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## The Steroidogenic Acute Regulatory Protein Homolog MLN64, a Late Endosomal Cholesterol-binding Protein\*

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MLN64 is a transmembrane protein that shares homology with the cholesterol binding domain (START domain) of the steroidogenic acute regulatory protein. The steroidogenic acute regulatory protein is located in the inner membrane of mitochondria, where it facilitates cholesterol import into the mitochondria. Crystallographic analysis showed that the START domain of MLN64 is a cholesterol-binding domain. The present work was undertaken to determine which step of the intracellular cholesterol pathway MLN64 participates in. Using immunocytofluorescence, MLN64 colocalizes with LBPA, a lipid found specifically in late endosomes. Electron microscopy indicates that MLN64 is restricted to the limiting membrane of late endosomes. Microinjection or endocytosis of specific antibodies shows that the START domain of MLN64 is cytoplasmic. Deletion and mutagenesis experiments demonstrate that the amino-terminal part of MLN64 is responsible for its addressing. Although this domain does not contain conventional dileucine- or tyrosine-based targeting signals, we show that a dileucine motif (Leu<sup>66</sup>-Leu<sup>67</sup>) and a tyrosine residue (Tyr<sup>89</sup>) are critical for the targeting or the proper folding of the molecule. Finally, MLN64 colocalizes with cholesterol and Niemann Pick C1 protein in late endosomes. However, complementation assays show that MLN64 is not involved in the Niemann Pick C2 disease which, results in cholesterol lysosomal accumulation. Together, our results show that MLN64 plays a role at the surface of the late endosomes, where it might shuttle cholesterol from the limiting membrane to cytoplasmic acceptor(s).

Cholesterol, the essential sterol found in vertebrates, has several functions, which include modulating the fluidity and permeability of membranes, serving as precursor for steroid hormones and bile acid synthesis, and covalently modifying proteins. Animal cells obtain cholesterol by *de novo* synthesis in the endoplasmic reticulum or receptor-mediated uptake of plasma lipoproteins (1). Most of cells acquire cholesterol from low density lipoprotein (LDL).<sup>1</sup> LDL is endocytosed and transported to early endosomes and then to late endosomes/lysosomes for degradation. Free cholesterol generated from LDL in late endosomes/lysosomes is then redistributed in the cell. Although the regulation of cholesterol content in cells has been extensively studied, little is known about the mechanisms of its intracellular transport. Only a few molecules involved in this pathway have been identified, including steroidogenic acute regulatory protein (StAR), whose expression is tissue-specific, or the recently identified Niemann Pick C1 protein (2, 3).

MLN64 cDNA was identified from a breast cancer-derived metastatic lymph node cDNA library by differential hybridization using malignant (metastatic lymph node) versus nonmalignant (breast fibroadenoma and normal lymph node) tissues. Chromosomal mapping showed that the *MLN64* gene is located in the q12-q21 region of the long arm of chromosome 17 (4). This region is altered in 20–30% of breast cancers, the most common modification being the amplification of the proto-oncogene *c-erbB-2* (5–7). The proto-oncogene *c-erbB-2* is a marker of poor prognosis and tumor aggressiveness in breast cancers; its overexpression in tumors has been correlated to hormone therapy failure (8). In breast cancers, an invariable coamplification and consequent overexpression of *MLN64* and *c-erbB-2* in 22.5% of the cases tested (98 cases) was observed, suggesting that overexpression of *MLN64* could be of clinical relevance for breast cancer development and/or progression (9).

MLN64 cDNA encodes for a protein of 445 residues containing four potential transmembrane regions at its amino-terminal part. In addition, MLN64 shares a conserved COOH-terminal region with StAR called the StAR homology domain (SHD) (10). Recently, a larger domain, including the SHD, has been defined as the StAR-related lipid transfer (START) domain (20). A wide variety of proteins involved in different cell processes possess a START domain, such as the phosphatidylcho-

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<sup>1</sup> The abbreviations used are: LDL, low density lipoprotein; StAR, steroidogenic acute regulatory protein; SHD, StAR homology domain; START, StAR-related lipid transfer; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; NPC, Niemann-Pick C.

line transfer protein, the signal-transducing protein p122-RhoGAP, or a putative acyl-CoA thioesterase. Interestingly, it has been shown that mutations in the StAR gene, which lead to COOH-terminal truncated proteins in which the SHD domain is deleted, are responsible for congenital adrenal hyperplasia, a disease characterized by severely impaired steroidogenesis (12–14). The functional relationship between MLN64 and StAR was previously investigated. It was shown that, like StAR, MLN64 can enhance steroidogenesis in an *in vitro* assay. Removal of the SHD domain resulted in the complete loss of steroidogenic activity, while removal of the NH<sub>2</sub>-terminal region of MLN64 increased this activity (15).

StAR is a mitochondrial protein that regulates the acute production of steroids in the adrenal glands and gonads in response to corticotropin and luteinizing hormone, respectively. StAR regulates the rate-limiting step of steroidogenesis, which is the transfer of cholesterol from the outer to the inner mitochondrial membrane, where it is converted into pregnenolone (16). Investigations into the mechanism of action of StAR have shown that StAR is a sterol transfer protein that acts directly on the mitochondria (17, 18). In addition, StAR has been shown to be a sterol transfer protein *in vitro* (19). Recently, the three-dimensional structure of the START domain of MLN64 was solved, and its ability to bind cholesterol at an equimolar ratio was reported (20).

MLN64 is likely to be involved in cholesterol transport, and cholesterol is the precursor of all steroid hormones. Since *c-erbB-2* and *MLN64* are coamplified and overexpressed in breast cancer, a role for MLN64 in facilitating intratumoral biosynthesis of steroid hormones can be postulated, which may relate to the hormonal resistance of part of the *c-erbB-2*-expressing tumors.

This study was aimed at identifying the exact subcellular localization of MLN64 to define where MLN64 is likely to play a key role during cholesterol trafficking.

#### EXPERIMENTAL PROCEDURES

**Cell Culture**—The MCF7 human breast cancer and the SK-OV-3 human ovarian cancer cell lines were provided by the American Type Culture Collection (ATCC, Manassas, VA) and routinely maintained in our laboratory and cultured as recommended. The Chinese hamster ovary (CHO) cell line was a kind gift of Dr. L. Liscum (Tufts University, Boston, MA).

The NPC2 fibroblast strain was obtained from case 16 in Vanier *et al.* (21). Human lipoprotein-deficient serum and human LDL were prepared in the laboratory as described previously (21).

