CHAPTER 3

Caenorhabditis elegans homologues of the CLN3 gene, mutated in Juvenile Neuronal Ceroid Lipofuscinosis (JNCL)

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Abstract

Neuronal ceroid lipofuscinoses (NCL) are the most common hereditary neurodegenerative disorders of childhood. The first symptom of this heterogeneous group of devastating lysosomal storage diseases is progressive visual failure. The different forms of NCL can be specified by age of onset, clinical features and characteristics of the accumulated materials. The juvenile form, Batten-Spielmeier-Vogt disease caused by mutations in the CLN3 gene, is the most frequent form of the disease in which loss of vision becomes apparent at the age 5 – 8 years. The gene was found to encode a novel integral membrane protein localising to the lysosomes, confirming that the primary defect in NCL is in lysosomal function. The CLN3 protein function is still unknown, and is examined in several model organisms. We are studying the nematode Caenorhabditis elegans, and identified three CLN3 homologues. In order to investigate the role of the CLN3 protein in C. elegans, CeCLN3 deletion mutants are being isolated from an ethyl methanesulphonate (EMS)-induced deletion mutant library. Examination of these mutants may provide us with information that will help dissecting the processes in which the CLN3 protein is involved. In this library two mutated C. elegans CLN3 loci have been identified, of which one mutant, NL748, was isolated. This mutant contains a deletion of the whole gene. The deletion mutant was characterised regarding the life span, and showed no prominent difference compared to wild-type.

Keywords: Neuronal ceroid lipofuscinosis – Batten disease – NCL – CLN3 – Caenorhabditis elegans
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Introduction

The neuronal ceroid lipofuscinoses form a group of lysosomal storage diseases with a devastating nature. This group of congenital children's disorders has an incidence of 1:12 500 births in Northern Europe and the USA, and 1:100 000 elsewhere. Clinical features of this diverse neurodegenerative disease are on-going ocular deterioration, epileptic fits, and gradual dementia. Ultimately the patients die in a vegetative state. On account of the differences among the disease-types, particularly starting age, one can discriminate between infantile, late infantile, juvenile, and adult NCL. A common feature of all forms of NCL is the accumulation of autofluorescent lipopigments in neurons and to a lesser extent in other cell types. The major accumulated component in most types of NCL, with the exception of the infantile form, was found to be Subunit c of the mitochondrial ATP synthase.

The most abundant form of NCL is the juvenile form, JNCL or Batten-Spielmeyer-Vogt disease. It has been linked to the CLN3 gene in which mutations, most often a 1.02 kb deletion, are causing the disease. The function of the CLN3 protein is still unknown, but it is situated in the lysosomes, and the Golgi apparatus, which supports the observation of failing lysosomal operation. So far, no direct link between the gene product and the accumulated Subunit c has been established.

To characterise the CLN3 protein, research was done in model organisms. Sequence comparison indicated the existence of CLN3 homologues in several organisms, including Mus musculus, Saccharomyces cerevisiae, and C. elegans. Natural mutants displaying NCL symptoms occur in a wide range of animal species. However none of these models are caused by mutations in the CLN3 gene, which should be the case for an ideal JNCL model. In order to study the function of normal and mutated CLN3 proteins two CLN3 knockout mouse models and one knockout yeast model have been developed. The murine models are expected to be of great use in the process of developing and testing new treatments, but may be too complex for functional studies. The knockout yeast model appears to be useful for investigations at the cellular level. On the other hand a yeast model can not provide any knowledge about the role of the CLN3 protein in a multicellular environment, e.g. in the nervous system, where the major defect in NCL patients is observed.

A relatively simple multicellular eukaryote is needed to fill the gap between these two model organisms, and to investigate the processes the CLN3 protein is involved in. The nematode C. elegans might fill this gap since it is a small, easy manageable organism with a distinct number of cells of known lineage and a completely described anatomy. Furthermore C. elegans has a nervous system, consisting of 302 neurons with 7600 mapped synaptic junctions. Probably the nematode will allow us to investigate the function of the CLN3 genes in different cell types, including the most affected cells in humans, the neurons. The availability of the CLN3 homologues, the nervous system, and the other advantages mentioned above make C. elegans a suitable model organism to research the neuronal ceroid lipofuscinoses.
Materials and Methods

Strains and growth conditions

We used Caenorhabditis elegans strains: wild-type Bristol N2, mutant mev-1 (kn1)\textsuperscript{15, 16}, kindly provided by Dr. N. Ishii, is a positive control on account of its increased accumulation of lipofuscin. CLN3 deletion mutant NL748 (pk479) which was derived from an N2 background. All nematodes were cultured at 20 °C as previously described\textsuperscript{17}.

