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Molecular background of progressive myoclonus epilepsy

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Research on human inherited diseases provides a powerful tool to identify an intrinsically important subset of genes vital to healthy functioning of the organism. Progressive myoclonus epilepsies (PMEs) are a group of rare inherited disorders characterized by the association of epilepsy, myoclonus and progressive neurological deterioration. Significant progress has been made in elucidating the molecular background of PMEs. Here, progress towards understanding the molecular pathogenesis of PMEs is reviewed using the most common single cause of PME, Unverricht–Lundborg disease, as an example. Mutations in the gene encoding cystatin B (CSTB), a cysteine protease inhibitor, are responsible for the primary defect in Unverricht–Lundborg disease. CSTB-deficient mice, produced by targeted disruption of the mouse *Cstb* gene, display a phenotype similar to the human disease, with progressive ataxia and myoclonic seizures. The mice show neuronal atrophy, apoptosis and gliosis as well as increased expression of apoptosis and glial activation genes. Although significant advances towards understanding the molecular basis of Unverricht–Lundborg disease have been achieved, the physiological function of CSTB and the molecular pathogenesis of the disease remain unknown.

Keywords: apoptosis/cystatin B/EPM1/ neurodegeneration/progressive myoclonus epilepsy

Introduction

Progressive myoclonus epilepsies (PMEs) are a heterogeneous group of inherited disorders defined by the association of myoclonus, epilepsy and progressive neurological deterioration (Berkovic *et al*., 1986; Marseille Consensus Group, 1990). Despite a common name, PMEs differ in clinical features, aetiology and pathogenesis. Five disease entities or disease groups, Unverricht–Lundborg disease, Lafora's disease, neuronal ceroid lipofuscinoses (NCLs), mitochondrial disorders and sialidoses, account for the majority of PME cases in the world. In addition, a number of quite rare disorders can cause the PME phenotype.

Recent advances in molecular genetics have significantly increased the understanding of the basic mechanisms involved in the PMEs. Positional cloning has been used successfully to identify genes underlying the major forms of PME (Table I). In addition, several genes for the more rare forms have been identified, e.g. the *DRPLA* gene underlying dentatorubral–pallidoluysian atrophy found predominantly in Japan (Koide *et al*., 1994; Nagafuchi *et al*., 1994). The research now aims at understanding the function of the proteins encoded by the PME genes as well as revealing the underlying disturbed metabolic pathways. In this review, progress towards these goals is described using Unverricht–Lundborg disease, the most common single cause of PME, as an example.

Unverricht–Lundborg disease (EPM1; OMIM254800) is an autosomal recessive inherited disorder characterized by onset at the age of 6–15 years, severe incapacitating stimulus-sensitive progressive myoclonus, tonic–clonic epileptic seizures and characteristic abnormalities in the electroencephalogram (EEG) (Koskiniemi *et al*., 1974a,b; Norio and Koskiniemi, 1979). EPM1 patients also develop other neurological symptoms such as ataxia, incoordination and dysarthria (Koskiniemi *et al*., 1974a; Norio and Koskiniemi, 1979). On histopathological examination of the brain, widespread non-specific degenerative changes, and loss of Purkinje cells, but no intracellular inclusions have been observed (Haltia *et al*., 1969; Koskiniemi *et al*., 1974a; Eldridge *et al*., 1983).