**Plasmids**—To produce stable cell lines expressing MLN64, a 1.5-kilobase *Bam*HI fragment corresponding to the open reading frame of the human MLN64 cDNA (GenBank™ accession number X80198) was cloned into the *Bam*HI site of the pCMVneo vector (22), thus generating the pCMVneo-MLN64 plasmid. To map the domain responsible for the sorting of MLN64, NH<sub>2</sub>-terminal deletion mutants of pSG5 MLN64 (10) were constructed. pSG5 MLN64-(30–445) was obtained in two steps. An intermediate plasmid was constructed by creating two in-frame *Nhe*I sites at amino acid positions 2 and 28 by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) and the synthetic oligonucleotides 1 (5'-GGGGCCACC AGGATGGCTA GCCTGCCAG GGAGCTGA) and 2 (5'-GGGCTCCTCA CTGTCCGCTA GCCAGAGCCT CTCCTCGC). Sequences corresponding to amino acids 2–29 were then excised by a *Nhe*I digest, and the remaining plasmid was recircularized to produce pSG5 MLN64-(30–445). pSG5 MLN64-(47–445) and pSG5 MLN64-(54–445) were obtained in a similar way. For pSG5 MLN64-(47–445), the two in-frame *Nhe*I sites at amino acid positions 2 and 45 were obtained by site-directed mutagenesis using the synthetic oligonucleotides 1 and 3 (5'-GCCTGAGAAG CGAAGGGCTA GCTCTGATGT CCGCCGCAC). For pSG5 MLN64-(54–445), the two in-frame *Nhe*I sites at amino acid positions 2 and 52 were obtained by site-directed mutagenesis using the synthetic oligonucleotides 1 and 4 (5'-CTCTGATGTC CGCCGCGCTA GCTGTCTCTT CGTCACT). pSG5 MLN64-(1–218) was obtained by insertion of an in-frame stop codon at amino acid position 219 by site-directed mutagenesis using the following oligonucleotide: 5'-

GCAGGGTCTG ACTAGTAATC AGATGAAG. pSG5 MLN64-(1–171) was obtained by insertion of an in-frame stop codon at amino acid position 172 by site-directed mutagenesis using the following oligonucleotide: 5'-GGTTCCTTGA CTTTAAATC CTACCCAGG. pSG5 MLN64-(1–145) was obtained by insertion of an in-frame stop codon at amino acid position 146 by site-directed mutagenesis using the following oligonucleotide: 5'-CTCTGAGCTG CTTTAAAAAG GGGCATTGTG.

pSG5 MLN64 LL1 was constructed by mutating Leu<sup>61</sup>-Leu<sup>62</sup> to Ala<sup>61</sup>-Ser<sup>62</sup> by site-directed mutagenesis using the following synthetic oligonucleotide: 5'-ACTTCGTCACC TTCGACGCTA GCTTCATCTC CCTGCCTC. Similarly, pSG5 MLN64 LL2 was constructed by mutating Leu<sup>66</sup>-Leu<sup>67</sup> to Ala<sup>66</sup>-Ser<sup>67</sup> (5'-CCTGCTCTTC ATCTCCGCTA GCTG-GATCAT CGAAGCTG), pSG5 MLN64 LL3 by mutating Leu<sup>109</sup>-Leu<sup>110</sup> to Ala<sup>109</sup>-Ser<sup>110</sup> (5'-CTCCGCTTC TCTGGAGCTA GCCTAGGCTA TGC-CGTGC), pSG5 MLN64 LL4 by mutating Leu<sup>144</sup>-Leu<sup>145</sup> to Ala<sup>144</sup>-Ser<sup>145</sup> (5'-GGTCATCTC TCTGAGGCTA GCAGCAAAGG GGCATTGTG), pSG5 MLN64 LL5 (and Y3) by mutating Tyr<sup>152</sup>-Leu<sup>153</sup> to Ala<sup>152</sup>-Ser<sup>153</sup> (5'-CAAAGGGGCA TTTGGCGCTA GCCTCCCAT CGTCTCTT), pSG5 MLN64 Y1 by mutating Tyr<sup>89</sup> to Val<sup>89</sup> (5'-GCAGGAGATC ATC-CAGGTTA ACTTTAAAC TTCC), pSG5 MLN64 Y2 by mutating Gly<sup>112</sup>-Tyr<sup>113</sup> to Ala<sup>112</sup>-Ser<sup>113</sup> (5'-CTCTGGACTG CTCCTAGCTA GCGCCGTGCT GCAGCTCC).

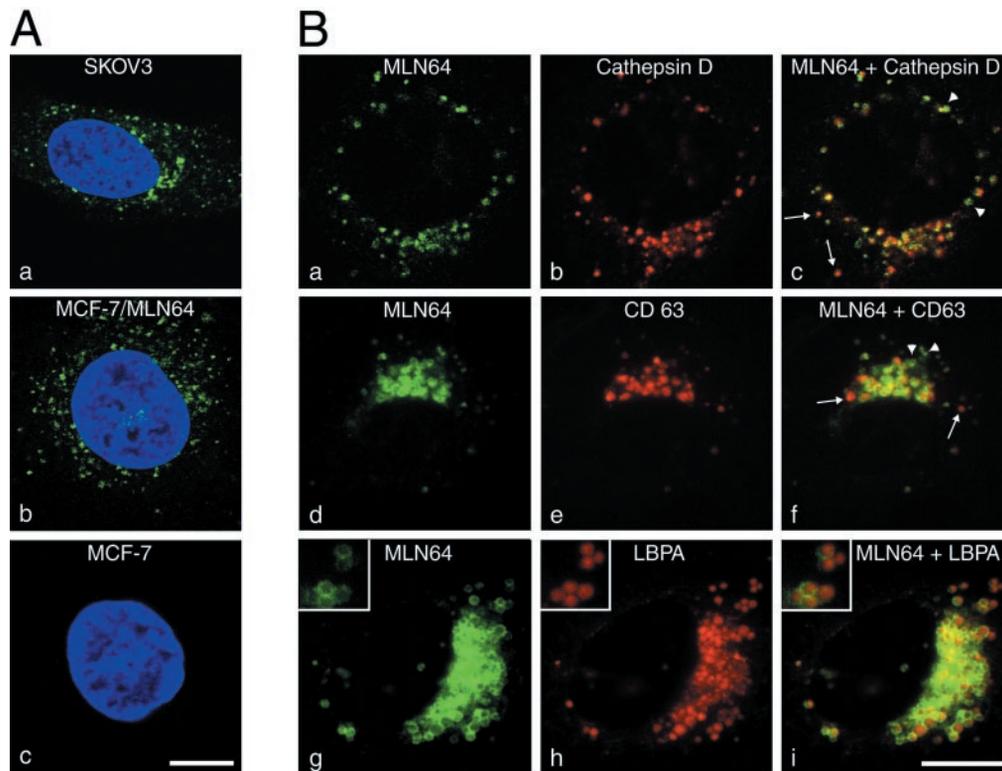
The pCR3.1 NPC1 expression plasmid was a kind gift of Dr. E. Ikonen (National Public Health Institute, Helsinki, Finland).

**Stable Cell Lines**—MCF7 cells were transfected by calcium phosphate coprecipitation with either the pCMVneo-MLN64 expression vector or the pCMVneo vector, both linearized by *Hind*III. The medium was changed after 20 h, and on the following day, the selection was begun in the same medium supplemented with 400 µg/liter G418 (Life Technologies, Inc.). After 2 weeks of selection, resistant clones were subcloned, and expression of MLN64 was assessed by Western blot as described previously (10).