Sequence comparison and alignment

The three C. elegans CLN3 homologues were identified by means of a Blastp search http://www.ncbi.nlm.nih.gov/BLAST/ using the human CLN3 protein sequence. The nematode’s CLN3 homologues were aligned to the human, Genbank Accession number U32680, canine L76281, murine U68064, rabbit U92812, Drosophila AE003522, and yeast Z49334 CLN3 protein sequences, using ClustalW v. 1.8 on the Baylor College of Medicine server http://gc.bcm.tmc.edu:8088/. Boxshade 3.21 was used to indicate conservation between species in the alignment http://www.ch.embnet.org/software/BOX_form.html. C. elegans CLN3 proteins were compared to each other concerning sequence identity and similarity.

Isolation of deletion mutants

A C. elegans ethyl methanesulphonate (EMS)-induced deletion mutant library was constructed by Jansen et al\textsuperscript{18}. The CLN3 deletion mutants were isolated by screening with nested PCR primers, and by using the protocol as previously described\textsuperscript{18}. Primers that are approximately 3 kb apart were designed from the genomic sequences of the CLN3 homologues. To isolate CeCLN3-1 deletion mutants, library pools were screened using a nested PCR. The first PCR was performed with primer pair: CeCLN3-1 F1 (5’ CGC GTT TCC AGT ATT CTC AG 3’) and CeCLN3-1 R1 (5’ CTG GAA CTA CGA ATT GAG GG 3’), this was nested with primer pair: CeCLN3-1 F2 (5’ TTC CAG AAG GAC AGT CTA GG 3’) and CeCLN3-1 R2 (5’ TAG ACA TGT CAA CGA GCT CC 3’). When after the PCR screening an aberrant band was detected, this band was cut out gel and sequenced using primer pair F2, and R2 and standard sequencing procedures. After isolation of a mutant subline, the mutation was transferred into a clean background by backcrossing six times with wild-type N2 to minimise the occurrence of mutations in other genes.
CeCLN3-2 deletion mutants were isolated by screening the library pools using primer pair: CeCLN3-2 F1 (5’ TTCTGCGAAAAATTGAATCCC 3’) and CeCLN3-2 R1 (5’ TTC GGG TTC GGT CAG TTA TC 3’), this was nested with primer pair: CeCLN3-2 F2 (5’AAT TCC AGA ATG GAT GTG GC 3’) and CeCLN3-2 R2 (5’ CAA CGG AGA TAT GGT TTC AAA G 3’).

Characterisation of longevity of C. elegans mutants

Hundred worms from pools of synchronously growing L1 wild-type N2, mutant NL748, and mutant mev-1 strains were plated individually on 5 cm petri-dishes, and were cultured under standard conditions at 20°C as described above. The nematodes were counted, and checked for movement almost daily, and placed onto a fresh dish in case any progeny appeared. If the worms were not moving, a tap on the head was used to examine their reflexes. When no movement was observed after a tap on the head, the worm was scored as being dead.
Results

Sequence comparison

The human CLN3 protein sequence was compared to *C. elegans* protein sequences using a Blastp search. Three matches were found, situated on cosmids F07B10 (Genbank Accession number Z77656), C01G8 (U80439), and ZC190 (AF078788). The genes have been named CeCLN3-1, CeCLN3-2, and CeCLN3-3 respectively, encoding putative proteins of 424, 435, and 375 amino acids respectively. Additional details about the nematodes CLN3 homologues can be seen in table 1. The protein sequences of CLN3-1 and CLN3-2 share 43% identity and 63% similarity, CLN3-1 and CLN3-3 49% identity and 62% similarity, and CLN3-2 and CLN3-3 show 37% identity and 52% similarity. Sequence analyses predict 9, 7, and 9 exons respectively for CeCLN3-1, CeCLN3-2, and CeCLN3-3 (Figure 1). To examine the sequences for conserved stretches an alignment was done with the amino acid sequences of human, canine, murine, rabbit, Drosophila, and yeast CLN3 proteins (Figure 2). Comparison of the CLN3 protein sequences indicates that the predicted proteins contain several conserved regions that may be important for CLN3 protein function.

<table>
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</tbody>
</table>

Figure 1 The genomic structure of the *C. elegans* CLN3 homologues CeCLN3-1, CeCLN3-2, and CeCLN3-3

The deletions are indicated by the thin line directly under the genes CeCLN3-1 and CeCLN3-2; the primers (F1, R1, F2, and R2) used to detect the deletion indicated by arrows. The exons are indicated by boxes and the introns are indicated by upside down V’s.
### Figure 2 Alignment of the sequences from human, canine, murine, rabbit, Drosophila, *C. elegans*, and yeast CLN3 proteins

To obtain maximum homology the Genefinder sequences of the genes, *C. elegans* and *Drosophila melanogaster* sequences have been adjusted with regard to reading frames. Conserved amino acid residues are shown in black boxes, neutral substitutions in grey boxes. The consensus sequence is indicated by asterisks when fully conserved, and by dots when more than 50% conserved between species.
Isolation of deletion mutants

In order to get more insight in gene functions CLN3 deletion mutants were isolated from an ethyl methanesulphonate (EMS)-induced deletion mutant library. The primary screening of this mutant library indicated that the library pools contain mutants with deletions in two out of three *C. elegans* CLN3 genes, CeCLN3-1, and CeCLN3-2. A mutant with a deletion in CeCLN3-1 was isolated from the mutant pools. This mutant was found to carry a 2.5 kb deletion (Figure 1), and has been designated NL748. Sequence analysis indicated that the region from 100 bp upstream of the start codon to 330 bp downstream of the stop codon is deleted. Therefore, the mutant NL748 carries a null allele (pk 479) of the CLN3 homologue CeCLN3-1.

