAG) and NPC1 truncated at TM2 (NPC-TM1/2) showed that full-length NPC1 reaches its vesicular destination exclusively via the direct TGN to endosome route, whereas NPC-TM1/2 reaches vesicles via the plasma membrane route only.
are present within the last transmembrane domain (TM13) and C-terminal cytoplasmatic tail, all of which are well conserved among known NPC1 orthologs. In addition, transmembrane domain 3 contains two potential tyrosine motifs and a dileucine-like motif in close succession. Two complementary approaches were used to analyze the role of the NPC1 transmembrane domains and tail in directing NPC1 to endosomes. Initially, mutations in the tail of full-length NPC1 provided insight into the functional consequences of these mutations in a physiologically relevant context. Wild-type and mutated CD32-NPC1 chimeras were then used to assess whether loss of function could be correlated with mislocalization of the NPC1 protein.

The Cytoplasmatic Tail of NPC1 Is Important for Efficient ER Maturation and Export—The ultimate 8 amino acids of NPC1 were found to be critical for directing the protein to late endosomes, because truncation of these amino acids abolished its E/L localization and NPC1-dependent cholesterol egress from endosomes/lysosomes. The disrupted ER-Golgi trafficking of NPC1-TR8 and CD32-TR8 proteins apparent from immunofluorescence suggests that truncation of the cytoplasmic tail by 8 amino acids results in deletion of an ER export/maturation signal. The transmembrane protein lysosomal acid phosphatase and a number of others require cytoplasmic (D/E)(X)(D/E) motifs for efficient exit from the ER, possibly via a COPPI-mediated mechanism (18, 26–28, 31, 32). It seems likely that the C-terminal 1270ERE1272 of NPC1 plays a similar role in the concentration of NPC1 in COPPII-coated vesicles prior to ER-Golgi transport and that deletion of these amino acids interferes with an essential interaction required for this process. The results of the plasma membrane localization of CD32-TR8, however, are in apparent conflict. It should be noted that ER export signals affect the efficiency of escape from the ER and not the movement of proteins out of the ER per se. Thus, TR8 mutants would still be expected to move out of the ER and to the plasma membrane, albeit at a much reduced rate. This still does not explain why plasma membrane levels of CD32-TR8 are so high. We therefore suggest that loss of theERE sequence leads to increased degradation of the protein retained in the ER, leading to a lower than expected amount of “total” CD32-TR8 protein in the cell and giving the appearance that CD32-TR8 is present in high levels at the PM. As can be seen from Fig. 5, very little CD32-TR8 is isolated from cells compared with the other constructs tested, thus strengthening the idea that a little CD32-TR8 exits the ER and makes its way to the PM and that CD32-TR8 remaining in the ER becomes rapidly degraded. In support of this hypothesis, the ABC transporter protein Yor1p shows increased sensitivity to ER degradation when both of its potential ER export signals are mutated (27).

TR4 mutants also show an ER-like distribution, and NPC1-TR4 is unable to correct the NPC1 phenotype, presumably because of a lack of protein reaching the late endosome. Most interestingly, however, TR4 mutants still contain the ER export motif discussed above and therefore should be capable of efficiently negotiating this step of the secretory pathway. In addition, CD32-TR4 does not appear to be degraded in the ER as CD32-TR8. To reconcile the apparent discrepancy of this result and to account for the observed lack of ER export of TR4 mutants, we suggest that although the ERE in the tail of TR4 mutants is still able to concentrate the protein in COPPII vesicles at sites of ER export, the lack of dileucine targeting information in TR4 mutants affects the COPPII budding process and results in the protein being stably sequestered at budding sites rather than transported away from the ER. In support of this, a study of vesicular stomatitis virus G protein has shown that the DXE export motif may form only part of a signal required for efficient protein progression out of the ER (33). Thus we conclude that the 1270ERE1272 and last 4 amino acids of the NPC1 tail are required for uninhibited movement of NPC1 out of the ER.

