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TMEM74, a lysosome and autophagosome protein, regulates autophagy

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Abstract

Autophagy is an intracellular degradation/recycling process in eukaryotic cells. It contributes to the turnover of cellular components by delivering portions of the cytoplasm and organelles to lysosomes for digestion. The molecular mechanisms of autophagy and vesicle trafficking, especially the biogenesis and turnover of autophagosomes, are poorly understood. In this report, we describe the biological activity of a novel autophagy-related molecule, FLJ30668, or Transmembrane protein 74 (TMEM74). Its transcript was identified by Northern blot and the open reading frame was found to encode 393 amino acids, which shared very little identity with other genetic products. Subcellular localization analysis showed TMEM74 localized to the lysosome and autophagosome. Overexpression of TMEM74 in HeLa cells resulted in autophagic vacuolization, increased the dotted distribution of MDC and GFP-LC3, and endogenous LC3-II levels. Wortmannin, an autophagy inhibitor, partially attenuated these effects. Moreover, knockdown of TMEM74 by small interference RNA abolished the autophagic characteristics induced by starvation. These findings demonstrate that TMEM74 may be involved in promoting functional autophagy during cell starvation and other stress conditions.

Keywords: Autophagy; Lysosome; Autophagosome; TMEM74

Autophagy is the primary process by which cells degrade their own cytoplasm [1]. This process begins with the formation of an isolation membrane or cup-shaped membrane known as autophagophore [2]. The origin of the isolation membrane still remains unknown. The isolation membranes engulf a portion of the cytoplasm, including the cytoplasmic macromolecules and organelles, to form a double membrane vesicle known as an autophagosome [3,4]. The outer membrane layer of autophagosomes fuses with endosomes and/or lysosomes to form amphisomes and/or autolysosomes, respectively, [2] and monolayered autophagic vesicles were released into the acidic vacuolar lumen [5,6]. Within the autolysosome, cytoplasmic contents are degraded by lysosomal enzymes at a low pH [3]. The degradation products are transported back into the cytoplasm in response to cellular stress. Under starvation conditions, autophagic degradation of the cytoplasmic contents is enhanced in order to provide amino acids for essential metabolic processes like gluconeogenesis and ATP production [7].

Transmission electron microscopy is an important method used to demonstrate the occurrence of autophagy

Abbreviations: MDC, monodansylcadaverine; siRNA, small interference RNA; LC3, microtubule-associated protein 1 light chain 3; GFP, green fluorescent protein; FCS, fetal bovine serum; TEM, transmission electron microscopy; EST, expressed sequence tag; PCR, polymerase chain reaction; PI3K, phosphatidylinositol 3-kinase; PI, propidium iodide; MTR, MitoTracker Red; LTR, LysoTracker Red.

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[8]. Another morphological method used to identify autophagy is monodansylcadaverine (MDC) staining. As a tracer for autophagic vacuoles including autolysosomes [9], MDC staining intensity and the number of MDC dots correlate with autophagic activity during starvation [10]. Microtubule associated protein light 3 (MAP-LC3, LC3) is the mammalian homolog of the yeast Atg8/Apg8/Aut7, and the level of membrane-associated 16 kDa LC3-II increases, while the level of soluble 18 kDa LC3-I decreases during autophagy [11]. After induction of autophagy, LC3-I transforms into LC3-II and localizes from the cytosol to both the inner and outer autophagosome membranes. Thus, GFP-LC3 localization is a relatively simple method to detect autophagy [12].

By searching the human Refseq and expressed sequence tag (EST) databases, we obtained sequences from hundreds of novel human genes whose open reading frames (ORF) were longer than 300 base pairs. The ORFs of these genes were cloned into pcDNA3.1-myc/his (-) B vectors for screening analysis [13]. By cotransfecting GFP-LC3 and plasmids encoding functionally unknown genes, we found a novel human gene, *TMEM74*, which resulted in dotted GFP-LC3 distribution. RT-PCR analysis revealed specific expression of *TMEM74* in various human tissues and cell lines, and subcellular localization analysis showed that TMEM74 localized to both lysosome and autophagosome. Further studies indicated that TMEM74 overexpression induced functional autophagy in HeLa cells, suggesting that TMEM74 may be involved in this cellular process.

Materials and methods

Materials. Earle's balanced salt solution (EBSS), bafilomycin A1, monodansylcadaverine (MDC), and a monoclonal antibody against β-actin were obtained from Sigma (USA). cDNA libraries of cancer tissues were obtained from Shanghai Genomics, Inc. Antibodies against cathepsinD and MAP-LC3 were purchased from Santa Cruz (Santa Cruz Biotechnology, Inc.). LysoTracker Red was obtained from Molecular Probes (USA). ER-DsRed plasmid was obtained from Clontech (USA). Alexa Fluor 780-labeled IgG secondary antibody was obtained from Odyssey (Odyssey, Lincoln, NE). The GFP-LC3 plasmid was kindly provided by Professor Zhenyu Yue (Mount Sinai School of Medicine, New York, USA).