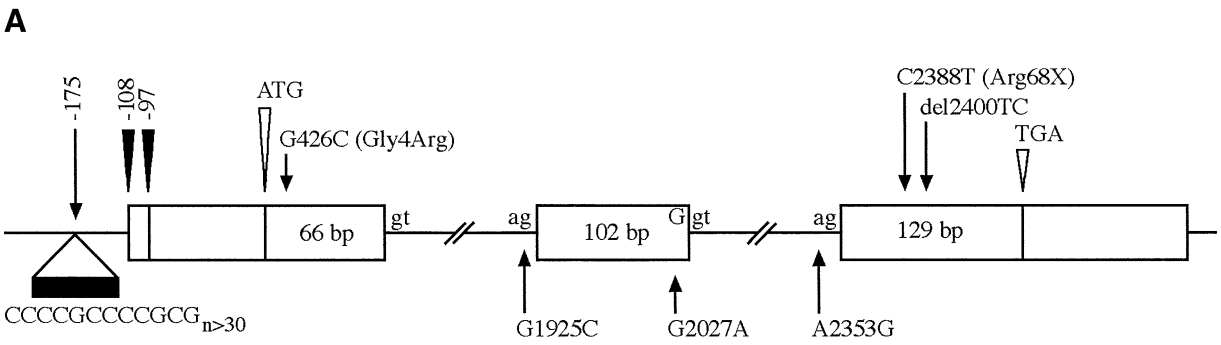
Molecular genetic basis of EPM1

The mutated gene on chromosome 21q22.3 responsible for EPM1 was identified using positional cloning (Lehesjoki *et al*., 1991, 1993; Pennacchio *et al*., 1996; Stone *et al*., 1996; Virtaneva *et al*., 1996). It encodes a previously described and characterized, but unmapped protein, cystatin B (CSTB), a cysteine protease inhibitor (Järvinen and Rinne, 1982; Ritonja *et al*., 1985). The *CSTB* gene is ubiquitously expressed with a transcript of ~0.8 kb in northern blot analysis (Pennacchio *et al*., 1996). Lalioti *et al*. (1997a) used an RNase protection assay to determine the transcription start site of *CSTB*, and found two sites, 97 bp and 108 bp upstream of the translation initiation codon and downstream of a dodecamer repeat unit (Figure 1). To date, seven *CSTB* gene mutations underlying EPM1 have been described (Pennacchio *et al*., 1996; Lafreniere *et al*., 1997; Lalioti *et al*., 1997a,b; Virtaneva *et al*., 1997; Kagitani-Shimono *et al*., 2002; Figure 1).

An unstable expansion of a dodecamer (12 bp) or minisatellite repeat unit of 5′-ccccgccccgcg-3′ located 175 bp upstream from the translation initiation codon in the putative promoter region of *CSTB* (Figure 1) is the most common EPM1-associated mutation and accounts for ~90% of disease alleles (Lafreniere *et al*., 1997; Lalioti *et al*., 1997a; Virtaneva *et al*., 1997). The *CSTB* minisatellite repeat is normally polymorphic, with 2–3 copies, but EPM1-associated expanded alleles contain at

Table I. The major forms of PME and their underlying genes

Disorder	Locus/chromosome	Gene product	References
Unverricht–Lundborg disease	EPM1/21q22.3	Cystatin B (CSTB) Cysteine protease inhibitor	Pennacchio *et al.* (1996)
Lafora's disease	EPM2A/6q24	Laforin; dual-specificity phosphatase	Minassian *et al.* (1998); Serratosa *et al.* (1999); Ganesh *et al.* (2000)
NCL			
Infantile	CLN1/1p32	Palmitoyl-protein thioesterase 1 (PPT1)	Vesa *et al.* (1995)
Late infantile	CLN2/11p15	Tripeptidyl peptidase 1 (TPP1)	Sleat *et al.* (1997); Rawlings and Barrett (1999); Vines and Warburton (1999)
Finnish variant late infantile	CLN5/13q22	Novel membrane protein (CLN5)	Savukoski *et al.* (1998)
Variant late infantile	CLN6/15q21–23	Novel membrane protein (CLN6)	Gao *et al.* (2002); Wheeler *et al.* (2002)
Juvenile	CLN3/16p12	Novel membrane protein (CLN3)	International Batten Disease Consortium (1995)
Northern epilepsy	CLN8/8p23	Novel membrane protein (CLN8)	Ranta *et al.* (1999)
MERRF	MTTK/mtDNA	$tRNA^{Lys}$	Shoffner *et al.* (1990); Yoneda *et al.* (1990)
Sialidosis	NEU1/6p21	Neuraminidase 1 (NEU1)	Bonten *et al.* (1996); Pshezhetsky *et al.* (1997)



