CLN3L, a novel protein related to the Batten disease protein, is overexpressed in Cln3\textsuperscript{−/−} mice and in Batten disease

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Summary

Batten disease is a severe autosomal recessive neurodegenerative disease which results from mutations in CLN3. Although the gene was cloned in 1995, the tissue distribution and subcellular localization of the CLN3 protein (CLN3P) remains inconclusive. We have demonstrated the presence of a novel 33 kDa protein in both normal human and wild-type mouse brain. This 33 kDa protein, which is overexpressed in brains of patients with Batten disease and in Cln3\textsuperscript{−/−} mouse brain, binds to the antibody raised against the peptide sequence of CLN3P and results in aberrant CLN3P localization studies. We expressed a novel 33 kDa protein that is highly similar to CLN3P. We showed that the 33 kDa protein is identical to that recognized in Batten disease and Cln3\textsuperscript{−/−} brain. These studies strongly suggest the presence of an alternative CLN3-like (CLN3L) product in Batten disease. Previous studies of CLN3P tissue distribution and intracellular localization will require extensive reanalysis in order to determine the true expression of CLN3P.

Keywords: Batten disease; CLN3 protein; Cln3 gene; cln3\textsuperscript{−/−} mice; neurodegenerative disease

Abbreviations: CLN3P = CLN3 protein; CLN3L = CLN3-like protein; NCL = neuronal ceroid-lipofuscinoses; JNCL = juvenile form of neuronal ceroid-lipofuscinoses; ORF = open reading frame


Introduction

The neuronal ceroid-lipofuscinoses (NCLs) are the most common group of autosomal recessive inherited neurodegenerative disorders of childhood, with an overall incidence of up to 1 in 12,500 (Santavuori, 1988). The clinical courses are progressive and characterized by loss of vision, seizures, loss of motor function, and behavioural alterations leading to a vegetative state and early death (Rapola, 1993). The juvenile form of NCL (JNCL; Batten or Spielmeyer-Vogt-Sjogren disease) is known as Batten disease and results from mutations in CLN3 (International Batten Disease Consortium, 1995). CLN3 encoding a 438 amino acid protein (CLN3P), a putative membrane protein predicted to have 5–11 transmembrane domains (Persaud-Sawin et al., 2002) with an estimated molecular weight of 48 kDa. A 1.02 kb deletion comprising exons 7 and 8 results in the removal of nucleotides 461–677 of the CLN3 coding region and accounts for the majority of alleles from patients with Batten disease. In addition, 33 mutations and three polymorphisms have been identified so far in Batten disease patients (Munroe et al., 1997; http://www.ucl.ac.uk/ncl/cln3.html).

Localization studies using northern analysis demonstrated the ubiquitous presence of CLN3 mRNA, with very low expression in the brain (International Batten Disease Consortium, 1995). Immunohistochemical studies show the presence of CLN3P in various organelles (Katz et al., 1997; Margraf et al., 1999). Immunological studies of subcellular fractions have shown that in non-neuronal cells CLN3P localizes to late endosomes and lysosomes (Jarvela et al., 1999; Haskell et al., 2000). In neuronal cell types co-localization of CLN3P to synaptic vesicles as well as to late endosomes and lysosomes has been observed (Dawson and Cho, 2000; Haskell et al., 2000). In a conflicting report CLN3P was localized to...
synaptosomes but not to synaptic vesicles, suggesting that CLN3P may be involved in neurotransmission (Luiro et al., 2001).

