MENTHO, a MLN64 Homologue Devoid of the START Domain*

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MLN64 is a late endosomal membrane protein containing a carboxy-terminal cholesterol binding START domain and is presumably involved in intracellular cholesterol transport. In the present study, we have cloned a human cDNA encoding a novel protein that we called MENTHO as an acronym for MLN64 N-terminal domain homologue because this protein is closely related to the amino-terminal half of MLN64. MLN64 and MENTHO share 70% identity and 83% similarity in an original amino-terminal part encompassing 171 amino acids that we designated as the MENTAL (MLN64 N-terminal) domain. By translation initiation scanning MENTHO is synthesized as two isoforms of 234 (α) and 227 (β) amino acids that can be phosphorylated. As MLN64, MENTHO is ubiquitously expressed and is located in the membrane of late endosomes, its amino and carboxy-terminal extremities projecting toward the cytoplasm. We show that MENTHO overexpression does not rescue the Niemann-Pick type C lipid storage phenotype. However, MENTHO overexpression alters severely the endocytic transport of cholesterol from the outer to the inner mitochondrial membrane, where it is converted into pregnenolone (7). Unlike StAR, which is therefore a mitochondrial protein, we have shown that MLN64 is located in late endosomes (8). The specific localization suggested that MLN64 is a transporter of cholesterol derived from low-density lipoprotein (LDL), a cholesterol-containing particle, which is endocytosed and transported to late endosomes where cholesterol is routed to different organelles (9). Among the known molecules involved in this pathway are the products of the causative genes for the Niemann-Pick C disease, NPC1 and NPC2. We have shown that MLN64 was colocalized with NPC1 on late endosomes. However, MLN64 overexpression failed to rescue the NPC phenotype that is characterized by the accumulation of cholesterol-loaded vesicles in patient fibroblasts (8).

To identify molecules involved in intracellular cholesterol vesicular traffic we have screened for novel MLN64-related protein. Homology searches using the whole human genome sequence database revealed the presence of a potential protein corresponding to the MLN64 amino-terminal domain only. This part of MLN64 is an original domain since no other protein with such domain was described so far. We have shown that this domain is responsible for the specific localization of MLN64 protein in late endosomes and anchors the protein in the limiting membrane of late endosomes (8). The finding of this novel protein suggests that the amino-terminal part of MLN64 could have an intrinsic function. In the present study we have cloned and characterized this novel protein that we have called MENTHO for MLN64 N-terminal domain homologue.

MATERIALS AND METHODS

Cell Culture and Tissues—Surgical specimens obtained at the Hôpitaux Universitaires de Strasbourg were frozen in liquid nitrogen for RNA extraction.

The Chinese hamster ovary (CHO) cells were maintained in Dulbecco's modified Eagles medium/Ham's F12 supplemented with 20 mm Hepes, 2 mm glutamine, 10% calf serum, and 1% gentamycin. Transfection of CHO cells was done with FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN). The COS simian kidney, the HeLa human cervix, and HEK293 human embryonic kidney cell lines were provided by the American Type Culture Collection (ATCC, Manassas, VA) and routinely maintained in our laboratory and previously named StAR homology domain (SHD) (4). 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 (5). START domains are found in distinct proteins; for most of which their function is still unclear (6). However among them, the steroidogenic acute regulatory (StAR) protein has a well characterized function. 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 (7). Unlike StAR, which is therefore a mitochondrial protein, we have shown that MLN64 was colocalized with NPC1 on late endosomes. However, MLN64 overexpression failed to rescue the NPC phenotype that is characterized by the accumulation of cholesterol-loaded vesicles in patient fibroblasts (8).

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The abbreviations used are: StAR, steroidogenic acute regulatory protein; START, StAR-related lipid transfer domain; PBS, phosphate-buffered saline; GFP, green fluorescent protein; MENTHO, MLN64 N-terminal domain homologue; MENTAL, MLN64 N-terminal domain; LDL, low density lipoprotein; NPC, Niemann-Pick Type C.
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Infinigen™ (hTERT-HME1), was purchased from Clontech (Palo Alto, CA). The NPC2 fibroblast strain was obtained from case 16 in Vanier et al. (10) and transfected with FuGENE 6 transfection reagent. Human lipoprotein-deficient serum and human LDL were prepared in the laboratory as described previously (10).