B

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1                       23                                      57                                      98
MMCGAPSATQPATAETQHIADQ VRSQLEEKENKKFPVFKAVSFKSQVVAGTNYFIK VHVGDEDFVHLRVFQSLPHENKPLTLSNYQTNKAKHDELTYF

G426C (Gly4Arg)
MMCRAPSATQPATAETQHIADQ VRSQLEEKENKKFPVFKAVSFKSQVVAGTNYFIK VHVGDEDFVHLRVFQSLPHENKPLTLSNYQTNKAKHDELTYF

C2388T (Arg68X)
MMCGAPSATQPATAETQHIADQ VRSQLEEKENKKFPVFKAVSFKSQVVAGTNYFIK VHVGDEDFVHL

del2400TC
MMCGAPSATQPATAETQHIADQ VRSQLEEKENKKFPVFKAVSFKSQVVAGTNYFIK VHVGDEDFVHLRVFQSPS

G1925C
MMCGAPSATQPATAETQHIADQ VHVGDEDFVHLRVFQSLPHENKPLTLSNYQTNKAKHDELTYF
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Fig. 1. Schematic overview of the *CSTB* gene structure, mutations and predicted amino acid sequences of mutant proteins. (**A**) The 98 amino acid CSTB protein is transcribed from three exons depicted as white boxes, with the corresponding lengths of coding sequences in base pairs. White arrowheads indicate the translation start (ATG) and stop (TGA) sites. Black arrowheads indicate the positions of the two transcription start sites. The positions of the EPM1-associated mutations are shown with arrows. (**B**) Predicted sequences of CSTB proteins representing EPM1 mutations. The major mutation underlying EPM1, the dodecamer repeat expansion in the promoter region, results in production of reduced amounts of CSTB with normal sequence, shown on top. The del2400TC mutation creates a frameshift with two out-of-frame amino acids before an early stop codon. Of the three mutations affecting exon–intron boundaries, the G1925C mutation has been shown to result in skipping of exon 2 (Bespalova *et al*., 1997b), with consequent in-frame deletion of 34 amino acids from the polypeptide. The consequences of the other two splice site mutations have not been studied.

least 30 repeat copies (Lalioti *et al*., 1998). The majority of EPM1 patients are homozygous for the minisatellite expansion. No correlation between the repeat size and the age of onset or the severity of the clinical phenotype has been observed (Lafreniere *et al*., 1997; Lalioti *et al*., 1997a, 1998; Virtaneva *et al*., 1997). Expanded, pathogenic alleles of the EPM1 minisatellite show a high (47%) mutation rate, with contractions or expansions of the minisatellite typically by a single repeat unit (Larson *et al*., 1999). The EPM1-associated dodecamer repeat has been shown to form stable secondary structures under physiological conditions, which may be, at least in part, responsible for the expansion (Pataskar *et al*., 2001; Saha and Ushdin, 2001).

The six further EPM1-associated mutations occur within the transcription unit of *CSTB* (Pennacchio *et al*.,

1996; Lalioti *et al*., 1997b; Kagitani-Shimono *et al*., 2002; Figure 1). Three mutations, G1925C, G2027A and A2353G (GenBank acccession No. U46692), affect conserved splice site sequences and predict severe splicing defects, while two mutations in exon 3 (C2388T and del2400TC; GenBank accession No. U46692) predict a truncated protein through either creating a nonsense codon or causing a frameshift. The sixth mutation is a G426C (GenBank accession No. U46692) transversion in exon 1 that results in the substitution of a highly conserved glycine by an arginine at amino acid position 4 (Gly4Arg). This is, so far, the only missense mutation reported in EPM1 patients.