**Antibodies**—The rabbit polyclonal 605 (pAbMLN64-Ct) and mouse monoclonal 2BE2F4 (mAbMLN64-Ct) antibodies were raised against the synthetic peptide HSAKPPTHKYVRGENG corresponding to residues 369–384 of human MLN64 as described previously (10, 23). In addition, a rabbit polyclonal antibody 1611 (pAbMLN64-Nt) was raised against the peptide MSKLPRELTRDLERSLPAV corresponding to residues 1–19 of human MLN64. The 6C4 monoclonal anti-LBPA antibody was described previously (24). The M1G8 monoclonal anti-cathepsin D antibody was a generous gift from Dr. M. Garcia and Prof. H. Rochefort (INSERM U148, Montpellier, France). The monoclonal anti-CD63 antibody was purchased from Chemicon (Temecula, CA). The rabbit polyclonal anti-NPC1 antibody was a kind gift of Dr. E. Ikonen (National Public Health Institute, Helsinki, Finland). Cy3-conjugated affinity-purified donkey anti-mouse IgG- and Cy2-conjugated affinity-purified goat anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Biotin-conjugated donkey anti-mouse antibody was purchased from Vector Laboratories (Burlingame, CA).

**Immunocytofluorescence**—SK-OV-3 and MCF7 cells were grown to 70% confluence on glass coverslips. After washing with phosphate-buffered saline (PBS), cells were fixed 10 min at room temperature in 4% paraformaldehyde in PBS and permeabilized for 10 min with 0.1% Triton X-100 in PBS. After blocking in 1% bovine serum albumin in PBS, cells were incubated at room temperature with the primary antibodies, either pAbMLN64-Ct or mAbMLN64-Ct together with anti-cathepsin D M1G8, anti-LBPA 6C4, anti-CD63, or anti-NPC1. Cells were washed three times in PBS and incubated 1 h with Cy3- and Cy2-conjugated secondary antibodies (1:400). Cells were washed three times in PBS, and in some cases, nuclei were counterstained with Hoechst-33258 dye. Slides were mounted in Aqua Poly/Mount (Polysciences Inc., Warrington, PA). Observations were made with a confocal microscope (Leica TCS4D; Heidelberg, Germany) or with a fluorescence microscope (Leica DMLB 30T).

**Immunoelectron Microscopy**—MCF7 cells were grown on glass coverslips to 70% confluence. After washing with PBS, cells were fixed 45 min at room temperature in 2.5% glutaraldehyde in PBS followed by a 15-min incubation in 1% sodium borohydride in PBS. Cells were then permeabilized for 20 min with 0.1% saponin in PBS. After blocking for 30 min in 5% normal donkey serum in PBS, cells were incubated overnight at 4 °C with mAbMLN64-Ct antibody (1:1000 dilution). After three washes with PBS, cells were incubated 1.5 h with biotin-conjugated donkey anti-mouse antibody. Cells were then processed for immunoperoxidase labeling using the Vectastain Elite ABC standard kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions, which was followed by incubation with 0.0125% diaminobenzidine and 0.005% H<sub>2</sub>O<sub>2</sub> in 0.05 M Tris buffer, pH 7.6. Cells were



**FIG. 1. MLN64 is a late endosomal protein.** A, SK-OV-3 (a), MCF7 (c), and stably transfected MCF7/MLN64 cell lines (b) were fixed, permeabilized, and then labeled with pAbMLN64-Ct antibody (green). Nuclei were counterstained with Hoechst-33258 dye (blue). SK-OV-3 and MCF7/MLN64 exhibited similar punctuate MLN64 staining, whereas MCF7 was not stained. B, MCF7/MLN64 cells were fixed, permeabilized, and then incubated with pAbMLN64-Ct antibody (B, a, d, and g) and anti-cathepsin D (B, b), anti-CD63 (B, e), or anti-LBPA (B, h). Overlays of B (a) and B (b), of B (d) and B (e), and of B (g) and B (h) are shown in B (c), B (f), and B (i), respectively. The yellow staining indicates colocalization. MLN64 colocalized with cathepsin D (B, c) or CD63 (B, f), although the relative quantity of both proteins from one vesicle to another one was variable (arrows, stronger signal for cathepsin D or CD63; arrowheads, stronger signal for MLN64). MLN64 colocalized completely with LBPA. Scale bar, 10  $\mu$ m except for insets, showing 2 times higher magnification in B, g, h, and i.

postfixed with 2.5% glutaraldehyde in PBS and then with 2% OsO<sub>4</sub> for 30 min. They were then dehydrated with graded concentrations of ethanol and conventionally embedded in epoxy resin. Glass coverslips were dissolved in hydrofluoric acid, and ultrathin sections were observed under a transmission electron microscope (Hitachi 7500, Japan).

**Endocytosis and Microinjection of Antibodies**—MCF7 cells, on glass coverslips, were washed three times with Dulbecco's modified Eagle's medium without serum.

For endocytosis of antibodies, cells were incubated for 1 h with either anti-cathepsin D M1G8, mAbMLN64-Ct, or pAbMLN64-Nt antibodies. Antibodies were used at the same concentration as for immunofluorescence.

Antibodies were microinjected into the cell cytoplasm together with lysine fixable dextran fluorescein 40000 MW (Molecular Probes, Inc., Eugene, OR) as described previously (25). Microinjections were followed by an incubation of the cells for 1 h. Antibodies were used 10 times more concentrated than for immunofluorescence.

Detection of either internalized or microinjected antibodies was performed by incubating fixed cells with the secondary antibody as described above (see "Immunocytofluorescence").

**Transfection of NPC2 Fibroblasts and Filipin Staining**—Niemann-Pick C2 fibroblasts were grown on glass coverslips to 70% confluence and were transfected with the expression vector pSG5 MLN64 with FuGENE6 transfection reagent (Roche Molecular Biochemicals). After transfection, cells were cultured in medium supplemented with 5% lipoprotein-deficient serum for 24 h and then changed to medium supplemented with 5% lipoprotein-deficient serum and 50  $\mu$ g/ml human LDL for an additional 24 h of culture. Cells were then processed for immunofluorescence to identify transfected cells with mAbMLN64-Ct anti-MLN64 as described above (see "Immunocytofluorescence") except that permeabilization with Triton X-100 was omitted. Staining of free cholesterol was performed after fixation using 50  $\mu$ g/ml filipin (Sigma) for 30 min.