**cDNA Library Screening**—A specific cDNA probe for MENTHO was obtained by RT-PCR using the two forward synthetic oligonucleotides (forward primer: 5′-ATG AAC CAC CTG CGA GGA GAC-3′ and reverse primer: 5′-TCA TAG TTC TAA AAG AAC GCT TCT ACC-3′) and total RNA extracted from human mammary tumors. Briefly, first strand cDNA synthesis was obtained using the reverse primer and AMV reverse transcriptase (Roche Molecular Biochemicals). This cDNA template was then amplified by PCR using forward and reverse primers. The amplified 661-bp fragment was used as a probe to screen a human fetal brain cDNA library constructed in the ZAPII vector. Briefly, 500,000 plaque-forming units were plated on LB agar and nylon filter replica (Hybond N; Amersham Biosciences) were hybridized at 42 °C in 50% formamide, 5× standard saline citrate (SSC), 0.4× Ficoll, 9.4% polyvinylpyrrolidone, 20 mmol/liter sodium phosphate (pH 6.5), 0.5% SDS, 10% dextran sulfate, and 100 μg/ml denatured salmon sperm DNA for 16 h with the 32P-labeled probe (11) diluted to 0.5–1× 106 cpm/ml. Stringent washes were performed at 60 °C in 0.1× SSC and 0.1% SDS. Filters were autoradiographed at ~80 °C for 24 h. Positive plaques were subjected to a secondary screening in the same hybridization conditions. Pure plaques were directly recovered as bacterial colonies using the pBlueScript/A-ZAPII in vivo excision system (Stratagene, La Jolla, CA). Three different clones containing the complete open reading frame of MENTHO were isolated.

**Plasmids**—A construct allowing the expression of wild type MENTHO was generated. A 672-bp fragment corresponding to the complete putative open reading frame of MENTHO was amplified by PCR using a MENTHO cDNA comprising plasmid as template and the synthetic oligonucleotides 5′-GAG AGA ATT CAA CTT TAC TGT CGA CAG GGA GCC GCC TCC-3′ and 5′-GAG AGA ATT CAA CTT TAC TGT CGA CAG GGA GCC GCC TCC-3′ incorporating EcoRI flanking sites. The PCR fragment was then digested by EcoRI and subcloned into the EcoRI site of the vector pGEM5Zf (Stratagene, La Jolla, CA) thus generating the vector pGEM5-MENTHO. Constructs allowing the expression of FLAG-tagged MENTHO protein were also generated. A 733-bp fragment was amplified by PCR using the synthetic oligonucleotides 5′-GAG AGA ATT CAA CTT TAC TGT CGA CAG GGA GCC GCC TCC-3′ and 5′-GAG AGA ATT CAA CTT TAC TGT CGA CAG GGA GCC GCC TCC-3′ incorporating EcoRI flanking sites. The PCR fragment was then digested by EcoRI and subcloned into the EcoRI site of the vector pK7G (Stratagene, La Jolla, CA) thus generating the vector pK7G5-MENTHO.

A vector construct allowing the expression of the MENTHO-EFGP fusion protein was generated. After PCR amplification using the synthetic oligonucleotides 5′-GAG AGA ATT CTT TAC TGT CGA GGT GTT GG-3′ and 5′-GAG AGA ATT GGA AGC ACC ACC ACC ACC ACC CAG-3′, the mixture MENTHO cDNA was digested by MnlI and cloned in frame into the EcoRI site of the pEGFP-N2 vector (BD Biosciences, Clontech) behind the EFGP cDNA sequence that is driven by the promoter pEGFP-N2 vector (BD Biosciences, Clontech). The pGEM5-MENTHO was used to generate mutated translation initiation sites. The first and second methionine have been mutated into phenylalanine (MENTHO ΔM1) and leucine (MENTHO ΔM8) respectively by site-directed mutagenesis (QuickChange site-directed mutagenesis kit, Stratagene, La Jolla, CA) using the synthetic oligonucleotides 5′-GTA AGA ATT CTA TAG TAA AAT GCT TGT GTT GGC-3′ and 5′-CAC CTG CCA GAA GAC GTC GAG AAC GCT TCT ACC-3′, respectively.

The Kozak sequence of the first translation initiation site of MENTHO has been mutated into a strong Kozak sequence by site-directed mutagenesis using pGEM5-MENTHO as template and the synthetic oligonucleotides 5′-CTG CCTG CCA GAA GAC GTC GAG AAC GCT TCT ACC-3′, generating the vector pGEM5-MENTHO SK. The pSG5 MLN64 expression vector was already described (8).