The Cln3 knock-out mouse model was developed using targeted gene disruption in order to establish an animal model that mimics the features of Batten disease (Mitchison et al., 1999). Cln3−/− mice showed neuronal storage material similar to that seen in Batten disease patients. However, despite the cellular pathology that developed in Cln3−/− mice, they did not show obvious clinical symptoms even by 12 months of age. This was attributed to the longer time interval needed for symptoms to appear in the murine lifespan. It is not clear whether the pathology present in these mice is sufficient to cause clinical symptoms. Similar results were reported in Cln33/7/8 knock-in mice, a genetic change identical to the one seen in most Batten disease alleles. However, Cln33/7/8 mice displayed degenerative changes in the retina, cortex and cerebellum, with neurological deficits and premature death (Cotman et al., 2002). Neurological changes in homozygous mice were detected as early as embryonic day 19.5, especially in the cerebellum and hippocampus. Similarly, the related mnd−/− mouse, a model for CLN8, developed clear severe neurodegeneration by the age of 9 months (Messer et al., 1992). The nclf−/− mouse, a murine model of CLN6, is also symptomatic (Bronson et al., 1998).

The literature regarding CLN3P expression by western blot analysis is confusing. Most groups have reported the presence of multiple bands with molecular weights ranging from 30 kDa in a study that examined expression of CLN3P (Golabeck et al., 1999) to 90 kDa in a study that indicated the presence of splice variants of Cln3 (Oswald et al., 1999). CLN3P also has multiple potential post-translation modification sites, which may account for some of the mass differences (Michalewski et al., 1999).

In our ongoing studies we are attempting to study the functions of CLN3P in order to define its potential role in neurotransmitter release. With this aim, we have raised three epitope-specific polyclonal antibodies (Margraf et al., 1999). In routine western blot analysis of human and mouse tissues, three proteins of 33, 66 and 90 kDa are detected (using all three different antibodies), a pattern that is in agreement with some other reports (Oswald et al., 1999).

Recently, a cDNA clone derived from a full-length mRNA preparation from human cerebellum, which closely resembles Cln3, was identified (Gif# 21748923: AK090709, 3081 bp). In addition, a genome-wide blast search provided additional clues indicating the presence of a repeat sequence in the chromosome 16, upstream of the Cln3 (Loftus et al., 1999; termed ‘duplicons’ by the authors).

In the present study, analysis of the full-length mRNA from the clone AK090709 indicated an open reading frame (ORF) that codes for a 33 kDa protein, which we have designated CLN3-like protein (CLN3L; accession number TPA: BK0001540). Our studies show that this novel protein is similar to CLN3P and may explain the discordant results, such as conflicting subcellular localization, reports seen in NCL studies (Katz et al., 1997; Jarvela et al., 1999; Margraf et al., 1999; Haskell et al., 2000).

**Methods**

**cDNA analysis and expression clone construction**

CDNA AK090709/FLJ33390/BRACE2006900 was obtained from National Biological Resource Center, NITE, Chiba, Japan (http://www.bio.nite.go.jp/nbrc/). BRACE2006900 is the clone derived as part of the human genome project and is generated using an oligo-capping method, containing the full-length mRNA. The gene insert (AK090709) in the cDNA was analysed for ORF using ORF finder software from the NCBI server (http://www.ncbi.nih.gov/orf/gorf/gorf.html). The largest ORF encoded a 316 amino acid polypeptide with a theoretical molecular weight of 33.5 kDa. The cDNA sequence was cloned into a pBAD/TOPO thiофusion plasmid (Invitrogen Life Technologies, Carlsbad, CA, USA) for expression studies. Protein expression was induced using various concentrations of arabinose ranging from 0.0002% to 20%.

**Northern analysis**

Northern analysis for the CLN3L mRNA was performed using Neverfail human northern blots from RNway (Seoul, Korea). A cDNA probe containing a DIG (digoxin)-labelled dUTP probe was generated using a DIG-labeling kit (Roche, USA). A plasmid containing the gene AK090709 was used as a template to generate the cDNA probe of 500 bp (forward primer 5'-TGGGAGCCCCCTTTTGGGA-3'; reverse 5'-ACAGGGTGGTCCCCACA-3'). Twenty-five nanograms of cDNA was used for hybridizations. Blots were hybridized in hybridization bags at 42°C with constant agitation at 50 r.p.m. Blots were washed several times using 2× standard saline citrate (SSC) at room temperature and washed with 0.5× SSC twice at 68°C for 30 min. Blots were developed using an alkaline phosphatase-labelled anti-DIG-antibody and chemiluminescence detection kit (Roche). Blots were exposed for 45 min to X-ray film and developed to visualize bands.