## RESULTS

**MLN64 Is a Late Endosomal Protein**—To assess the subcellular localization of the MLN64 protein, we performed indirect

immunofluorescence experiments using two cancer-derived cell lines. A human ovarian cancer cell line, SK-OV-3, that overexpresses MLN64 at both the RNA and protein levels and a human breast cancer cell line, MCF7, that does not express detectable levels of MLN64 mRNA were chosen (4, 10). A punctuate cytoplasmic staining was observed using an anti-MLN64 antibody in the SK-OV-3 cells (Fig. 1A, a), whereas no protein expression could be detected in MCF7 cells (Fig. 1A, c). MCF7 cells overexpressing MLN64 (MCF7/MLN64) showed a similar punctuate staining as observed for SK-OV-3 cells (Fig. 1A, b). To identify the subcellular structures where the MLN64 protein resides, MCF7/MLN64 cells were colabeled with antibodies directed against known organelle-resident proteins or lipids. Double staining experiments using MLN64 and cathepsin D or Lamp1, two markers of both endosomes and lysosomes (26, 24), showed a large overlap of both signals, the relative quantity of both proteins being variable from one vesicle to another (Fig. 1B, a–c, and data not shown). Double labeling experiments with MLN64 and CD63, a marker for all types of endosomes (27), showed also a large overlap of both signals, with the relative quantity of both proteins again being variable from one vesicle to another (Fig. 1B, d–f). Colocalization of MLN64 and LBPA, a lipid restricted to late endosomes (24), showed that both signals completely overlapped (Fig. 1B, g–i). Thus, among the endosome/lysosome vesicles, MLN64 appeared to specifically reside in the late endosomes. In addition, we noted that the immunocytofluorescence staining of MLN64 appeared as a ring on most of the endosomes (Fig. 1B, g–i). When the cells are costained with anti-LBPA, a lipid exclusively located in late endosomes (24), MLN64 staining surrounded LBPA-stained spheres (Fig. 1B, i).

TABLE I

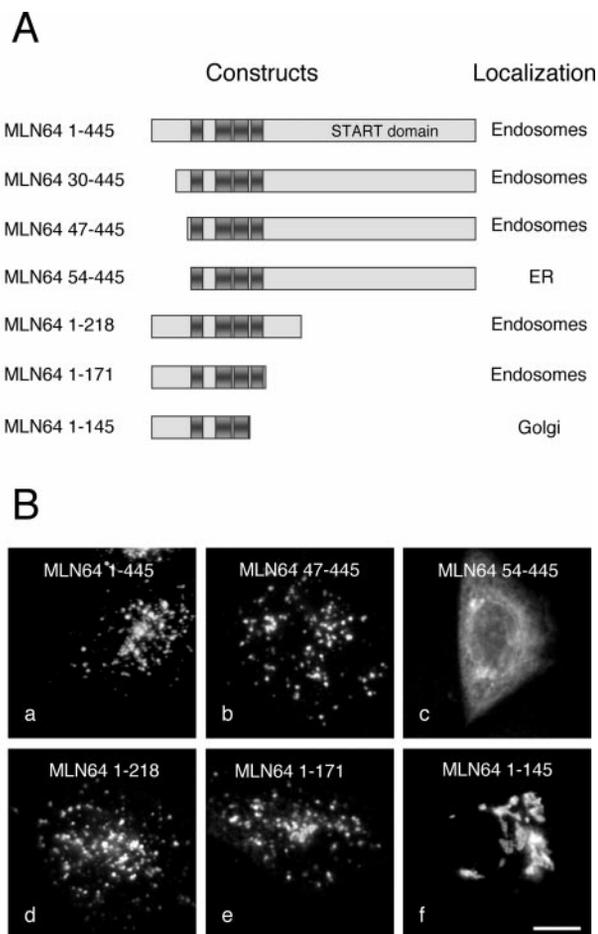
Sequences and positions of the potential *N*-glycosylation sites and GYXXZ type sorting motif of MLN64

Mutagenesis of these sites on the cDNA of MLN64 cloned in the pSG5 expression vector and the subcellular localization of the resulting mutant proteins are indicated.

Mutagenesis	Localization of the mutant protein
Asn <sup>219</sup> → Ala	Endosomes
Asn <sup>311</sup> → Ser	Endosomes
Asn <sup>219</sup> → Ala, Asn <sup>311</sup> → Ser	Endosomes
GY <sup>113</sup> AVL → ASAVL	Endosomes

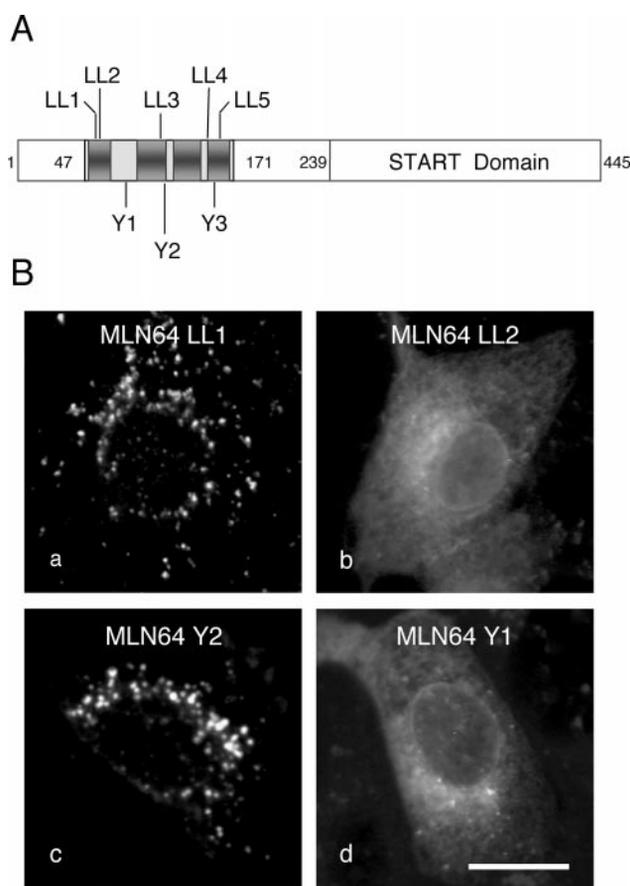
*The Transmembrane Helices-containing Domain Is Responsible for the Addressing of MLN64 to Late Endosomes*—To identify endosomal addressing signals, we analyzed the MLN64 protein sequence for known motifs. MLN64 possesses four putative transmembrane domains (10). As a control of the functionality of these transmembrane domains, we decided to analyze potential *N*-glycosylation sites, which have been shown to direct luminal proteins to endosomes/lysosomes (28). MLN64 contains two potential sites of *N*-glycosylation at positions 219 and 311; we performed site-directed mutagenesis of these amino acids (Asn<sup>219</sup> → Ala<sup>219</sup> and Asn<sup>311</sup> → Ser<sup>311</sup>) (Table I). The localization of mutated proteins was compared with a marker of late endosomes (data not shown). Neither single mutants nor the double mutant have a modified subcellular localization. These data indicate that putative *N*-glycosylation of MLN64 are not involved in the sorting of MLN64, which strongly suggests that the transmembrane domains of the protein are functional. The contribution of another sorting signal typical of endosomal transmembrane protein was therefore tested. The GYXXZ motif (where *Z* represents an aliphatic or aromatic residue) is responsible for the sorting of the Lamp proteins to lysosome and endosome membranes (29). MLN64 contains one such motif at positions 112–116. Site-directed mutagenesis of this motif did not alter the endosomal localization of the protein (Table I). Therefore, this motif is not involved in the sorting of MLN64. Since common signals were not responsible for the addressing of MLN64, we decided to map the MLN64 endosomal sorting domain using recurrent deletions in the NH<sub>2</sub> and COOH termini of the protein.