RNA Preparation and Northern Blot—RNAs from cell lines and from surgical specimens were extracted using the single step procedure of Chomczynski and Sacchi (12). RNAs were fractionated by electrophoresis on 1% agarose, 2.2× formaldehyde gels (13), transferred to nylon membrane (Hybond N, Amersham Biosciences), and immobilized by baking for 2 h at 80 °C.

**Generation of Anti-MENTHO Antibodies**—The rabbit polyclonal 1546 antibody (pAbMENTHO-Ct) was raised against the synthetic peptide AGSEEAAERQDKSEPKLLE corresponding to residues 216–234 of human MENTHO. The peptide was coupled to bovine albumin through an additional amino-terminal cysteine residue and injected into New Zealand rabbits. Immunoactive serum was affinity purified against the synthetic peptide coupled to sulfo-link-coupling gel (Pierce) using conditions recommended by the manufacturer.

**Immunocytofluorescence**—HeLa and CHO 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, pAbMENTHO-Ct alone or together with anti-MLN64 (mouse IgG1 clone 14, BD Bioscience, Le pont de Claire, France). Cells were washed three times in PBS and incubated 1 h with Alexa Fluor488 conjugated antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). 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 TCS SP1; Heidelberg, Germany) or with a fluorescence microscope (Leica DMLB 30T). Staining of acidic compartments of the cell was performed with LysoTracker Red (Molecular Probes, Inc.) according to the manufacturer’s instructions.

**Transfection of NPC-deficient Fibroblasts and Filipin Staining**—Niemann-Pick C2 fibroblasts were grown on glass coverslips to 70% confluence and were transfected with the expression vector pRK7N-MENTHO with FuGENE 6 transfection reagent. After transfection, cells were cultured in medium supplemented with 5% lipoprotein-deficient serum for 24 h and then changed to medium with 5% lipoprotein-deficient serum and 50 μg/ml human LDL for an additional 24 h of culture. Cells were then processed for immunofluorescence to identify transfected cells with anti-FLAG M2 antibody (Sigma) as described above except that permeabilization with Triton X-100 was omitted. Staining of free cholesterol was performed after fixation using 50 μg/ml filipin (Sigma) for 30 min.

**Endocytosis and Microinjection of Antibodies**—CHO 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 anti-FLAG M2 antibody 0.4 mg/ml. Anti-FLAG antibodies (4 mg/ml) were microinjected into the cell cytoplasm together with a fixable dextran-500 (Molecular Probes, Inc.) and a Filipin microinjector (Eppendorf, Hamburg, Germany). Microinjections were followed by an incubation of the cells for 1 h in growing medium. Detection of either internalized or microinjected antibodies was performed by incubating fixed cells with the secondary antibody as described above.

**Sequence Analysis**—Plasmid DNAs were sequenced with Taq polymerase and dye-labeled deoxyxynucleotide triphosphate for detection on an Applied Biosystems 373A automated sequencer. Searches of nucleotide and protein data bases were performed using the programs from the Wisconsin package, version 9.1 (Genetics Computer Group, Madison, WI). Alignments were performed with the Eclustalw program. The multiple sequence alignment tool program of the ExPasy Molecular Biology Server was used to obtain the molecular weight and the pH, of the protein (14). Putative phosphorylation sites of MENTHO were predicted with the ScanProsite software (15). The transmembrane helices were predicted with the TMHMM program (16). The Postscript files from Eclustalw alignment results were generated with the ESPript program (17). The accession number AJ492267 has been deposited in the GenBank™/EMBL nucleotide sequence data base under accession number AJ492267.

**SDS-PAGE and Western Blotting**— Cultured cells were washed with PBS and homogenized in lysis buffer (50 mM Tris, pH 7.6, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% deoxycholate). After centrifugation at 10,000 rpm for 15 min at 4 °C, the supernatant was conserved. Protein concentration was measured using the Bradford assay (Bio-Rad). Proteins were then directly, without boiling step, loaded onto a 10% SDS-PAGE gel. After migration, proteins were electrotransferred to nitrocellulose (Schleicher & Schuell, Dassel, Germany). The membrane...
Cloning of MENTHO—Searches for MLN64 homologous sequences in the human genome sequence DNA data base identified a human potential cDNA encoding for a protein referred to as AAD15552 (GenBank™ accession no. AC006033). Besides its homology with the amino-terminal half of MLN64 (Fig. 2, A and C), based on this result, we named this protein MENTHO because it is an acronym for MLN64 N-terminal domain homologue.