The Dileucine Is a Direct Targeting Signal and Can Influence the Rate of Plasma Membrane Internalization—Mutation of the C-terminal dileucine (NPC1-AA) did not prevent cholesterol mobilization in NPC1 fibroblasts, demonstrating that this motif is not essential for directing NPC1 to its proper subcellular location, an assertion supported by immunofluorescence data localizing CD32-AA to late endosomes. Increased trafficking of CD32-AA via the plasma membrane compared with CD32-Wt indicated that the dileucine motif can act as a direct trans-Golg/network to late endosome targeting signal as suggested previously (15); however, clearly other signals can substitute for this motif given that the protein localized correctly and rescued the NPC1 phenotype when the dileucine was mutated. Many dileucine motifs capable of targeting proteins to the endosomal system also function as plasma membrane internalization signals (for a review see Ref. 1). The similarity between the levels of CD32-Wt and CD32-AA detected at the plasma membrane in overexpressing COS7 cells indicated that the tail dileucine motif of NPC1 is not such a signal. However, further analysis revealed an accumulation of CD32-AA at the plasma membrane compared with CD32-Wt in the trafficking assay (Fig. 7) implying that although the dileucine is not the sole determinant of NPC1 endocytosis, it can influence the rate of plasma membrane internalization, a phenomenon also seen with the insulin receptor (34). Dileucine-based motifs function through their interaction with adapter proteins that assist trafficking between different cellular compartments, including the plasma membrane. The exact amino acids influencing the specificity of adapter protein signal motif binding and whether a motif will signal direct targeting or internalization are not always clear. Signals vary among proteins, and subtle differences in the amino acids surrounding a familiar motif can greatly influence the way a protein behaves, e.g. the presence of acidic amino acids, arginine residues, or sites of phosphorylation, together with the proximity of signal to transmembrane domains or the C terminus (23, 35, 36). Analysis of the internalization properties of double NPC1-tail mutants in which the dileucines are mutated together with upstream residues such as Glu1272 as well as an assessment of the relative interaction of the tail with the different adapter proteins involved in trafficking between specific cellular compartments (e.g. AP-1, -2, -3, and -4, GGAs, and adaptins) will help confirm the role of the dileucine signal in NPC1.

The NPC1 Cytoplasmatic Tail Is Not Required for NPC1 Transport to Late Endosomes—Most surprisingly, deletion of the terminal 16 amino acids of the cytoplasmatic tail (NPC1-TR16) or the entire tail (NPC1-TM13) restored the cholesterol mobilizing function of NPC1, confirming that targeting signals exist elsewhere within the protein. A recent report (37) demonstrated that polar transmembrane domains are able to target proteins to the interior of the yeast vacuole. Similarly in yeast, the transmembrane domain and adjacent sequence of the exocytic SNARE Sec1p is required for targeting of the protein to the Golgi (38). In mammals, two regions have been identified within the transmembrane domain of the major histocompatibility complex class II invariant chain that are capable of lysosomal targeting (39). These regions contain a dileucine and two tyrosines that may be important, but the exact residues necessary for targeting have not been identified. Similarly, a transmembrane dileucine is involved in the late endosome targeting of the cholesterol-mobilizing protein MLN64 (40). Transmembrane domain 13 of NPC1 has three
potential dileucine motifs (1234LL1235, 1241LL1242, and 1247LL1248). The decreased levels of CD32-TM13 localizing to late endosomes compared with CD32-Wt and CD32-AA indicate that the dileucine motifs present within TM13 are not late endosome targeting signals. Instead, the finding that transport of CD32-TM13 to endosomes occurs via the plasma membrane rather than by the direct TGN to late endosome route suggests that the signal within transmembrane domain 13 acts as a plasma membrane internalization signal. Whether the signal within transmembrane domain 13 is functional in wild-type NPC1 or whether it becomes cryptic in the presence of the NPC1 tail remains to be determined.

Transmembrane Domain 3 Alone Allows Direct and Indirect Trafficking to Vesicles but Not Late Endosome Localization—NPC1 transmembrane domain 3 also contains significant targeting information and directs traffic efficiently to vesicular structures, in particular lysosomes. Transmembrane domain 3 is the first transmembrane domain of the NPC1 putative sterol-sensing domain. It contains two tyrosine-based sequences (628YAIMFLY634 and 634YISLY637) that, individually or combined, may signal vesicle targeting; however, the actual sequence involved and its role as an active targeting signal in full-length NPC1 have yet to be determined. The high levels of CD32-TM3 found at the plasma membrane combined with the observation that this chimera exists entirely in vesicular intracellular compartments indicate that the signal within transmembrane domain 3 is an internalization signal, for which the tyrosine motifs seem a likely candidate. Most surprisingly, CD32-TM3 reaches its vesicular destination by both direct trans-Golgi network to late endosome trafficking and the indirect plasma membrane route. Thus the signals within transmembrane domain 3 are capable of acting as targeting motifs as well as promoting internalization at the plasma membrane. Which of these functions is most prominent in wild-type NPC1 remains to be determined, but because of the inability of CD32-TM3 to be retained in late endosomes, it seems most likely that the primary function of transmembrane domain 3 is to redirect NPC1 mislocalized to the plasma membrane back into the endocytic system and that other signals within NPC1 are more important for late endosome retention of the protein. Tyrosine-based signals of the type YXY are able to act as plasma membrane internalization signals as well as direct endosomal targeting signals (41–45). These motifs are generally found in the cytoplasmic portion of proteins however, where they are recognized and bound by adapter proteins of the types already discussed. If the tyrosine motifs of NPC1 transmembrane domain 3 are indeed involved in protein internalization and targeting, a novel mechanism must exist capable of detecting sequences embedded within the membrane. To our knowledge no such mechanism has been identified within mammals; however the yeast integral membrane protein Rer1p has been shown recently to facilitate retrieval of the ER membrane proteins through interactions with their transmembrane domains (46, 47). Significantly the motif recognized by Rer1p consists of a highly hydrophobic region flanked by polar amino acids as is present in transmembrane domain 3 of NPC1 (626YAIMFLY634) (47). Thus a similar mechanism may operate with NPC1.