Three NH<sub>2</sub>-terminal deletions were constructed by site-directed mutagenesis in the eukaryotic expression vector pSG5 MLN64 (Fig. 2A). Subcellular localization was assayed by transient transfection in MCF7 cells followed by immunofluorescence using pAbMLN64-Ct. The localization of mutated proteins was compared with a marker of late endosomes (data not shown). Deletions of the NH<sub>2</sub>-terminal part to position 29 or 46 (pSG5 MLN64-(30–445) and pSG5 MLN64-(47–445)) showed a localization (Fig. 2B, b) similar to the wild-type protein (Fig. 2B, a). Further NH<sub>2</sub>-terminal deletion (pSG5 MLN64-(54–445)) involving the transmembrane domains of the protein led to a mislocalization of the protein, which then exhibited a reticular pattern characteristic of the endoplasmic reticulum (Fig. 2B, c). Similarly, three COOH-terminal deletions were constructed (Fig. 2A), and subcellular localization was assayed using pAbMLN64-Nt. The localization of mutated proteins was compared with a marker of late endosomes (data not shown). When the COOH-terminal part of the protein is deleted from amino acids 219 or 172 to 445 (pSG5 MLN64-(1–218), pSG5 MLN64-(1–171)), the protein remained in endosomes (Fig. 2B, d and e). Further deletion (pSG5 MLN64-(1–145)) including the transmembrane helices-containing domain led to a modified localization of the protein, which then resided in the Golgi apparatus (Fig. 2B, f). NH<sub>2</sub>- and COOH-terminal deletions showed that the minimal domain for sorting to the endosome was included within amino acids 47–171 of the MLN64 protein.



**FIG. 2. The transmembrane helices-containing domain is responsible for the targeting of MLN64 to late endosomes.** A, scheme of the deletion constructs of MLN64 and subcellular localization of the resulting proteins. The numbers correspond to the first and the last amino acid of the MLN64 wild type and mutant constructs. The four potential transmembrane domains are represented by dark boxes. ER, endoplasmic reticulum. B, immunofluorescence analysis of the subcellular localization of MLN64-(1–445) (a), MLN64-(47–445) (b), MLN64-(54–445) (c), MLN64-(1–218) (d), MLN64-(1–171) (e), and MLN64-(1–145) (f) revealed by immunocytofluorescence with pAbMLN64-Ct (a–c) or with pAbMLN64-Nt (d–f) antibodies. Scale bar, 10  $\mu$ m.

*Mutation of Dileucine 66–67 or of Tyrosine 89 Impairs the Localization of MLN64*—Further deletions of the protein would include the transmembrane helices-containing domain and could therefore modify the overall structure of MLN64 and its anchorage to the membrane. Thus, we decided to perform point mutations within the sequence located from residue 47 to 172 to identify amino acids involved in the sorting. Most of the membrane proteins located in the endosome/lysosome exhibit dileucine or tyrosine type addressing signals (30). We therefore decided to systematically mutate tyrosine and dileucine motifs within the minimal region necessary to target the protein to endosomes. Five dileucine motifs (LL) are present in this region of the protein (Fig. 3A, LL1–LL5). Mutations of four of them (LL1 and LL3–LL5) did not change the localization of the protein (Fig. 3B, a; data not shown) when compared with a marker of late endosomes (data not shown). In contrast, mutation of LL2 located at positions 66 and 67 led to a mislocalization of the protein to the endoplasmic reticulum (Fig. 3B, b). Three different tyrosines (Y1–Y3) are present between amino acids 47 and 172. Y2 is included in the previously studied GYXXZ motif (Table I). Mutations of Y2 or Y3 did not change the subcellular localization of MLN64 (Fig. 3B, c; data not



**FIG. 3. Mutations of dileucine 66–67 or of tyrosine 89 impairs the localization of MLN64.** *A*, positions of the five dileucine motifs (LL1–LL5) and of the three tyrosines (Y1–Y3) that were mutated within the MLN64 transmembrane helices-containing domain. The domain responsible for the targeting of MLN64 (amino acids 47–171) is represented by the gray box. Transmembrane helices are represented by the four dark boxes. The numbers indicate the amino acid residues. *B*, immunofluorescence analysis of the subcellular localization of four of the mutants revealed with pAbMLN64-Ct antibody. MLN64 LL1 (*B*, *a*) and MLN64 Y2 (*B*, *c*) had a similar subcellular localization as the wild type MLN64 (Fig. 2*B*, *a*). On the opposite MLN64 LL2 (*B*, *b*) and MLN64 Y1 (*B*, *d*) mutants showed an endoplasmic reticulum staining. Scale bar, 15  $\mu$ m.

shown) when compared with a marker of late endosomes (data not shown). On the contrary, the mutation of Y1 (residue 89) modified the subcellular localization of the protein, which then resided in the endoplasmic reticulum (Fig. 3*B*, *d*). Thus, two motifs, the dileucine motif 66–67 and the tyrosine 89 are important for proper addressing of MLN64 to late endosomes.

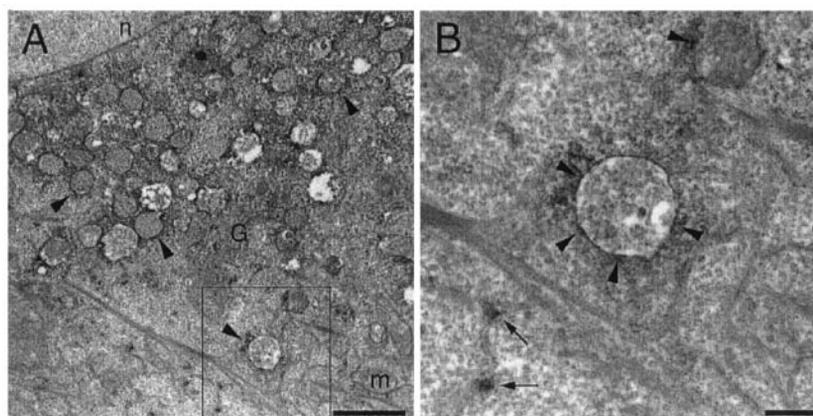
**MLN64 Is Located on the Limiting Membrane of Late Endosomes**—The immunocytofluorescence staining of MLN64 (Fig. 1*B*, *g–i*) suggested that MLN64 localizes to the limiting membrane of endosomes. To confirm this localization of MLN64, we performed immunoelectron microscopy on MCF7/MLN64 cells using the mAbMLN64-Ct antibody. The immunolabeling was restricted to round vesicles with an endosome shape (31) that were about 700 nm in diameter (Fig. 4*A*). These labeled vesicles were scattered through the cytoplasm, with frequent perinuclear accumulation (Fig. 4*A*). The labeling of MLN64 was restricted to the cytoplasmic surface of the vesicles. No internal membranous structures of the endosomes were labeled. Moreover, the labeling on the limiting membrane of endosomes appeared as patches (Fig. 4*B*), which showed an uneven localization of the protein within the membrane. Additional punctuate labeling, which was not associated with clear vesicular structures, appeared in the cytoplasm of cells (Fig. 4*B*). The

labeling of MLN64 is mainly observed on the cytoplasmic face of the limiting membrane of endosomes. No immunoreactivity was detected in the parental MCF7 cells, indicating that the staining was specific for MLN64 (data not shown).