The alignment of the human MLN64 and the human MENTHO showed that both proteins share an overall 75% similarity and 60% identity. The amino-terminal extremity of both proteins is highly conserved with the amino-terminal half of MLN64 (Fig. 2, A and C). The amino-terminal half of MENTHO shares an overall 75% similarity with the amino-terminal half of MLN64 (Fig. 2, A and C). Within this region, MENTHO sequence analysis predicted the presence of four transmembrane helices implicating amino acids 54-142, and 152-171, respectively (Fig. 2, A and C). In contrast, the presence of four transmembrane helices implies that the amino and the carboxyl termini of the protein are on the same side of the membrane. The highly conserved region by comparison with sequences available in databases showed that the amino-terminal half of MLN64 and MENTHO was designated as the MENTAL domain (Fig. 2, A and C).

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**Fig. 1.** cDNA and protein sequences of human MENTHO and expression analysis. A, nucleotides and amino acids are numbered on the right. Amino acids of the open reading frame are in the one-letter code. Underlined sequences correspond to Kozak sequences and to the polyadenylation sequence. The sequence of the synthetic peptide used to generate the pAb-MENTHO-Ct antibody is boxed. This sequence has been deposited to the GenBank™/EMBL nucleotide sequence data base under accession number AJ492267. B, total RNA (10 μg) extracted from normal human breast (NB, lane 1), fibroadenoma (FA, lane 2), primary breast tumors grades I, II, and III (lanes 3 and 4, 5 and 6, and 7 and 8) breast cancer-derived metastatic lymph node (lane 9), colon cancer (lane 10), normal mammary epithelial cells (NMEC, lane 11), and NPC-2-deficient human fibroblasts (NPC-2, lane 12) were loaded, transferred, and successively hybridized with 32P-labeled probes specific for MENTHO and GAPDH as RNA loading control. The approximate size of each transcript is indicated on the left in kilobases.

bran was blocked in PBS containing 0.1% Tween 20 (PBST) and 3% nonfat dried milk and washed and incubated with anti-MENTHO antibody in PBST. After washing, the blot was incubated with horseradish peroxidase-conjugated AffiniPure goat anti-rabbit secondary antibody (Jackson Immunoresearch Laboratories, Inc.). Protein-antibody complexes were visualized by an enhanced chemiluminescence detection system (SuperSignal West Pico; Pierce).

**RESULTS**

Cloning of MENTHO—Searches for MLN64 homologous sequences in the human genome sequence DNA data base identified a human potential cDNA encoding for a protein referred to as AAD15552 (GenBank™ accession no. AC006033). Besides its homology with the amino-terminal half of MLN64, no known protein domain or biological function was proposed for this potential protein. Using this potential cDNA sequence, we designed two 21 bp oligonucleotides as primers to amplify for this potential protein. Using this potential cDNA sequence, no known protein domain or biological function was proposed for this potential protein.
MEMENTHO is conserved in mammals and fish. Expressed Sequence Tags (EST) from different species including mouse, rat, cow and zebrafish showed at the nucleotide level an average score of 90% homology to the human MEMENTHO sequence. The murine MEMENTHO protein sequence (shown in Fig. 2) has been deduced from a compilation and translation of 3 overlapping murine EST sequences (GenBank™ accession numbers: BG084215, BC003334, AA261427). The protein is well conserved since human and mouse MEMENTHO proteins were shown to have an overall score of 97% identity and 99% similarity.

We studied MEMENTHO expression in different normal, benign, and malignant tumor tissues. MEMENTHO mRNA was expressed at a basal level in normal tissues (placenta, lung, lymph node, and colon, not shown), in normal breast tissue (Fig. 1B, lane 1), in normal breast epithelial cells (Fig. 1B, lane 11) and in NPC2-deficient human fibroblasts. A similar level of expression was detected in benign, malignant and metastatic tumors (Fig. 1B, lane 10 and not shown). Consistent with this wide tissue distribution, human ESTs corresponding to the MEMENTHO gene were found in many tissues including bone, brain, breast, heart, kidney, liver, lung, muscle, and ovary.