**RT-PCR for CLN3L and CLN3 mRNA**

Tissues were collected and stored in liquid nitrogen and total RNA was isolated from thawed tissues using Qiagen RNA extraction kit (Qiagen, Valencia, CA, USA). The RNA preparation was incubated with RNAase free DNase (Invitrogen, USA) to eliminate DNA contamination. The first-strand DNA was synthesized using oligo dT primers and the Superscript kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. ABL, the gene for tyrosine kinase, present universally, was used as housekeeper gene for monitoring the reverse transcription PCR (RT-PCR). The primers for ABL amplify exons 3 and 4 with approximate size of 100 bp. RT-PCR was performed separately for CLN3 and CLN3L using the primers spanning the entire length of the respective mRNAs. The PCR products were separated using 1% agarose gel electrophoresis and visualized using ethidium bromide. Primers for RT-PCR were as follows: ABL, forward 5'-TGATTGTGGCCAGTGGAG3' reverse 5'-CCATTTTGGTGGGGGCTTC-3'; CLN3L, forward 5'-TGGGAGCCCCCTTTTGGGA-3', reverse 5'-ACAGGGTGGTCCCCACA-3'; CLN3, forward 5'-GAACCCCTACCTCCCGGAGG-3', reverse 5'-GTGAACCCCTACCAAGAAGG-3'.

**Tissue**

Tissues were obtained from 4- and 16-month-old Cln3−/− mice and wild-type controls (Mitchison et al., 1999). The Cln5−/− and wild-type mice were of the 129S6/SvEv strain. The Batten disease brain tissue sample was a generous donation from the Human Brain and Spinal
Fluid Resource Center, Los Angeles, CA, USA. Normal human brain samples were purchased from the National Neurological Research Specimen Bank, Vame, Los Angeles, CA, USA.

**Antibodies**

CLN3P antibodies were produced as described (Margraf et al., 1999). We previously raised these antisera against three peptide fragments corresponding to amino acids 2–18, 64–76 and 250–264 of CLN3P. In the present study we used only the antisera raised against peptide fragment 250–264, referred to as Q438. CLN3P-specific (CLN3*) antiserum was raised against amino acids 321–340 (WYQMLY-QAGV FASRSSLRCC) and CLN3L-specific antibody was raised against amino acids 290–310 (ACSPGPPLI SSAFSRVCCG) (SynPep, Dublin, CA, USA).

The antiserum was purified using Sulfolink columns according to the manufacturer’s instructions (Pierce Biotechnology, Rockford, IL, USA). The fractions containing protein were dialysed overnight at 4°C in phosphate-buffered saline.

**Western blots**

The western blots were performed on samples containing 10 μg of protein each. 4–20% linear gradient precast gels (Biorad Laboratories, Hercules, CA, USA) were used for electrophoresis. They were then transferred onto nitrocellulose membranes at a constant voltage (100 V for 1 h). The membranes were incubated in blocking buffer (5% membrane blocking agent in 1 × TBS-T (Tris-Buffered Saline with Tween 20); Amersham Biosciences, Piscatoway, NJ, USA) overnight at 4°C in or 1% gelatin in 1 × TBS-T. The next day, the membranes were incubated with the CLN3P primary antibody at concentration of 5 μg/ml (TBS-T containing 1% blocking reagent) at 4°C overnight. Immunoreactivity was visualized with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (Amersham Pharmacia Biotech, Piscatoway, NJ, USA and Pierce Biotechnology, Rockford, IL, USA), followed by enhanced chemiluminescence detection (Amersham Pharmacia Biotech, Piscatoway, NJ, USA).