**The START Domain and the NH<sub>2</sub> Terminus of MLN64 Are Cytoplasmic**—Immunoelectron microscopy strongly suggested that the START domain of MLN64 is facing the cytoplasm and not the lumen of endosomes. To confirm this hypothesis, we first performed endocytosis of mAbMLN64-Ct antibody or anti-cathepsin D by MCF7/MLN64 living cells. Cathepsin D is a protease located in the lumen of endosomes/lysosomes (32). Antibodies directed against cathepsin D can be taken up by living cells permitting the visualization of the protein in endosomes/lysosomes (26). Indeed, a punctuate staining typical of cathepsin D was observed (Fig. 5*A*, *a*). On the opposite, no staining could be detected for MLN64 (Fig. 5*A*, *b*). The complementary experiment consisting of microinjection, into the cytoplasm of MCF7/MLN64 living cells, of anti-cathepsin D or mAbMLN64-Ct antibodies was performed. Cells microinjected with anti-cathepsin D exhibited no staining (Fig. 5*B*, *a–c*). On the opposite, cytoplasmic microinjection of mAbMLN64-Ct led to a punctuate cytoplasmic signal (Fig. 5*B*, *d–f*). Since the antibody used is directed against the COOH terminus of MLN64, these findings indicate that the START domain of MLN64 is cytoplasmic (Fig. 5*C*). MLN64 possesses four putative transmembrane domains, which implies that if the COOH terminus of the protein is cytoplasmic then the NH<sub>2</sub> terminus is also in the cytoplasm. We performed the same experiment using the pAbMLN64-Nt antibody directed against the NH<sub>2</sub> terminus of the protein. No staining was obtained when the pAbMLN64-Nt antibody was taken up by endocytosis by MCF7/MLN64 living cells (Fig. 5*A*, *c*), while cytoplasmic microinjection of pAbMLN64-Nt antibody led to a punctuate cytoplasmic signal (Fig. 5*B*, *g–i*). No staining was present when mAbMLN64-Ct antibodies were neutralized with their cognate antigen prior to microinjection in MCF7/MLN64 cells or when mAbMLN64-Ct or pAbMLN64-Nt antibodies were microinjected into MCF7 cells (data not shown). Taken together, these results show the NH<sub>2</sub> terminus and the COOH terminus of MLN64 are cytoplasmic (Fig. 5*C*).

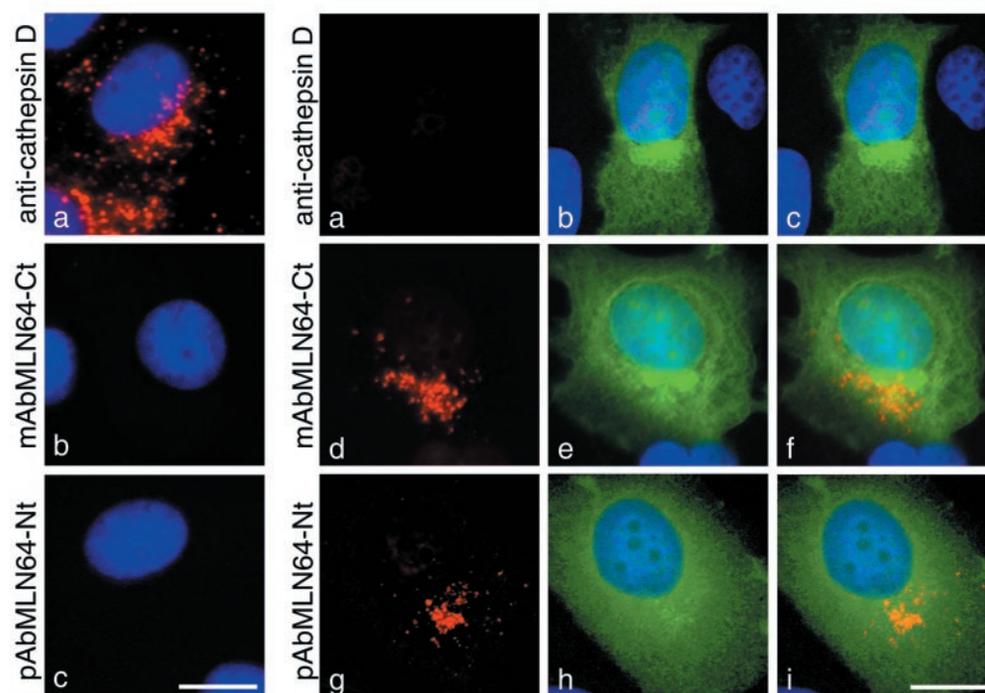
**MLN64 Colocalizes with Cholesterol and NPC1 but Does Not Correct Late Endosomal Accumulation of Cholesterol in NPC2 Patient Fibroblasts**—Niemann-Pick C disease is characterized by a late endosomal accumulation of LDL-derived cholesterol (2, 33, 34). Two complementation groups were identified, and NPC1, the protein defective in the first complementation group of NPC disease, is located in the endosomes/lysosomes (35, 36). Since MLN64 is located in the late endosomes, we postulated that MLN64 might be a candidate gene for the defect of the second complementation group of Niemann-Pick C disease (NPC2). We first performed colocalization experiments in CHO cells between MLN64 and cholesterol using filipin, a fluorescent molecule that specifically binds free cholesterol (33). The majority of MLN64-positive vesicles were also positive for cholesterol (Fig. 6*A*, *a–c*). We also examined the effect of drugs that block the transport of cholesterol out of lysosomes on the localization of MLN64 and cholesterol. We treated CHO cells with U18666A, a hydrophobic amine that induces the accumulation of cholesterol in lysosomes (1). In these cells, MLN64 was present on the surface of cholesterol-loaded vesicles (data not shown). We next performed colocalization experiments between MLN64 and NPC1. MLN64 and NPC1 colocalized in CHO cells, the relative quantity of both proteins being variable from one vesicle to another (Fig. 6*A*, *d–f*). Since MLN64 and NPC1 were located in the same subset of endosomes, we checked if the expression of MLN64 in NPC2 fibroblasts could

FIG. 4. **MLN64 is located on the limiting membrane of late endosomes.** MCF7/MLN64 cells were labeled with the mAbMLN64-Ct antibody. The labeling was detected by electron microscopy. *A*, perinuclear concentration of immunolabeled endosomes (arrowheads). The labeling was restricted to the cytoplasmic face of endosomes. The Golgi apparatus (*G*), nucleus (*n*), and mitochondria (*m*) were unlabeled. *B*, higher magnification of the area boxed in *A* showed the patchy distribution (arrowheads) of immunolabeling on the cytoplasmic surface of the limiting membrane of endosomes. The arrows indicate small punctuate staining within the cytoplasm. Scale bar, 2  $\mu$ m (*A*) and 500 nm (*B*).