Cystatin B, a cysteine protease inhibitor

CSTB belongs to family 1 of a large superfamily of protease inhibitors, the cystatins, which are known to inhibit *in vitro* several papain-family cysteine proteases, cathepsins, by tight and reversible binding (Barrett, 1986; Barrett *et al*., 1986; Rawlings and Barrett, 1990; Turk and Bode, 1991). Cystatins of family 1 are small molecular weight proteins comprising a single polypeptide chain with no disulfide bonds or carbohydrates. Human CSTB, also known as neutral cysteine protease inhibitor and stefin 1, was first identified and characterized from lymphatic tissue (Rinne *et al*., 1981; Järvinen and Rinne, 1982) and later from liver (Ritonja *et al*., 1985). It is a ubiquitously expressed 98 amino acid protein and has a mol. wt of 11 kDa (Järvinen and Rinne, 1982; Ritonja *et al*., 1985; Jerala *et al*., 1988). CSTB has isoelectric variants and it often forms dimers by intermolecular disulfide bond formation. CSTB binds *in vitro* tightly to cathepsins H, L and S, and less tightly to cathepsin B (Green *et al*., 1984; Abrahamson *et al*., 1986; Brömme *et al*., 1991; Machleidt *et al*., 1991; Lenarcic *et al*., 1996).

The crystal structures of chicken cystatin B (Bode *et al*., 1988) and of recombinant human CSTB–papain complex (Stubbs *et al*., 1990; PDB entry 1STF) have been determined. Both the chicken and human CSTB consist of a five-stranded β-sheet wrapped around a five-turn α-helix. In addition, the human CSTB contains a C-terminal strand that runs along the convex side of the sheet. CSTB interacts with papain through a tripartite wedge formed by conserved residues at the most N-terminal part of CSTB, the first hairpin loop containing the highly conserved QVVAG sequence, and the second hairpin loop (Stubbs *et al*., 1990). The extended C-terminus of human CSTB provides an additional binding site relative to chicken CSTB (Stubbs *et al*., 1990). Although CSTB has been characterized in detail *in vitro*, its physiological function is unknown.

Consequences of EPM1 mutations on CSTB mRNA and protein

CSTB mRNA expression in EPM1 patients has been studied both by northern analysis and by RNase protection assay, with somewhat controversial results. In northern analysis of lymphoblastoid cell mRNA, dramatically reduced levels of *CSTB* mRNA were observed in patients either homozygous or compound heterozygous for the minisatellite expansion mutation (Pennacchio *et al*., 1996; Lalioti *et al*., 1997b; Bespalova *et al*., 1997a,b; Lafreniere *et al*., 1997; Alakurtti *et al*., 2000). Contrary to the northern data, Lalioti *et al*. (1997a) showed with an RNase protection assay that the level of *CSTB* mRNA was markedly reduced in blood leukocytes, but was either normal or only slightly reduced in fibroblasts and lymphoblastoid cell lines of patients. These data suggested a cell-specific reduction in *CSTB* gene expression and/or modulation of the expression of *CSTB* by the repeat expansion in some cell types.

Consistent with the results in northern analyses of *CSTB* expression, Rinne *et al*. (2002) showed that the papain inhibitory (cystatin) activity was significantly decreased or absent in lymphoblastoid cells of EPM1 patients. This reduction correlated with a significant increase in general cathepsin activity, and in particular of cathepsin B, L and S activities. Taking these data together with the *in vitro* kinetic data, which have shown very strong binding of CSTB to cathepsin S, intermediate binding to cathepsins H and L, and relatively weak binding to cathepsin B (Green *et al*., 1984; Abrahamson *et al*., 1986; Brömme *et al*., 1991; Machleidt *et al*., 1991; Lenarcic *et al*., 1996), it was suggested that the consequences of decreased CSTB activity in EPM1 pathogenesis may be, at least in part, due to increased activity of cathepsins S and L.