Cell lines and culture. HeLa cell was obtained from the American Type Culture Collection (Manassas, VA) and cultured (37 °C, 5% CO_2 humidified atmosphere) in RPMI 1640 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Hyclone, Logan, UT) and supplemented with 2 mM L-glutamine (Invitrogen). All other cell lines used for RT-PCR were also from the American Type Culture Collection. All experiments were performed on logarithmically growing cells. Cell autophagy was induced by EBSS.

Cloning, plasmid construction, and transfection. The full-length TMEM74 (NM_153015) cDNA was amplified from a human esophagus cDNA library (Clontech, USA) by PCR with the forward primer P1 (5′-tttctccgggaaccgtgc-3′) and reverse primer P2 (5′-ggcagactctcaagatattc acaacc-3′). The purified PCR product was ligated into the pGEM-T Easy vector (Promega, USA). The insert was released by EcoRI and subcloned into the EcoRI site of pcDNA.3.1/myc-His (-) B (Invitrogen, USA) to construct pcDB-TMEM74 plasmid. To construct TMEM74-GFP plasmid, we performed PCR with the forward primer P1 and the reverse primer P3 (5′-actgaattctactctgtacagcaagcgca-3′), and then fused the PCR product with a C-terminal GFP tag in the pEGFP-N1 vector (Clontech) by BamHI. All plasmids were confirmed by DNA sequencing. DNA transfection was performed by electroporation at a single 120 V, 20 ms pulse with 10 μg plasmid per 10^6 cells in 2 mm gap cuvettes using an ECM 830 square wave electroporation system (BTX, USA).

Northern blot and RT-PCR. The mRNA expression profiles of TMEM74 were analyzed by Northern blot and RT-PCR as described previously [14]. A 552-bp PCR product of TMEM74 and GAPDH cDNA were labeled with fluorescein using a Gene Images Random Prime Labeling Kit (Amersham Biosciences, Sweden) according to the manufacturer's protocol. Total RNA of human tissues and cell lines were extracted with the TRIzol reagent (Invitrogen, USA). RT-PCR was performed using the ThermoScript™ RT-PCR System (Invitrogen, USA) with primer P1 and P4 (5′-ttgggaccactcatttggattt-3′), and primers of GAPDH.

Fluorescence and confocal microscopy. Cells transfected with GFP-LC3 plasmid were observed for LC3 distribution under fluorescence microscopy. LC3 punctate distribution was assessed in five non-overlapping fields and statistical data were obtained from three independent experiments. Cells were grown on coverslips and acidic vacuoles were labeled with 0.05 mM MDC at 37 °C for 30 min. After incubation, cells were washed three times with PBS, fixed with 4% paraformaldehyde, and observed using a Leica SP2 Confocal System (Germany) equipped with an appropriate filter. Transiently transfected HeLa cells expressing TMEM74-GFP were cultured on coverslips, stained with 200 nM LysoTracker Red, and observed by fluorescence confocal microscopy.

Electron microscopy. For transmission electron microscopy (TEM), cells were fixed in 0.1 M sodium phosphate buffer containing 2.5% glutaraldehyde, pH 7.4. The cells were then fixed in 0.1 M sodium phosphate buffer containing 1% OsO_4, pH 7.2, for 2 h at 4 °C, and dehydrated in graded series of ethanol. Cells were embedded into Ultracut (Leica, Germany) and sliced into 60 nm sections. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined using a JEM-1230 transmission electron microscope (JEOL, Japan).

TMEM74 siRNAs synthesis and electroporation transfection. Specific siRNA against TMEM74 with the targeting sequence, 5′-GGAGGATG ATACAAGTTCA-3′, and non-silencing siRNA with the sequence, 5′-U UCUCCGAACGUGUCACGU-3′, were designed, chemically synthesized, and PAGE purified in the absence of RNase contamination according to the manufacturer's instructions (Genechem Corporation, Shanghai, China). The non-silencing siRNA sequence with no sequence homology to any known human gene was used as the control. All siRNAs were dissolved to 20 μM in buffer containing 20 mM KCl, 6 mM Hepes, pH 7.5, and 0.2 mM $MgCl_2$. and 6 μl of the indicated siRNA was added to 1×10^6 cells in 350 μl RPMI 1640, and transfected by electroporation as described above.

Results

Cloning, bioinformatics analysis and expression profile of human TMEM74

The human TMEM74 cDNA clone [15] was directly isolated from a human esophagus cDNA library and found to be 2087 base pairs long with an in-frame stop codon upstream of the putative ATG start codon. The sequence surrounding the ATG start codon largely followed the Kozak consensus rule. The open reading frame encoded 305 amino acids with a predicted molecular mass of 33.33 kDa and an isoelectric point of 4.87. The full-length cDNA and predicted amino acid sequence of TMEM74 are shown in Fig. 1A. Human *TMEM74* is located on chromosome 8q23.2 and encompasses two exons and one intron. Database analysis reveals that TMEM74 is conserved in humans, *Macaca mulatta*, *Bos taurus*, *Rattus norvegicus*,