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Secretion and function of Cln5 during the early stages of *Dictyostelium* development

Robert J. Huber*, Sabateeshan Mathavarajah

Department of Biology, Trent University, Peterborough, Ontario, Canada

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ABSTRACT

Mutations in *CLN5* cause neuronal ceroid lipofuscinosis (NCL), a currently untreatable neurodegenerative disorder commonly known as Batten disease. Several genetic models have been generated to study the function of CLN5, but one limitation has been the lack of a homolog in lower eukaryotic model systems. Our previous work revealed a homolog of CLN5 in the social amoeba *Dictyostelium discoideum*. We used a Cln5-GFP fusion protein to show that the protein is secreted and functions as a glycoside hydrolase in *Dictyostelium*. Importantly, we also revealed this to be the molecular function of human CLN5. In this study, we generated an antibody against Cln5 to show that the endogenous protein is secreted during the early stages of *Dictyostelium* development. Like human CLN5, the *Dictyostelium* homolog is glycosylated and requires this post-translational modification for secretion. Cln5 secretion bypasses the Golgi complex, and instead, occurs via an unconventional pathway linked to autophagy. Interestingly, we observed co-localization of Cln5 and GFP-Cln3 as well as increased secretion of Cln5 and Cln5-GFP in *cln3*$^{-}$ cells. Loss of Cln5 causes defects in adhesion and chemotaxis, which intriguingly, has also been reported for *Dictyostelium* cells lacking Cln3. Finally, autofluorescence was detected in *cln5*$^{-}$ cells, which is consistent with observations in mammalian systems. Together, our data support a function for Cln5 during the early stages of multicellular development, provide further evidence for the molecular networking of NCL proteins, and provide insight into the mechanisms that may underlie CLN5 function in humans.

1. Introduction

The neuronal ceroid lipofuscinoses (NCLs), commonly known as Batten disease, are a group of rare, currently incurable forms of neurodegeneration that affect all ages and ethnicities [1]. Clinical manifestations of the NCLs include intellectual impairment, progressive loss of vision and motor function, epileptic seizures, and a reduced lifespan [2]. The mechanisms underlying these devastating neurological disorders are poorly understood, mainly because the proteins linked to the NCLs are not well characterized. Mutations in any one of the 13 genetically distinct NCL genes (*CLN1-8*, *CLN10-14*) cause the accumulation of autofluorescent material within cells and results in nearly identical clinical phenotypes [3]. As a result, NCL proteins are thought to participate in shared or convergent biological pathways [4]. Thus, studying the function of any one NCL protein is highly likely to enhance our knowledge of the mechanisms underlying the neurodegenerative process; knowledge that can then be applied to all subtypes of the disease.

Mutations in ceroid lipofuscinosis neuronal protein 5 (*CLN5*) cause late-infantile, juvenile, and adult forms of Batten disease [5–9]. CLN5 has been studied using animal models such as mice, dogs, sheep, and cattle, as well as a variety of mammalian cell lines. Unfortunately, homologs of CLN5 are absent in many lower eukaryotes including budding and fission yeast (*S. cerevisiae* and *S. pombe*), the nematode *C. elegans*, and the fruit fly *D. melanogaster* [10]. Following translation, CLN5 is processed by members of the SPP/SPPL intramembrane protease family to form the mature CLN5 protein, which has been reported to localize to the lysosomal matrix and extracellularly [11–18]. The molecular function of CLN5 has been resolved as our previous work revealed that the protein functions as a glycoside hydrolase [18]. It is speculated that CLN5 may function in adhesion, apoptosis, autophagy, biometal and pH homeostasis, differentiation, migration, neurogenesis,

Abbreviations: Acb, acyl-coenzyme-binding protein; BFA, brefeldin A; *bsr*, blasticidin resistance; cAMP, cyclic adenosine monophosphate; CTS, cathepsin; CLN, ceroid lipofuscinosis neuronal; CM, conditioned media; CMF, conditioned media factor; CV, contractile vacuole; CQ, chloroquine; G418, geneticin; NCLs, neuronal ceroid lipofuscinoses; PFA, paraformaldehyde; PNGase F, peptide-N-glycosidase F; PPT1, palmitoyl-protein thioesterase 1; SM, Sussman Maurice; TPP1, tripeptidyl peptidase 1; WT, wild-type

* Corresponding author at: Trent University, Department of Biology, 1600 West Bank Drive, Peterborough, Ontario K9L 0G2, Canada.
E-mail address: roberthuber@trentu.ca (R.J. Huber).