**MEMENTHO Has an Alternative Translation Initiation**—We cloned the cDNA of MEMENTHO in a vector allowing the expression of the protein under its translation initiation and termination control sequences (pSG5-MEMENTHO). Protein extracts of COS cells transfected with the pSG5-MEMENTHO or with the empty vector were analyzed by Western blot (Fig. 3A). To study the MEMENTHO protein, we have generated a rabbit polyclonal antibody, named pAbMEMENTHO-Ct, directed against a synthetic peptide corresponding to the carboxyl-terminal end of MEMENTHO (residues 216–234). Using pAbMEMENTHO-Ct antibody, control COS cells transfected with the empty vector showed no signal while in COS cells transfected with pSG5-MEMENTHO 4 bands of different molecular weights were detected (Fig. 3A, lanes 2 and 3).

As MEMENTHO possesses two potential favorable initiation codons in the same open reading frame, we tested whether both were functional and whether we could assign some of the distinct immunoreactive bands to a specific isoform. In the pSG5-MEMENTHO expression vector, the first ATG (methionine 1) and the second ATG (methionine 8) codons were mutated into a unique start codon (Fig. 3B, lanes 2–9). Using pAbMEMENTHO-Ct antibody, a specific isoform was detected in COS cells transfected with the pSG5-MEMENTHO expression vector as a doublet with a lower molecular weight than the control COS cells transfected with the empty vector. This result indicated that the second ATG codon (methionine 8) was not functional. Similarly, a doublet was obtained with COS cells transfected with pSG5-MEMENTHOΔM1 and pSG5-MEMENTHOΔM8. Synthetic mutant proteins MEMENTHOΔM1 and MEMENTHOΔM8. Synthesis of the mutant proteins in transfected COS cells was tested by Western blot. MEMENTHOΔM1 mutation resulted in the synthesis of two immunoreactive bands of high molecular weight (Fig. 3B, lane 2). Similarly, MEMENTHOΔM8 mutation resulted in the synthesis of two immunoreactive bands of high molecular weight (Fig. 3B, lane 3). These data showed that, in COS cells, the two first initiation codons are functional and that two protein isoforms can be synthesized. These isoforms of 234 and 227 amino acids were named α and β, respectively. In addition, the level of protein translated from both sites is rather similar. We have observed that the resulting protein isoforms appear as doublets indicating the presence of a post-translational modification of both proteins.
The first initiation codon of MENTHO is a weak Kozak sequence, the nucleotide in +4 position being an adenine (Fig. 3C). In contrast the methionine 8 of MENTHO is a strong Kozak sequence. It is likely that the poor recognition of the first initiation codon allows the anchorage of the ribosome onto the second AUG of the mRNA. In order to verify this hypothesis, we have modified the first Kozak motif into a strong Kozak motif by mutating the adenine +4 into a guanine (mutant MENTHO SK). Transfection of MENTHO SK in COS cells resulted in the synthesis of only two immunoreactive bands corresponding to the α isoform (Fig. 3B, lane 4). A slight increase of the molecular weight of these two forms was observed. In this construct the second amino acid, a glutamine was replaced by an aspartic acid residue. The two ATG sequences are underlined (Fig. 3B, lane 3) and in COS cells transfected either with an empty vector (lane 2) or with an expression vector encoding for MENTHO (lane 3). B, Western blot analysis of COS cells transfected with an expression vector encoding wild type MENTHO (lane 1), or MENTHO ΔM1 (lane 2), MENTHO ΔM8 (lane 3), and MENTHO SK (lane 4) mutants. C, sequence context of the two first ATG codons. The consensus Kozak sequence required for an efficient translation initiation is indicated at the bottom of the figure. Important nucleotides are printed in bold characters. The two ATG sequences are underlined. Note that the first methionine Kozak sequence is weak while the second methionine has a strong Kozak sequence.

The first initiation codon of MENTHO is a weak Kozak sequence, the nucleotide in +4 position being an adenine (Fig. 3C). In contrast the methionine 8 of MENTHO is a strong Kozak sequence. It is likely that the poor recognition of the first initiation codon allows the anchorage of the ribosome onto the second AUG of the mRNA. In order to verify this hypothesis, we have modified the first Kozak motif into a strong Kozak motif by mutating the adenine +4 into a guanine (mutant MENTHO SK). Transfection of MENTHO SK in COS cells resulted in the synthesis of only two immunoreactive bands corresponding to the α isoform (Fig. 3B, lane 4). A slight increase of the molecular weight of these two forms was observed. In this construct the second amino acid, a glutamine was replaced by an aspartic acid residue and the modification of the nature of the second amino acid of the protein, which is now an acidic residue can explain the observed increased molecular weight of the α isoform. Indeed, acidic residues in proteins induce a retarded migration that has been attributed to the poor binding of SDS to the acidic region (19).