### A Endocytosis

### B Micro-injection



### C

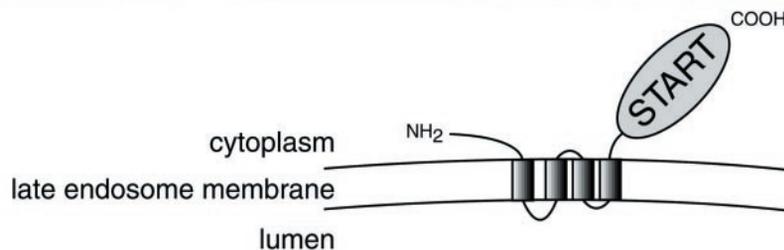
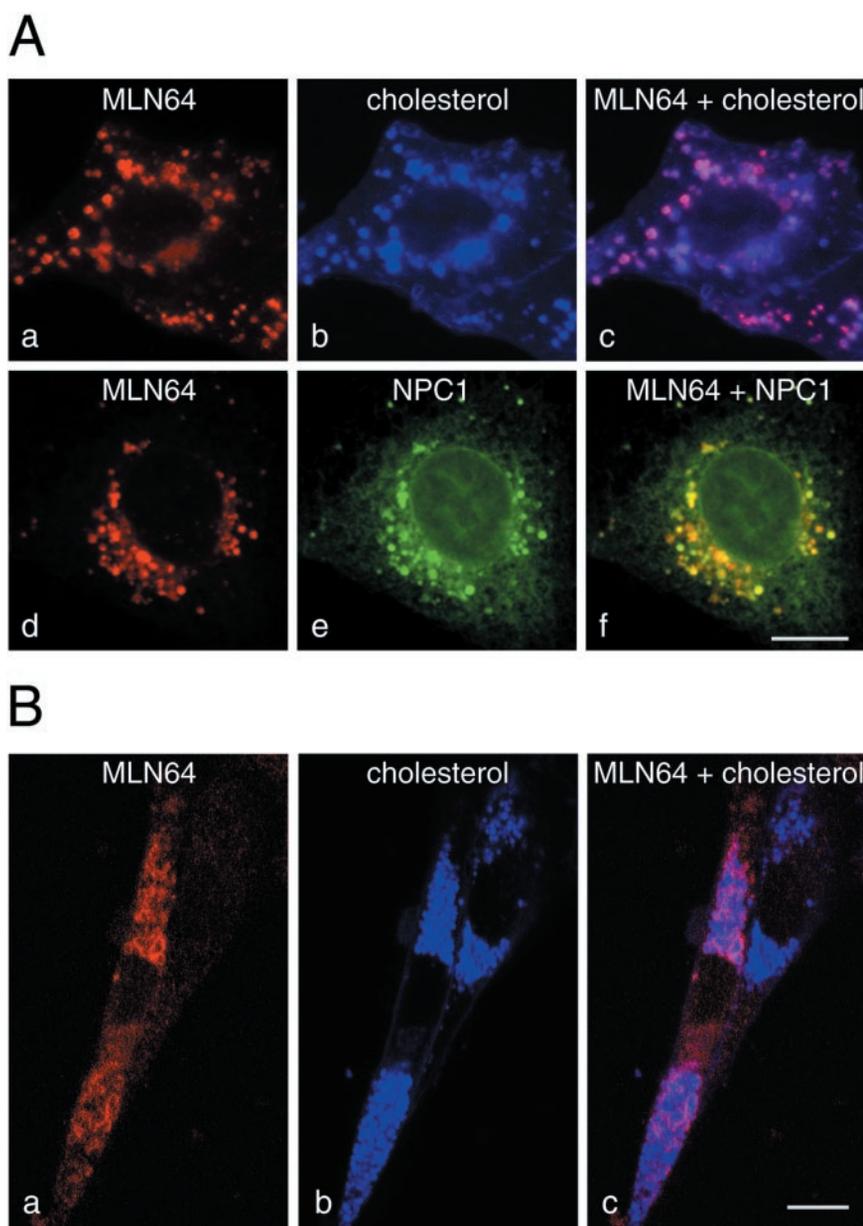


FIG. 5. **The START domain and the NH<sub>2</sub> terminus of MLN64 are cytoplasmic.** *A*, MCF7/MLN64 cells were incubated in a medium containing either anti-cathepsin D monoclonal antibody M1G8 (*a*), mAbMLN64-Ct antibody (*b*), or pAbMLN64-Nt antibody (*c*). Cells were then fixed, permeabilized, and incubated with Cy3-conjugated secondary antibody (red). Cells incubated with anti-cathepsin D antibody exhibited a punctuate staining (*a*), whereas cells incubated with mAbMLN64-Ct (*b*) or pAbMLN64-Nt (*c*) were not stained. Scale bar, 15  $\mu$ m. *B*, MCF7/MLN64 cells were microinjected with either M1G8 (*a-c*), mAbMLN64-Ct (*d-f*), or pAbMLN64-Nt (*g-i*) conjointly with dextran fluorescein. Cells were then fixed, permeabilized, and incubated with Cy3-conjugated secondary antibody (red; *a*, *d*, and *g*). Microinjected cells were visualized with dextran fluorescein (green; *b*, *e*, and *h*). Nuclei were counterstained with Hoechst-33258 dye (blue; *b*, *e*, and *h*). Overlays of *a* and *b*, *d* and *e*, and *g* and *h* are shown in *c*, *f*, and *i*, respectively. By microinjection of the antibodies in the cells' cytoplasm, a punctuate staining could be observed with mAbMLN64-Ct (*d*) and pAbMLN64-Nt (*g*) but not with anti-cathepsin D antibody (*a*). Scale bar, 15  $\mu$ m. *C*, model of topology for MLN64 on the limiting membrane of the late endosome.

complement the phenotype of accumulation of cholesterol in late endosomes. NPC2 fibroblasts were transiently transfected with MLN64, fed with LDL, processed for immunofluorescence, and stained with filipin. MLN64 was present at the surface of

cholesterol-loaded late endosomes in NPC2 cells (Fig. 6*B*, *a-c*). None of the 50 cells transfected with the MLN64 expression vector exhibited a corrected late endosomal accumulation of cholesterol (Fig. 6*B*, *a-c*). In addition, the cDNA encoding

**FIG. 6. MLN64 colocalizes with cholesterol and NPC1 but does not correct late endosomal accumulation of cholesterol in NPC2 patient fibroblasts.** *A*, CHO cells were transfected either with pSG5 MLN64 alone (*a–c*) or with pSG5 MLN64 and pCR3.1 NPC1 (*d–f*). Cells were then processed for immunofluorescence either with mAbMLN64-Ct antibody (*a*) together with filipin staining (*b*) or after classical Triton X-100 permeabilization with mAbMLN64-Ct antibody (*d*) and anti-NPC1 antibody (*e*). Overlays of *a* and *b* and of *d* and *e* are shown in *c* and *f*, respectively. A large majority of MLN64 positive vesicles were filled with cholesterol (*c*). MLN64 colocalized with NPC1, although the relative quantity of both proteins from one vesicle to another one was variable (*f*). Scale bar, 10  $\mu$ m. *B*, NPC2 fibroblasts were transfected with the expression vector pSG5 MLN64, incubated with LDL, and processed for immunofluorescence with the pAbMLN64-Ct antibody (*a*; red) and filipin staining (*b*; blue). Overlay of *a* and *b* is shown in *c*. MLN64 expression (*c*; left cell versus right cell) could not correct cholesterol accumulation in late endosomes (*c*). Scale bar, 10  $\mu$ m.