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proliferation, protein transport, synaptic recycling, and the regulation of sphingolipid levels and transport [16, 19–27]. However, the mechanisms by which CLN5 may affect these processes are not known.

For over 80 years, the social amoeba *Dictyostelium discoideum* has served as an inexpensive and high-throughput model system for studying a variety of fundamental cellular and developmental processes, including adhesion, chemotaxis, and intracellular trafficking [28]. The *Dictyostelium* life cycle has both single-cell and multicellular phases. During growth, haploid cells feed on their bacterial food source. When cells are starved, they halt their growth and enter a multicellular developmental program that begins with chemotaxis and aggregation. Aggregated cells then form a multicellular slug. Finally, cells within the slug terminally differentiate to form a fruiting body composed of a slender stalk that supports a mass of viable spores.

Dictyostelium has emerged as a unique and powerful model system for studying a variety of human neurological disorders including prion diseases, lissencephaly, epilepsy, Huntington's disease, Alzheimer's disease, and Parkinson's disease [29–35]. The organism has also been used with great success to study the *Dictyostelium* homologs of human tripeptidyl peptidase 1/ceroid lipofuscinosis neuronal protein 2 (TPP1/CLN2), ceroid lipofuscinosis neuronal protein 3 (CLN3), and CLN5 [18, 36–40]. Our previous work used a Cln5-GFP fusion protein to reveal the localization, molecular function, and interactome of Cln5 in *Dictyostelium* [18]. Specifically, we showed that Cln5 is a glycoside hydrolase that localizes to the ER during growth and starvation prior to being secreted. Also, consistent with the molecular networking of NCL proteins in human cells, Cln5 interacts with other homologs of NCL proteins in *Dictyostelium* (e.g., Tpp1, CtsD, and CtsF) [18, 41–43].

In the current study, we used an antibody against Cln5 to show that the protein is glycosylated and secreted during the early stages of *Dictyostelium* development via an unconventional pathway that is linked to autophagy. Cln5 co-localizes with GFP-Cln3 at the contractile vacuole (CV) system and the secretion of Cln5 is linked to Cln3 function. *cln5*$^-$ cells display defects in adhesion and chemotaxis, which intriguingly, has also been observed in *Dictyostelium* cells lacking Cln3 [38]. In total, our study provides new insight into the localization and function of Cln5 in *Dictyostelium*.

2. Materials and methods

2.1. Cells, chemicals, and antibodies

Cells were grown and maintained at room temperature on SM (Sussman Maurice) agar with *Klebsiella aerogenes* [44, 45]. AX3 was used as the wild-type (WT) cell line. Cells grown axenically in HL5 were incubated at room temperature and 150 rpm. Cultures were supplemented with ampicillin (100 μg/ml) and streptomycin sulfate (300 μg/ml). Cell lines carrying the extrachromosomal vector pDM323 were maintained under selection with geneticin (G418, 10 μg/ml) [46]. Knockout cells carrying a blasticidin-resistance cassette were maintained under selection with blasticidin (10 μg/ml) [47]. Unless otherwise indicated, all cells used for experiments were harvested in the mid-log phase of growth (1–5 × 10^6 cells/ml). HL5 and low-fluorescence HL5 were purchased from Formedium (Hunstanton, Norfolk, UK). Mouse monoclonal anti-GFP and mouse monoclonal anti-β-actin were purchased from Santa Cruz Biotechnology Incorporated (Santa Cruz, California, USA). Rabbit polyclonal anti-CMF and rabbit polyclonal anti-countin were generously provided by Dr. Richard Gomer (Texas A &M University, College Station, Texas, USA). A polyclonal antibody against *Dictyostelium* Cln5 was raised in rabbits against a synthetic peptide corresponding to the epitope ^{231}CTEKPQPIDFTEHYD^{244}, which is located near the C-terminus of the protein (Genscript, Piscataway, New Jersey, USA). The antibody was affinity-purified prior to use in western blotting and immunofluorescence. HRP-conjugated and Alexa Fluor-conjugated secondary antibodies were purchased from Fisher Scientific Company (Ottawa, Ontario, Canada). Tunicamycin and brefeldin A (BFA) were purchased from Santa Cruz Biotechnology Incorporated (Santa Cruz, California, USA). Chloroquine was purchased from Sigma Aldrich Canada (Oakville, Ontario Canada). Ammonium chloride was purchased from Bioshop Canada Incorporated (Burlington, Ontario, Canada). Vectors were obtained from the Dicty Stock Center [48].