An alternative mechanism that may account for the internalization capabilities of CD32-TM3 is ubiquitination of Lys643. Lys643 exists 3 amino acids C-terminal to the predicted end of the NPC1 transmembrane domain 3 and is the very last amino acid in the CD32-TM3 chimera. Ubiquitination, and in particular monoubiquitination of a single lysine residue, has been implicated in endocytosis of proteins at the plasma membrane as well as endosomal sorting of membrane receptors (for reviews see Refs. 1 and 48). Signal motifs for ubiquitination vary and are still under investigation. Most significantly, however, a transmembrane ubiquitin ligase (Tu1l) has been identified in yeast that interacts with polar residues in transmembrane domains and stimulates ubiquitination of an accessible cytosolic lysine (49). Tu1l ubiquitination results in the tagged membrane protein being selectively sorted to multivesicular bodies, the yeast equivalent to the lysosome, thus a similar phenomenon could apply with NPC1. It would be interesting to determine whether transmembrane domain 3 of NPC1 is involved in a similar mechanism to elicit either its sorting to late endosomes or its internalization at the plasma membrane. As of yet, no mammalian counterpart to Tu1l has been uncovered.

Transmembrane Domain 13 Together with the NPC1 Tail Are Not Sufficient to Mimic Trafficking of the Full-length Wild-type NPC1 Protein—Although CD32-Wt was able to localize to late endosomes, it is important to note that not even this chimera was able to mimic the targeting of full-length NPC1. This was indicated by the results of the trafficking assay where full-length NPC1 trafficked to late endosomes exclusively via the TGN-to-late endosome pathway, whereas CD32-Wt trafficked using both the direct TGN-to-late endosome and indirect plasma membrane routes. Thus targeting signals upstream of transmembrane domain 13, such as that identified in transmembrane domain 3, must contribute to NPC1 targeting in vivo. Multiple targeting and/or internalization signals are common among proteins that traffic through the secretory pathway, and such signals can be synergistic, interchangeable, or redundant (23, 39, 50–52). The data presented here suggest that transmembrane domain 3 works together with the NPC1 tail to direct NPC1 to its proper compartment. In addition we demonstrate that alone, the dileucine motif in the cytoplasmic tail of NPC1 is predominantly a TGN-lysosome trafficking signal rather than a plasma membrane retrieval signal but that it is able to influence the efficiency of plasma membrane internalization, even if it is not essential for the process of internalization. We also show that transmembrane domain 3 of the sterol-sensing domain of NPC1 contains significant plasma membrane internalization information, together with late endosomal targeting information, but that this domain does not contain sufficient information to retain the chimeric CD32-TM3 construct in late endosomes, leading instead to its accumulation in lysosomes. Finally, we demonstrate that an acidic region in the NPC1 tail aids its progress from the ER.

The rules for targeting signals are becoming increasingly complicated as more signals are identified that do not conform to "traditional motifs." Signals have been identified in transmembrane domains and, in the case of the lysosomal membrane protein cystinosin, in a cytoplasmic loop (53). In some proteins multiple signals function to localize them to the correct cellular domain, and in addition secondary structure and correct spacing of signals can be important (39, 51, 54, 55). In this study, we mutated only one potential NPC1 targeting signal at a time, and the construction of double mutants may reveal more information about how this protein is targeted and retained in late endosomes. Although it is likely that multiple signals contribute to delivery of NPC1 to late endosomes, the data presented here rule out common motifs such as the cytoplasmic tail tyrosine and palmitoylation of the cytoplasmic tail cysteine. Studies of NPC1 trafficking are essential to understand how the protein mediates lysosomal cholesterol egress to other intracellular compartments and the role NPC1 plays, if any, in cholesterol homeostasis.

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Targeting of NPC1 to Late Endosomes Involves Multiple Signals, Including One Residing within the Putative Sterol-sensing Domain
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J. Biol. Chem. 2004, 279:48214-48223.
doi: 10.1074/jbc.M406090200 originally published online September 3, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M406090200

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