In conclusion, the MENTHO cDNA possesses two functional translation initiation sites leading to the synthesis of the MENTHO α (234 amino acids) and MENTHO β (227 amino acids) isoforms. Each isoform appears as several immunoreactive bands suggesting the presence of additional post-translational modifications.

**MENTHO Is a Phosphoprotein**—The analysis of the protein sequence of MENTHO revealed that it possesses numerous serine that can be phosphorylated by protein kinases (Table 1). The treatment with phosphatase of protein extracts of COS cells transfected with an expression vector encoding for wild type MENTHO led to the disappearance of some of the immunoreactive bands on the behalf of others (Fig. 4, lanes 1 and 2). To discriminate between the different isoforms, protein extracts of COS cells expressing the α (MENTHO ΔM8 and MENTHO SK) and β (MENTHO ΔM1) isoforms were dephosphorylated. For each construct, phosphatase treatments of the protein extracts led to the disappearance of the highest molecular weight form and to the intensification of the lowest molecular weight band (Fig. 4A, lanes 3–8). These data showed that the different immunoreactive bands detected in cell synthesizing either the α or the β isoform correspond to phosphorylated and unphosphorylated proteins.

Endogenous MENTHO protein was also detected in non-transfected HEK293 cells. Protein extracts from these cells were analyzed by Western blot using pAbMENTHO-Ct antibody. In these cells two immunoreactive bands were detected. The upper band having a molecular weight corresponding to the α isoform was predominant (Fig. 4A, lane 1). This protein extract was treated with phosphatase prior to Western blot analysis. Phosphatase treatment led to a complete shift to a lower molecular weight of the two immunoreactive bands (Fig. 4, lanes 9 and 10). These results indicate that HEK293 cells synthesize mostly phosphorylated MENTHO protein. Moreover, despite the presence of a weak consensus Kozak sequence, the MENTHO α form is mostly synthesized in these cells (Fig. 4A, lane 1 and Fig. 4, lane 9). Finally, MENTHO α and MENTHO β can be phosphorylated. The main isoform synthesized in HEK293 cells is the phosphorylated MENTHO α.

**MENTHO Is an Endosomal Protein**—The subcellular localization of MENTHO protein was assessed by indirect immunofluorescence experiments using CHO or HeLa cells transiently transfected with MENTHO-expressing vectors. Transfected CHO and HeLa cells showed a punctate cytoplasmic staining using pAbMENTHO-Ct antibody (Fig. 5A, a and j), whereas no staining could be detected in non-transfected cells (data not shown). CHO cells were co-transfected with MENTHO- and MLN64-expressing vectors. Detection of MENTHO and MLN64 using specific antibodies in transfected cells showed that both signals completely overlapped (Fig. 5A, a–c). Double staining experiments using MENTHO and the endogenous lipg95 protein (20), a marker of both endosomes and lysosomes showed overlap of both signals (Fig. 5A, d–f). CHO cells were transfected with the pGFP-MENTHO expression vector and then incubated with lystotracker red, a membrane-diffusible probe accumulating in acidic organelles. GFP-MENTHO fusion protein signal partially co-localized with lystotracker positive vesicles, the vast majority of GFP-MENTHO positive vesicles contain also MENTHO.

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**Table 1. Putative phosphorylation sites of human MENTHO protein**

| Amino acid | Position | Protein kinase |
|------------|----------|---------------|
| Serine     | 21       | Protein kinase C |
| Serine     | 96       | Casein kinase II |
| Serine     | 193      | Protein kinase C |
| Serine     | 210      | Casein kinase II |
| Serine     | 227      | Protein kinase C |
being negative for lysisotracker staining (Fig. 5A, g–i). Only a marginal proportion of MENTHO is localized in acidic lysosomal structures. Finally double staining experiments in HeLa cells transfected with MENTHO expression vector and the endogenous EEA1 protein, a marker of early endosomes (21), showed only a weak uniform signal (Fig. 5A, a–f) or GFP signal (Fig. 5A, g–i). MENTHO co-localized only rarely with lysisotracker-positive vesicles (Fig. 5A, i–l). In contrast, lysisotracker-positive vesicles (i) were shown in c, f, i, l, respectively. The yellow staining indicates co-localization. MENTHO co-localized with MLN64 (c) and with lgs95 (f). MENTHO co-localized only rarely with lysisotracker-positive vesicles (i) and EEA1 (l). Scale bar, 10 μm except for insets showing 2× higher magnification in a–f. All images were obtained using a confocal microscope and are representatives of transfected cells present in the majority of the fields.

