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A novel role of the Batten disease gene CLN3: Association with BMP synthesis

Judith A. Hobert [a,*], Glyn Dawson [b,c,*]

[a] Committee on Molecular Metabolism and Nutrition, Biological Sciences Division, The University of Chicago, Chicago, IL, USA
[b] Department of Pediatrics, The University of Chicago, Chicago, IL, USA
[c] Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, IL, USA

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Abstract

Juvenile Neuronal Ceroid Lipofuscinosis (JNCL) results from a deficiency of CLN3, a protein recently identified within detergent-resistant membranes (DRMs). To study the function of CLN3 within these domains we isolated DRMs from control and JNCL-brain and noted that JNCL-derived DRMs are less buoyant than control. Analysis of DRM phospholipids derived from JNCL-brain revealed a reduction of bis(monoacylglycerol)phosphate. Metabolic labeling of JNCL-fibroblasts demonstrated a reduction in the synthesis of bis(monoacylglycerol)phosphate which was restored following complementation with wild-type-CLN3, substantiating our initial observation in brain. Metabolic labeling of cell lines overexpressing wild-type-CLN3 resulted in increased bis(monoacylglycerol)phosphate synthesis, while overexpression of mutant CLN3-L170P decreased bis(monoacylglycerol)phosphate synthesis. These data illustrate a new finding, a strong correlation between CLN3 protein expression and synthesis of bis(monoacylglycerol)phosphate.

Keywords: Neuronal Ceroid Lipofuscinosis; Batten disease; CLN3; Bis(monoacylglycerol)phosphate; Lysobisphosphatidic acid; Detergent-resistant microdomains

Deficiency of CLN3 protein is the underlying cause of Juvenile Neuronal Ceroid Lipofuscinosis (JNCL). This lysosomal storage disease typically manifests between the ages of 4 and 10 and is characterized by progressive vision loss, seizures and dementia. JNCL belongs to a group of disorders known as the Neuronal Ceroid Lipofuscinoses (NCLs) that share a common pathology of lysosomal accumulation of autofluorescent lipopigment and collectively are one of the most common causes of inherited childhood neurodegeneration. The genes associated with NCL encode a diverse group of both soluble and membrane bound proteins that reside in multiple cellular compartments. Single deficiencies in any of four soluble proteins, palmitoyl-protein thioesterase-1 [1], tripeptidyl peptidase-1 [2], cathepsin D [3] or cathepsin F [4], or of six membrane bound proteins, CLN3 [5], CLN5 [6], CLN6 [7], CLN8 [8], CLC-3 [9,10] or CLC-7 [11], have been associated with the development of NCL in either human or animal models.

CLN3 protein is highly hydrophobic and its expression has been detected in multiple human tissues including brain, pancreatic islets, peripheral nerve, spleen, and testis [12]. Topology prediction studies suggest that the CLN3 protein contains 5–6 transmembrane spanning domains [13], and detergent partitioning experiments revealed that it spontaneously integrates with cellular membranes [14]. Western blot analysis of lipid rafts, or detergent-resistant microdomains (DRMs), isolated from bovine brain demonstrated that CLN3 protein exists within these hydrophobic domains [15]. To further examine the role of CLN3 protein within DRMs we isolated DRMs from normal and JNCL-brain and noted that DRMs derived from

* Corresponding authors. Present Address: Genomic Medicine Institute, The Cleveland Clinic, 9500 Euclid Avenue NE5-216, Cleveland, OH 44195, USA. (J.A. Hobert). Fax: +1 2166360009 (J.A. Hobert), +1 7737029234 (G. Dawson).
E-mail addresses: hobertj@ccf.org (J.A. Hobert), dawg@uchicago.edu (G. Dawson).

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JNCL-brain were less buoyant than control. As the buoyancy of DRMs is directly related to their lipid composition, we analyzed the phospholipid composition of DRMs and total lipids isolated from CLN3-deficient human brain. In addition, we studied the ability of CLN3-deficient fibroblasts and LA-N-5 cell lines overexpressing wild-type or mutant CLN3-L170P protein to synthesize phospholipids. Our data demonstrate that expression of CLN3 protein is directly correlated with the synthesis of the phospholipid bis(monoacylglycerol)phosphate.