**Immunocytochemistry**

The tissues were fixed and processed as previously described (Boriack and Bennett, 2001). The primary CLN3P antibody incubation was done overnight at 4°C at concentrations ranging from 5.0 to 10.0 μg/ml. The negative control was replaced by pre-immune rabbit serum and/or negative control rabbit immunoglobulin normal fraction (Dako Laboratories, Carpenteria, CA, USA).

**Morphological and immunostaining intensity analysis**

The immunohistochemical reactivity intensity was graded under a light microscope (1+ to 3+).

**Results**

**Immunohistochemistry shows the presence of CLN3 protein in Cln3*–/– tissue sections**

Immunohistochemistry using antibody Q438 (directed towards amino acids 250–264 of CLN3P) showed the presence of CLN3P reactivity in various regions of the brain and testis from young (4 months) wild-type and Cln3*–/– mice (Fig. 1). It is evident from the figure that there is increased reactivity to the Q438 antibody raised against CLN3P in the Cln3*–/– mice. This is particularly clear in sections of the cerebellum, dentate gyrus, hippocampus and testis. The specificity of the antibody was confirmed by the elimination of activity by adding 1 μg/ml antigen peptide to the section during immunostaining.

**Western blot analysis shows the expression of CLN3L in both human and mouse tissues**

The western blots were developed with newly developed CLN3P-specific (CLN3*) and CLN3L antibodies. The antibodies used in the study bind specifically to human and mouse CLN3P and CLN3L with equal affinity. All the anti-CLN3P antibodies currently available react with equal affinity to both human and murine CLN3P due to the high degree (82%) of sequence identity (Lee et al., 1996). A 33 kDa protein was seen in brain homogenates from both the patient with Batten disease and from control human brain using the CLN3L-specific antibody, confirming that the 33 kDa protein is CLN3L (Fig. 2A). Further confirmation is demonstrated in Fig. 2A, which shows the absence of this protein when blots were developed using CLN3*. Brain homogenate from wild-type mice (young and old) demonstrated the presence of a 33 kDa protein of equal intensity, while the western blots for the Cln3*–/– mouse brain showed a prominent 33 kDa protein in both old (16–17 months)
and young (4 months) mice, with increased expression in the young Cln3–/– mice. The overexpression of the 33 kDa protein can be seen in the Batten brain tissue homogenates. The 33 kDa protein is completely effaced in a blot developed using CLN3* antibody. A 66 and 44 kDa protein can also be seen in these blots. Increased expression of 44 kDa protein can be seen in Batten’s disease brain, in both anti-CLN3L and anti-CLN3 immunoblots. (B) Western immunoblots using anti-CLN3L and anti-CLN3* in mouse brain. Western blot with wild-type or knock-out mouse brain homogenate were performed. The young mice were 4 months old and the old mice were 16–17 months old when they were killed. A 33 kDa protein is seen distinctly in the wild-type mouse brain homogenates; it is more intense in the knock-out brain homogenates in the anti-CLN3L immunoblot and completely effaced in the CLN3* blot. A 66 and 44 kDa protein can also be seen in these blots. A 44 kDa protein is seen in the anti-CLN3L immunoblot and is clearly increased in Cln3–/– brain. A prominent 66 kDa protein can also be seen in both immunoblots.

Expression profile of CLN3L mRNA by northern analysis

Northern blot analysis was performed using a DIG-labelled cDNA probe amplified from the plasmid containing the full-length Cln3. The probe was homologous to both CLN3 and CLN3L mRNA. The results of replicate northern blots show an approximately 3.0 kb transcript from various human tissues. CLN3 mRNA (1.6 kb transcript) can be seen with similar tissue distribution. The northern blot clearly shows the expression of CLN3L and CLN3 mRNA in testis and brain. CLN3L and CLN3 mRNA expression could also be seen in other tissues, such as lung, spleen, heart, small intestine, ovary, breast, kidney, liver, uterus and placenta (Fig. 3). The transcript was not detectable by northern blotting in some regions of brain, including the thalamus and cerebellum, but was detectable by RT-PCR (data not shown). The mRNA expression of CLN3L has not been reported previously.