MLN64 was sequenced from NPC2 fibroblasts, and no mutations were found (data not shown). Therefore, alteration of MLN64 is not responsible for NPC2 disease.

#### DISCUSSION

MLN64 and StAR belong to a novel protein family involved in cholesterol transport. They share a specific domain, the START domain. This 200–210-amino acid domain is present in a wide range of proteins involved in diverse cell functions (11), including potential cholesterol transfer. Among the START domain-containing proteins, MLN64 and StAR are the most closely related in sequence (11). Mutations or truncations within the START domain of StAR cause congenital adrenal hyperplasia, an autosomal recessive disease characterized by markedly impaired gonadal and adrenal steroid hormone synthesis. This finding suggests that this domain is functionally relevant. In addition, the START domain of MLN64 and StAR was shown to bind cholesterol at an equimolar ratio (20). The crystal structure of the START domain of MLN64 was solved, revealing that the protein encompasses a hydrophobic tunnel large enough to bind a

single cholesterol molecule (20).

In animal cells, cholesterol can be obtained either by *de novo* synthesis or receptor-mediated lipoprotein uptake. Cholesterol can be used as a structural component of membranes, be converted into cholesteryl esters, be effluxed to extracellular acceptors, or be metabolized into steroid hormones or bile acids depending on the cell type (1, 2). Mechanisms of cholesterol transport between intracellular compartments remain unclear. Only a few proteins involved in these processes have been identified. StAR has been shown to be implicated in the transport of cholesterol into the mitochondria (37). In contrast to StAR, MLN64 was not present in the mitochondria but in distinct undefined vesicular structures (10). Besides human cancers that show abnormal overexpression, MLN64 is expressed at basal levels in all cells and tissues, and its promoter harbors the classical feature of a housekeeping gene promoter.<sup>2</sup> Therefore, MLN64 fulfills the criteria of a protein involved in an essential cellular function such as cholesterol transport.

<sup>2</sup> F. Alpy, manuscript in preparation.

Here, we have studied where MLN64 is likely to play a role in intracellular cholesterol movement.

MLN64 protein was found to colocalize largely with cathepsin D, LampI, CD63, and LBPA, different markers of endosomes/lysosomes. More precisely, the staining of MLN64 completely overlapped with the staining of LBPA, a late endosomal lipid, demonstrating that MLN64 is localized to late endosomes. Addressing protein to endosomes/lysosomes can be achieved by several means (30, 38), and redundant pathways have been suggested in eukaryotic cells (39, 40). We show in this study that putative *N*-glycosylation of MLN64 is not the sorting determinant of the protein as is common with luminal proteins of endosomes/lysosomes (28), which strongly suggests that the potential transmembrane domains of the protein are functional. MLN64 contains several potential conventional endosome/lysosome targeting signals typical of membranous proteins; using serial deletion constructs, we defined a minimal addressing domain. This domain, spanning amino acids 47–171, which comprises the four putative transmembrane domains, is necessary for correct sorting to late endosomes. Among the classical endosomal/lysosomal determinants that contain tyrosine- or dileucine-based motifs (30), five dileucines (LL1–LL5) and three tyrosines (Y1–Y3) are present in the minimal targeting domain of MLN64. Alteration by mutagenesis of the dileucine motif LL2 (amino acids 66 and 67) or of the tyrosine Y1 (amino acid 89) resulted in the modification of the subcellular localization. The mutated protein exhibited a reticular pattern specific to the endoplasmic reticulum. Although mutations of LL2 and Y1 modify the subcellular localization of MLN64, these two sites do not fulfill the criteria of targeting motifs. Typical dileucine- and tyrosine-based signals are located in the cytoplasmic region of proteins; when these signals are mutated, the protein is routed to the plasma membrane. Within the MLN64 protein structure, LL2 is located in the first putative transmembrane helix and Y1 in a luminal loop; when mutated, the protein is localized in the ER, suggesting a misfolding of the protein. Thus, we can conclude that within the endosomal addressing minimal region of MLN64, no conventional targeting signals could be identified. However, the dileucine motif LL2 and the tyrosine residue Y1 are critical for the proper folding of this domain and/or for the targeting of the protein to late endosomes.

By immunoelectron microscopy, MLN64 labeling shows a patchy pattern on the limiting membrane of endosomes. This uneven distribution reveals a compartmentalization within the same continuous membrane of endosomes, as is the case for Rab proteins (41).

Interestingly, late endosomes have recently been shown to be an important organelle for the trafficking of cholesterol derived from LDL. Indeed, multivesicular internal membranes of late endosomes, which contain high amounts of LBPA, regulate cholesterol transport (34).

Immunoelectron microscopy images obtained using an antibody against MLN64 COOH terminus, which contains the START domain, suggest that this part of the protein might be cytoplasmic. We have further investigated the localization of the MLN64-START domain by microinjection and endocytosis experiments using the mAbMLN64-Ct antibody. Antibodies incorporated by endocytosis allowed the detection of intraendosomal proteins such as cathepsin D (26). By contrast, antibodies directly microinjected into the cytoplasm of cells recognized antigens present in the cytoplasm that are not sequestered in subcellular vesicles. A specific signal was obtained when the antibody raised against the COOH terminus was directly injected into the cytoplasm, whereas no signal could be detected when the same antibody was endocytosed by

cells. Therefore, the functional START domain of MLN64 is cytoplasmic. MLN64 possesses four putative transmembrane domains, which implies that both the COOH and the NH<sub>2</sub> termini of the protein are cytoplasmic. By endocytosis and microinjection of the NH<sub>2</sub> terminus-specific antibody, we showed that both the NH<sub>2</sub> and the COOH termini of MLN64 are cytoplasmic.

In the autosomal recessive Niemann-Pick type C disease, a neurodegenerative endosomal/lysosomal storage disorder, LDL-derived cholesterol accumulates in the late endosomes (33, 34). In over 95% of the cases of NPC disease, the recently identified gene *NPC1* is mutated. There is evidence for the existence of a second complementation group, which concerns a few families of NPC patients (21, 42). The gene inactivated in this complementation group, named the *NPC2* gene, remains unknown. Therefore, *MLN64* appeared as a potential candidate gene for this disease. We have shown that MLN64 and cholesterol as well as NPC1 colocalized in CHO cells, showing that the two proteins might act in the same compartments. However, transient transfection of an expression vector encoding for MLN64 could not restore a normal phenotype to fibroblasts from NPC2 patients. Transfected fibroblasts still accumulated LDL-derived cholesterol in the late endosomes. Therefore, inactivation of MLN64 is not responsible for the NPC2 disorder.

Taken together, these observations suggest that MLN64 acts on late endosome cholesterol traffic. The MLN64 amino-terminal transmembrane-containing domain is anchored in the limiting membrane of late endosomes, and its COOH-terminal START domain is cytoplasmic. The START domain of MLN64 can interact with the membrane of late endosome, where it could deplete cholesterol by shuttling it to a cytoplasmic acceptor site.

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