**Fig. 5.** MENTHO is a late endosomal protein. CHO (a–i) and HeLa (j–l) cells were transfected with pSG5-MENTHO (a–f and j–l), fixed, permeabilized, and then incubated with pAGMANTEDHO-Ct antibody (a, d, and j), and anti-MLN64 (b), anti-lgp95 (e), or anti-EEA1 (k). CHO cells were transfected with the pGFP-MENTHO vector (g) and incubated with lysisotracker red (h) 1 h before observation. Overlays of a and b, d and e, g and h, and j and k together with nuclei counterstained with Hoechst-33258 dye (blue except for i) are shown in c, f, i, l, respectively. The yellow staining indicates co-localization. MENTHO co-localized with MLN64 (c) and with lgs95 (f). MENTHO co-localized only rarely with lysisotracker-positive vesicles (i) and EEA1 (l). Scale bar, 10 μm except for insets showing 2× higher magnification in a–f. All images were obtained using a confocal microscope and are representatives of transfected cells present in the majority of the fields.

**The Amino and the Carboxyl Termini of MENTHO Are Cytoplasmic.** We have previously shown that MLN64 amino- and carboxyl-terminal ends are cytoplasmic (8). The conservation between MENTHO and MLN64 suggested that MENTHO has a similar topology. To confirm this hypothesis, we performed endocytosis and cytoplasmic microinjection of antibodies directed against epitopes located at the amino or at the carboxyl terminus of the MENTHO protein. Antibodies directed against an endosomal luminal epitope can be taken up by living cells, permitting the visualization of the protein into endosomes. CHO cells were transfected either with a vector allowing the expression of an amino-terminal N-FLAG MENTHO fusion protein or with a carboxyl-terminal MENTHO C-FLAG fusion protein. To visualize transfected cells, these constructs were co-transfected with a vector allowing the expression of the GFP protein.

Living CHO cells synthesizing N-FLAG MENTHO were incubated with antibodies directed against the FLAG epitope. After fixation and detection with the secondary antibody no staining was observed (Fig. 6Aa). In contrast, the complementary experiment consisting of anti-FLAG antibodies microinjection into the cellular cytoplasm led to the detection of a punctate cytoplasmic signal (Fig. 6Ab, a and d). Thus, the amino terminus of MENTHO is cytoplasmic (Fig. 6C).

We next performed the same set of experiments with the MENTHO C-FLAG fusion protein expression vector. No staining was obtained when the anti-FLAG antibody was taken up by endocytosis by transfected CHO living cells (Fig. 6Ab), while cytoplasmic microinjection of anti-FLAG antibody led to the detection of a punctate cytoplasmic signal (Fig. 6B, e and h). Only a weak uniform staining background was present when anti-FLAG antibodies were microinjected into non-transfected CHO cells (Fig. 6B, i–l) or when anti-FLAG antibodies were neutralized with their cognate antigen prior to microinjection in transfected CHO cells (data not shown).

Taken together, these results show that the amino and the carboxyl extremities of MENTHO that correspond to a size of 45/53 and 65 amino acids, respectively, are projecting toward the cytoplasm (Fig. 6C).

**MENTHO Overexpression Does Not Correct Cholesterol Accumulation in NPC2 Patient Fibroblasts.** As late endosomes are involved in intracellular cholesterol transport, we studied...
MENTHO, lgp95, and Hoechst signals are shown in staining (b, no fluorescence with anti-FLAG antibodies (a). GFP expression vectors, incubated with LDL and processed for immunoblotting obtained in cells synthesizing low and high level of nuclear-GFP are green (c). Transfected with pSG5-MENTHO (a) and pGFP-Net-NLS (–, transfected cell (c), top cell). By filipin staining does not differ between un-transfected cell and vector (Fig. 7b, a and d). Double labeling experiments using MENTHO and the endosomal protein lgp95 specific antibodies further confirmed that the giants vesicles are endosomes since a complete overlap of both signals is observed (Fig. 7b, g and h). In conclusion, this phenotype is reminiscent of some aspects of the Niemann-Pick C phenotype and was observed when high levels of MENTHO protein were synthesized in transfected cells.