2.2. Creation of a cln5 knockout cell line

The c*ln5* gene in *Dictyostelium discoideum* was knocked out by homologous recombination using an approach that has been previously described [47]. Targeting arms were PCR amplified from gDNA using the AccuPrime Pfx DNA Polymerase (Fisher Scientific Company, Ottawa, Ontario, Canada) and the primers listed in Table S1. Targeting arms were then cloned into vector pLPBLP that contained a blasticidin resistance (*bsr*) cassette. The 5′ targeting arm incorporated *Kpn*I and *Hin*dIII sites to facilitate directional cloning into pLPBLP, upstream of the *bsr* cassette. The 3′ targeting arm incorporated *Pst*I and *Spe*I sites to facilitate directional cloning into pLPBLP, downstream of the *bsr* cassette. A MicroPulser electroporator (Bio-Rad Laboratories Limited, Mississauga, Ontario, Canada) was used to electroporate AX3 cells with 35 μg of linearized gene-targeting DNA. Cells were allowed to recover for 7 days in HL5 supplemented with ampicillin (100 μg/ml), streptomycin sulfate (300 μg/ml), and blasticidin (10 μg/ml). Clonal cell lines were isolated by plating transformants onto SM agar with *Klebsiella aerogenes*. Individual clones were isolated from distinct plaques with a pipette tip and then transferred to separate wells of a 12-well dish containing HL5 supplemented with ampicillin (100 μg/ml), streptomycin sulfate (300 μg/ml), and blasticidin (10 μg/ml). Clones were grown to confluency after which time the gDNA from each clone was isolated using the DNeasy Blood and Tissue Kit (Qiagen Incorporated, Toronto, Ontario, Canada). Targeted gene disruptions were validated by PCR using a series of primers (Table S2, Table S3, Fig. 1). Positive clones, as determined by PCR analysis, were further validated by RT-PCR to confirm the absence of the *cln5* transcript in these clones (Fig. 1). For this analysis, total RNA was extracted from pelleted cells using the RNeasy Plus Mini kit (Qiagen Incorporated, Toronto, Ontario, Canada). RT-PCR was performed using the OneTaq One-Step PCR kit (New England Biolabs Limited, Whitby, Ontario, Canada). One of these clones, hereafter referred to as *cln5*$^-$, was used in all experiments described in this paper. Restriction enzymes were purchased from New England Biolabs Limited (Whitby, Ontario, Canada). Amplicon insertion was validated by agarose gel electrophoresis and DNA sequencing (The Centre for Applied Genomics, Hospital for Sick Children, Toronto, Ontario, Canada).

2.3. Cell fixation, immunolocalization, and imaging

Cells (5 × 10^5 total) were deposited onto coverslips placed in separate wells of a 12-well dish and grown overnight in low-fluorescence HL5 at room temperature. Cells were fixed in either −80 °C methanol for 45 min or 4% paraformaldehyde (PFA) diluted in KK2 buffer (2.2 g/l KH_2PO_4, 0.7 g/l K_2HPO_4, pH 6.5) for 30 min [49]. Cells fixed in PFA were also incubated in −20 °C methanol for 5 min. The following primary and secondary antibodies were used for immunolocalization: anti-Cln5 (1:50), anti-rabbit Alexa Fluor 488 (1:100), and anti-rabbit Alexa Fluor 555 (1:100). Coverslips were mounted onto slides using Prolong Gold Anti-Fade Reagent with DAPI (Fisher Scientific Company, Ottawa, Ontario, Canada), and then sealed with nail polish. Fixed cells were viewed with a Leica DM6000B microscope equipped with a Leica DFC350FX digital camera (Concord, Ontario, Canada). Images were captured and viewed with Leica Application Suite Advanced Fluorescence version 2.4.1 build 6384. Images were merged with Fiji/ImageJ.