Materials and methods

Chemicals and reagents. [^{3}H]Palmitic acid (43 Ci/mmol) was purchased from New England Nuclear (Boston, MA). The protein assay kit was from Bio-Rad (Hercules, CA). Human autopsy brains HSB 635 (control, 15-year-old male, frontal cortex) and HSB 3187 (CLN3-deficient, 16-year-old male, frontal cortex) were obtained from Human Brain and Spinal Fluid Resource Center, Los Angeles California. CLN3-deficient fibroblasts were a kind gift of Dr. K.E. Wisniewski, Staten Island, New York. The neuron-like LA-N-5 cell line was obtained from Dr. J. Kanfer, University of Manitoba, Canada [16].

CLN3 expression plasmid construction and generation of stable cell lines. The complete wild-type-CLN3 cDNA was amplified by PCR using oligonucleotide primers 5′-ATGGGAGGCTGTGCA-3′ and 5′-TCAGGAGA GCTGGCAGAGGAA-3′ designed to allow for directional cloning into the p3XFLAG-CMV-14 vector multiple cloning site (Sigma) to produce a C-terminal FLAG CLN3. Likewise, complete WT CLN3 cDNA was amplified by PCR using oligonucleotide primers 5′-TTGAATTCGGCCA CCATGGGAGGCTGTGCA-3′ and 5′-TAAGGATCCTCAGGAGAG CTGGCAGAGG AA-3′ designed to allow for directional cloning into the p3XFLAG-CMV-9 vector multiple cloning site (Sigma) to produce a N-terminal FLAG CLN3. LA-N-5 cells were electroporated with 5 μg of either p3XFLAG-CMV-14-CLN3 or p3XFLAG-CMV-9-CLN3 expression vectors. Stable clones were selected in Dulbecco's modified Eagle's medium (DMEM) supplemented with neomycin (500 μg/mL), and individual clones isolated by limiting dilution. Clones were screened for CLN3 protein expression by western blot analysis using anti-FLAG antibody (Sigma) according to manufacturer's specifications.

The CLN3-L170P point mutant was generated using the megaprimer method [17]. Briefly, WT CLN3 cDNA was amplified by PCR using oligonucleotide primers 5′-ATGGGAGGCTGTGCA-3′ and 5′-AGGCAGT GAGGGAGGGGAAGG-3′ to generate a megaprimer containing the point mutation. This megaprimer was then gel purified and utilized in a subsequent PCR reaction with 5′-TCAGGAGAGCTGGCAGAGGAA-3′ to amplify the entire CLN3-L170P cDNA. The PCR product was cloned into pGEM-T (Invitrogen) and subsequently cloned into the p3XFLAG-CMV-14 expression vector. Stable cell lines were generated as described above.

All plasmid constructs were assessed for correct orientation by restriction digestion analysis, and all PCR-amplified DNA sequences were verified by DNA sequencing.

Cell culture and transient transfection. Fibroblasts and LA-N-5 cells were grown in monolayers on 100 mm tissue culture dishes in DMEM supplemented with 10% fetal bovine serum and 1% gentamicin. LA-N-5 cells were originally derived from a human adrenal gland tumor and resemble neurons [16]. The LA-N-5 cell lines expressing FLAG-tagged CLN3 and CLN3-L170P were grown in DMEM supplemented with 10% fetal bovine serum, 1% gentamicin and 500 μg/mL neomycin. All cells were maintained in a humidified incubator at 37 °C and 10% CO_2.

CLN3-deficient patient derived fibroblasts were transiently transfected with p3XFLAG-CMV-14 vector or the p3XFLAG-CMV-14 vector containing wild-type (WT) CLN3 cDNA. Surviving cells were allowed to adhere for 6 h at 37 °C, at which time media was removed, cells were gently washed with PBS, and used in experiments.