**DISCUSSION**

As an essential constituent of biological membranes and as a precursor for steroid hormones and bile acids, cholesterol is a major biological component. Cellular cholesterol can be obtained either by de novo synthesis using the acetyl-CoA pathway or by salvage through the LDL receptor pathway. The latter pathway involves vesicular transport, and endosomes and lysosomes play a critical role in this transport (9, 25). Indeed, mutations affecting genes involved in the endosomal/lysosomal traffic of cholesterol are responsible for the NPC autosomal recessive lipid storage disorders (22, 26). Causative genes involved in these disorders, NPC1 and NPC2, have recently been characterized and the study of their protein products has provided insights into the mechanism of intracellular cholesterol traffic (27, 28). Mutation of NPC1 or NPC2 impairs the transport of LDL-derived cholesterol leading to the accumulation of cholesterol in lysosomes while the transport of neo-synthesized cholesterol from the endoplasmic reticulum to the plasma membrane is not altered (Ref. 29 and references therein).

It is noteworthy that, as NPC1, MLN64 is a late endosomal membrane protein and, as NPC2, MLN64 is a cholesterol-binding protein. However, we have shown that MLN64 overexpression does not correct cholesterol accumulation in NPC-mutant fibroblasts suggesting that NPC1, NPC2, and MLN64 could function sequentially in a common pathway (8). To identify other molecules that may be involved in this pathway we have performed homology searches for MLN64-related proteins using the whole human genome data base. This allowed us to identify a potential gene coding for a protein homologous to the amino-terminal half of MLN64. We cloned the corresponding cDNA from a human fetal brain cDNA library and characterized the encoded protein that we named MENTHO for MLN64-MLN64 amino-terminal (MLN64 amino-terminal) domain.
The MENTHO cDNA encodes two isoforms of 234 or 227 amino acids, that we named MENTHO α and β, respectively. The first initiation codon of MENTHO is in a weak Kozak context while the second one is in a strong Kozak context (30). By mutagenesis experiments and transfection of MENTHO wild type or mutated vectors in COS cells, we have shown that an initiation scanning mechanism is responsible for the synthesis of the α and β isoforms. In addition, the presence of many consensus sites for phosphorylation in MENTHO primary sequence is responsible for the detection of the different molecular weight species in protein extracts. Study of endogenously synthesized MENTHO using untransfected HEK293 cells showed that these cells synthesized preferentially the phosphorylated α isofrom.

MENTHO subcellular localization was assessed by immunofluorescence using co-labeling experiments. MENTHO was found to co-localize with both MLN64 and lgp 95, two late endosomal proteins, demonstrating that it is a late endosomal-resident protein. We have previously shown that the amino-terminal half of MLN64 was responsible for the addressing of MLN64 to the late endosomes. Although this region does not contain conventional dileucine or tyrosine-based targeting signals, we have shown that a dileucine motif (Leu<sup>66-Leu<sup>67</sup></sup>) and a tyrosine residue (Tyr<sup>89</sup>) were critical for the targeting or the proper folding of the molecule (8). In good agreement with the co-localization of MENTHO with MLN64, both motifs are conserved in MENTHO. As for MLN64, MENTHO is located in the membrane of late endosomes. Although this region does not contain conventional dileucine or tyrosine-based targeting signals, we have shown that a dileucine motif (Leu<sup>66-Leu<sup>67</sup></sup>) and a tyrosine residue (Tyr<sup>89</sup>) were critical for the targeting or the proper folding of the molecule (8). In good agreement with the co-localization of MENTHO with MLN64, both motifs are conserved in MENTHO. As for MLN64, MENTHO is located in the membrane of late endosomes from where both its amino- and carboxyl-terminal extremities project toward the cytoplasm and may interact with other cytoplasmic proteins and/or membranes.

MENTHO, like the MLN64 gene, is well conserved in mammals, and displays an ubiquitous pattern of expression. In normal cells both genes are expressed at a low level. In contrast to MLN64, MENTHO is not overexpressed in breast cancers. This result can be explained by their different chromosomal localization. The gene coding for MLN64 is located on chromosome 17q11-12 a region commonly amplified in breast cancer (31). The first initiation codon of MENTHO is in a weak Kozak context while the second one is in a strong Kozak context (30). By mutagenesis experiments and transfection of MENTHO wild type or mutated vectors in COS cells, we have shown that an initiation scanning mechanism is responsible for the synthesis of the α and β isoforms. In addition, the presence of many consensus sites for phosphorylation in MENTHO primary sequence is responsible for the detection of the different molecular weight species in protein extracts. Study of endogenously synthesized MENTHO using untransfected HEK293 cells showed that these cells synthesized preferentially the phosphorylated α isofrom.

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