RT-PCR shows the presence of CLN3L mRNA in both Cln3–/– and wild-type mice

RT-PCR analysis showed the presence of CLN3L mRNA in testis, liver, brain and pancreas in wild-type as well as Cln3–/– mice (Fig. 4A). The expression of CLN3 mRNA was upregulated in Cln3–/– mouse tissues, notably in brain. CLN3 mRNA was not present in Cln3–/– mice (Fig. 4B). The expression profile of CLN3 mRNA was similar to the mRNA expression profile in human tissues reported previously (International Batten Disease Consortium, 1995). However, the smaller splice products described in a recent study (Cotman et al., 2002) were not seen in our studies. These RT-PCR studies confirm the presence of a novel variant of Cln3 or a related gene.

CLN3L is expressed as an insoluble protein in an E. coli expression system

The ORF from the clone AK090709 was cloned into the pBAD/TOPO expression kit (Invitrogen Life Technologies). The clone had an insert of full-length mRNA (3081 bp), the largest ORF coding for a 316 amino acid polypeptide with a molecular weight of 33 kDa. The mRNA contained a large 5’-untranslated region of 1354 bp and also a 3’-untranslated region of 700 bp. The ORF was amplified and cloned into the pBAD/TOPO plasmid.
expression system. The insert flanked by a 5’ thio redox gene and 3’ histidine tag for purification produced an insoluble protein product of ~50 kDa (the 5’ thio redox gene and 3’ histidine tag add approximately 16.5 kDa to the actual molecular weight of the expressed protein). The maximum response was seen with 0.002% arabinose. The identity of the protein was confirmed by western blot analysis using Q438 antibody. The sequence of the 316 amino acids aligned with CLN3P shows that the expressed protein had 1–284 amino acids identical to CLN3P, whereas amino acids 285–316 were unique to the CLN3L protein (Fig. 5).

Discussion

Our immunohistochemical and western blot analysis demonstrates the expression of a novel protein that is highly similar to CLN3P in wild-type and Cln3+/- mice. The Cln3+/- mouse was developed by targeted gene deletion of the first six exons of the Cln3. It is not uncommon to have a truncated or novel protein product that is formed in targeted gene deletion experiments (Joyner, 2001). Our present studies suggest that the Cln3+/- mice make a novel protein in the absence of CLN3P. It is possible that the new protein made in Cln3+/- mice is functionally active and compensates for the absence of CLN3P, resulting in less profound changes than those seen in mnd+/- and nefl+/- mice (Pardo et al., 1994; Bronson et al., 1998; Cooper et al., 1999; Mitchison et al., 1999). This hypothesis cannot be validated until the functions of the CLN3P and CLN3L proteins have been determined.

It has been 8 years since the isolation and cloning of Cln3 (International Batten Disease Consortium, 1995) and 4 years since the establishment of the murine model of Batten disease (Mitchison et al., 1999). No one group has been able to show convincingly the presence of CLN3P by western blot analysis or to purify CLN3P from the tissue sources. Although numerous studies have been published that have looked at the overexpression of Cln3, studies have in general failed to localize CLN3P unequivocally (Kida et al., 1999; Haskell et al., 2000).