Sucrose gradient fractionation. Detergent-resistant microdomains, were isolated by their insolubility in Triton X-100 at 4 °C as previously described [18,19]. Briefly, cells and brain tissue were lysed in 1.5 mL of 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.5, 150 mM NaCl, 1% Triton X-100, supplemented with protease inhibitor cocktail (leupeptin, phenylmethylsulfonyl fluoride, and aprotinin) (Pierce) and incubated on ice for 1 h. The lysates were homogenized by 10–20 strokes in a loose-fit Dounce homogenizer. Homogenates were mixed with 1.5 mL of 80% sucrose in MBS (25 mM MES, pH 6.5, 150 mM NaCl) and overlaid with 3 mL of 30% sucrose in MBS followed by 3 mL of 5% sucrose in MBS. Homogenates were centrifuged for 18 h at 31,000 rpm in an SW41 swinging-bucket rotor and 1 mL fractions were collected from the top down for Western blot analysis. The DRM fraction was typically found in fractions 3 and 4.

Western blot analysis. Sucrose gradient fractions were separated on 12% SDS–PAGE gels, transferred to Immobilon-P membranes (Milli-Q) and blocked as directed by the antibody manufacturer. The Flotillin-2/ESA monoclonal antibody (BD Biosciences) was detected with horseradish peroxidase coupled anti-mouse secondary antibody and protein bands were detected by chemiluminescence.

To determine the relative expression of CLN3-FLAG protein in LA-N-5 cell lines whole cell lysates containing 20 μg of total protein were separated on 12% SDS–PAGE gels. Following electrophoresis, proteins were transferred to Immobilon-P membranes (Milli-Q) and blocked as directed by the antibody manufacturer. M2-antibody (Sigma) was detected using horseradish peroxidase-coupled anti-mouse secondary antibody protein bands were detected by chemiluminescence.

Lipid synthesis, extraction, and HPTLC. Cells were cultured for 18 h in media containing 1 mCi/mL [^{3}H]palmitate. Under these conditions, sphingolipids are labeled and palmitate is converted into acetate and recycled such that *de novo*-synthesized cholesterol is also labeled. Lipids were extracted as previously described [20]. Briefly, for total lipid analysis, cells were washed three times with phosphate buffered saline, pH 7.4 and collected, centrifuged, and the cell pellet resuspended in Milli-Q water. Following sonication, lipids were extracted according to Folch [20]. For analysis of DRM lipids, sucrose fraction four was dialyzed overnight against 5 L water at 4 °C to remove sucrose. A Folch extraction was then performed using the total volume after dialysis.

Lipids were analyzed by 2D-high performance thin layer chromatography (HPTLC) using 10 × 10 cm LHP-K TLC plates (Whatman, Inc.). Lipids from cell extracts were separated in the first dimension using chloroform:methanol:acetic acid:water (75:25:8.8:4.5 v/v) and second dimension using chloroform:methanol:ammonium hydroxide:water (92:36:2.8:3.1 v/v). TLC plates were sprayed with EN^{3}HANCE (Perkin-Elmer) to facilitate autoradiography and bands were excised for radioactivity determination by liquid scintillation counting.

Lipids from brain extracts were separated two times in the first dimension using chloroform:methanol:ammonium hydroxide (65:20:4 v/v) and second dimension using chloroform:acetone:methanol:acetic acid:water (50:20:10:10:5 v/v). Brain lipids were visualized by charring; HPTLC plates were sprayed with a solution of 10% $CuSO_4$, 8% H_3PO_4 and heated to 180 °C.

Individual lipids were unequivocally identified by the migration of authentic lipid standards in two independent solvent systems.

Lipid phosphorus assay. Lipid phosphorus assay was done as previously described [21].

Results

CLN3-deficient DRMs are less buoyant than control

Following centrifugation of sucrose gradients during the isolation of DRMs we visually observed that DRMs derived from JNCL-brain appeared to be more diffuse than control. To verify this observation, we assessed the localization of Flotillin-2 ESA, a known DRM protein marker.