Phenotypic characterisation of NL748

The NL748 mutants exhibit no obvious phenotypic alterations. Examination in closer detail at the behavioural and the cellular level is required to detect an aberrant phenotype. In a life-span experiment longevity of the N2 wild-type, the *mev-1* mutant, and the NL748 mutant nematodes was determined. The *mev-1* mutant strain is a positive control because it has been shown to age faster than the wild-type (N2) strain. Figure 3 was created by dividing the number of living worms at the moment of counting by the total number of worms at the beginning of the experiment. No dramatic difference in longevity has been observed between the N2 wild-type and the NL748 deletion mutant, whereas the *mev-1* mutant clearly exhibits a shorter life span.

![Figure 3 Life-span of N2 wild-type, NL748 deletion mutant, and mev-1 mutant](image)

The NL748 mutant strain shows no different longevity compared to the wild-type.
Discussion

Although Batten disease has been studied extensively, and the CLN3 gene was identified in 1995 no protein function has been established yet. The CLN3 protein is under survey in different eukaryotic species. In the mouse, targeted disruption of the CLN3 gene was used to create a model for JNCL. When these mice are homozygous for their CLN3 null alleles, they display a neuronal storage disorder with the same characteristics seen in Batten disease patients. For instance, the Cln3/- mice exhibit progressive accumulation of autofluorescent material in the neurons. In addition, inclusions were found that contained Subunit c of mitochondrial ATP-synthase. Although the mice show characteristic features of JNCL, they did not develop obvious clinical symptoms by 12 months of age. Furthermore, the naturally occurring mouse models for NCL display more severe cellular changes than the Cln3/- knock-out. Whereas additional research in the mouse model is necessary, another model organism, yeast, provides an abundance of information at the cellular level. In yeast a CLN3 orthologue, BTN1, was knocked out resulting in a viable mutant that is able to degrade mitochondrial ATP-synthase. The BTN1 protein localises to the vacuole, which functions as a lysosome, and regulates osmosis in the yeast cell. BTN1 knockout yeast are resistant to ANP (D-(-)-threo-2-amino-1-[p-nitrophenyl]-1,3-propanediol) since they have improved their ability to acidify the growth medium. In fact the pH of the mutant vacuole is lowered early in the growth phase, and this effect can be rescued by introducing the human CLN3 gene, indicating a connection between CLN3 and cellular pH regulation. Although yeast proves to be a suitable model for investigation at the cellular level, it unfortunately is a unicellular organism which makes it arduous to extrapolate the results provided by the experiments with this organism to the clinical picture of the disease. The nematode C. elegans is a multicellular very elaborately described organism, and has the advantages that its genome and anatomy of the nervous system have been well characterised. Which make it a more attractive model organism to study the functions of homologues of genes involved in human neurodegenerative disorders.

In C. elegans we identified three CLN3 homologous genes CeCLN3-1, CeCLN3-2, and CeCLN3-3, allowing functional studies at the protein level, and of the processes the protein is involved in. The CLN3 protein sequence alignment reveals several conserved areas present in all examined species, suggesting that they are essential for protein function. The different number of exons and the different predicted exon-intron boundaries of the CLN3 genes of C. elegans, point to ancient duplications in the nematode.

The next logical step is analysis of mutants. To do so, we analysed a C. elegans mutant library and identified deletion mutants for two of the genes. For CeCLN3-1 a mutant, NL748, was isolated. For CeCLN3-2 a mutant was localised in a mutant pool, and
The \textit{cln-3} genes of \textit{Caenorhabditis elegans}

will be isolated in the near future. Mutant NL748 did not show an obvious phenotype although the whole gene was deleted. This could be due to redundancy, because three CLN3 homologues are available. Another possibility may be that CeCLN3-1 is not a functional \textit{C. elegans} CLN3 gene but only a rudiment, and one of the other genes is functional. Also, CLN3 might prove to be a gene involved in stress situations, therefore the phenotype will be examined under different stress conditions. We will continue the phenotypic characterisation studies of the NL748 deletion mutants, focussing on sensory behaviour because we expect to find neurological defects since NCL mainly manifests itself in the neurons. Similar experiments will be performed for the other mutants as well as for double and triple mutants.

To study the disability of the mutants in closer detail it is necessary to know in which cells, and in what life stage the CeCLN3 genes are being transcribed. That is why CeCLN3 expression studies are being performed. These experiments should make cellular differences between wild-type and NL748 mutant easier to detect. We expect that analysis of \textit{C. elegans} CLN3 mutants will provide us with information about the processes in which the CLN3 proteins play a role, and will give further insight into the functions of the CLN3 protein and in the mechanism of neurodegeneration in NCL.

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References