The multiple protein variants reacting with CLN3P antibodies have been observed consistently by several researchers. Some groups believe that extensive post-translational modifications and proteolytic cleavage of CLN3P are responsible for the wide variations in molecular weight (Golabek et al., 1999). Others consider the presence of Cln3 splice variants at the 5’ end as reported in the original description of human genomic structure (International Batten Disease Consortium, 1995; Mitchison et al., 1997). Palmer and Colleagues have isolated four different splice products from sheep liver, placenta at the 3’ end (Oswald et al., 1999). Our data favour the presence of multiple splice variants and also support the existence of a possible homologue of Cln3. This explains the presence of products with lower molecular weights and also the presence of multiple mRNAs of varying lengths. It is possible that these CLN3P-like products are being transcribed from a homologous gene different from Cln3. Loftus et al. (1999) have described the presence of extensive repeat sequences on chromosome 16, where Cln3 resides. A 20 kb repeat of Cln3 has been identified very close to it. This chromosome-16 low-abundance repeat has been shown to be located at chromosome 16p11.2 and is 99.5% identical to Cln3 (Mitchison et al., 1997) located at chromosome 16p12.1. The concept of homologous gene and alternative splice products provides a clear explanation for all the results of multiple Cln3 products observed by many investigators. The Cln35/7/8 knock-in mouse model developed by MacDonald and colleagues shows a number of alternative splice variants with lower molecular weights (Cotman et al., 2002). The immunohistochemical analysis described in this study further provides evidence for our view that there are multiple splice variants. The two antibodies used in Cotman’s study have varying degrees of affinity for different splice variants, one antibody (Battenin1, similar to Q438) having broad specificity and the other (Battenin2 similar to CLN3*) having the highest specificity for CLN3. Increased intensity of staining was observed with Battenin1 and intensity was decreased with Battenin2 in brain sections of Cln35/7/8 knock-in mice (Cotman et al., 2002).

The RT-PCR studies confirm that the CLN3L mRNA is expressed in both normal and Cln3+/- mouse brain. RT-PCR data show that CLN3L mRNA expression is increased in Cln3+/- mouse tissues. Expression of CLN3L in our laboratory clearly demonstrates the presence of multiple CLN3-related products. Using antibodies against the more selective, unique
CLN3L-peptide sequence, we have shown the existence of a novel CLN3L protein. We have also demonstrated the presence of CLN3L in the brain and testis of both wild-type and Cln3–/– mice. CLN3L is overexpressed both in Cln3–/– mice and in Batten disease brain. These antibodies will allow us to further study interactions of CLN3P and CLN3L, the regulation of CLN3P and CLN3L in Cln3–/– mice, and their involvement in the pathogenesis of Batten disease.

We have been able to show the presence of a novel 33 kDa protein in Batten disease brain homogenates using specific antibodies. Our western blots using CLN3P confirm that the 33 kDa protein is in fact not CLN3P but CLN3L. These results indicate that the existing analyses of CLN3P localization and function need to be revisited. Based on our observations and those of others (Oswald et al., 1999), we assert that there are other alternative splice products of Cln3 that need to be characterized and purified.

Palmer and colleagues have suggested the presence of one species of Cln3 alternative splice product that may have pathophysiological significance in neurons (Oswald et al., 1999). Epitope-specific deletion experiments within motifs of CLN3P by Boustany and colleagues have suggested the presence of several important domains that modulate the growth of the cells and are also involved in the regulation of apoptosis (Persaud-Sawin et al., 2002). We believe that the 33 kDa CLN3L is a variant protein present in cells that have lost some of these functional epitopes. The novel protein, which is upregulated in the disease process, may be only partially functional.

We have also shown that CLN3L is a CLN3P-homologous protein that is present at low levels in the normal brain, with increased expression in human Batten disease brain and Cln3–/– mouse tissues. We have expressed a tagged CLN3L protein in E. coli which, when isolated from the insoluble fraction, has a molecular weight of approximately 33 kDa. A molecular modelling program (http://www.cbs.dtu.dk/services/TMHMM/) has predicted the presence of around six transmembrane domains and the model favours the intracellular orientation for both N-terminal and C-terminal ends. The structure of CLN3L seems to be similar to that of CLN3P using structure prediction programs. We believe that the search for CLN3-related proteins and their molecular partners will be facilitated by our finding of CLN3L.

In conclusion, we report the presence of a novel CLN3L protein in normal human brain which is overexpressed in human Batten disease patients and in the Cln3–/– mouse brain. The true localization of CLN3P can be determined only by using antibodies that are specific to CLN3P and do not cross-react with CLN3L. It is important to determine whether the absence of CLN3P in Batten disease results in a compensatory increase in CLN3L and whether the interactions of CLN3L are similar to those of CLN3P.

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