The consequences of the only EPM1-associated missense mutation (Gly4Arg) have not been experimentally tested. However, Gly4 is known to be highly conserved in cystatins from various species (Rawlings and Barrett, 1990; Turk and Bode, 1991; Lalioti *et al*., 1997b). Based on the known crystal structure of the CSTB–papain complex (Stubbs *et al*., 1990), the Gly4Arg substitution that is located in the N-terminal interacting part is likely to modify the papain-binding pocket. Three-dimensional modelling suggests that the Gly4Arg mutation, by bringing in an amino acid with a long and charged side chain, which causes major steric hindrance, is likely to strongly affect the papain-binding capacity of CSTB (Lalioti *et al*., 1997b).

Characteristics of the CSTB gene promoter

As the expansion mutation is located upstream from the transcription initiation sites of *CSTB* in the putative promoter region, two studies have aimed to characterize the *CSTB* promoter (Lalioti *et al*., 1999; Alakurtti *et al*., 2000). Alakurtti *et al*. (2000) limited the promoter analysis to downstream of an Alu-rich repeat region starting at ~0.7 kb upstream from translation initiation and used different promoter–luciferase reporter constructs in transient transfection experiments in COS-1 cells to map the promoter within 670 bp from the translation initiation codon. Sixteen extra copies of the dodecamer in the 670 bp promoter construct resulted in 10-fold reduced luciferase expression, indicating that the repeat expansion downregulates transcription *in vitro*, compatible with northern analysis of lymphoblastoid cell RNA of patients (Alakurtti *et al*., 2000). Using electrophoretic mobility shift assays, active binding to five Sp1 and four AP1 sites as well as weak binding to an androgen response element half-site were demonstrated (Alakurtti *et al*., 2000).

Lalioti *et al*. (1999) used a 3.2 kb fragment upstream of the translation initiation codon in characterization of the

promoter. They showed that a 600 bp repeat expansion in this promoter fragment reduced luciferase activity 2- to 4-fold compared with an identical fragment with a normal sized repeat. This reduction was only seen in some cell types (SK-N-BE neuroblastoma and HeLa), whereas in others (CHP neuroblastoma and COS-7) either a small decrease or an increase in luciferase activity was observed (Lalioti *et al*., 1999). These data were compatible with the observations on the cell type-specific reduction in mRNA expression in EPM1 patients using the RNase protection assay (Lalioti *et al*., 1997a). Introduction of heterologous DNA fragments of 730 and 1000 bp into the normal promoter instead of the repeat expansion showed similarly reduced activity, suggesting that altered spacing of promoter elements due to the dodecamer repeat expansion contributed to reduced *CSTB* gene expression (Lalioti *et al*., 1999).

In addition to altered spacing of promoter elements, the possibility of abnormal hypermethylation of the promoter region in the presence of the expansion mutation as a cause of reduced transcription has been considered. However, restriction analysis of the CSTB promoter region with methylation-sensitive enzymes has revealed no significant hypermethylation (Lalioti *et al*., 1997b; own unpublished findings), although the methylation status of the dodecamer repeat itself has not been determined. It is possible that the stable secondary structures that the dodecamer repeat forms under physiological conditions (Pataskar *et al*., 2001; Saha and Usdin, 2001) affect transcription or even translation, which has been shown to occur, for example, in association with trinucleotide repeat expansions (Feng *et al*., 1995; Parsons *et al*., 1998).

Cellular localization and molecular interactions of CSTB

Two studies have reported the results of immunofluorescence analysis of endogenous CSTB in a variety of cultured cell types (Calkins *et al*., 1998; Riccio *et al*., 2001), with somewhat controversial results. Calkins *et al*. (1998) used a monoclonal anti-CSTB antibody in confocal immunofluorescence analysis of CSTB and cathepsin B in two liver cell lines. CSTB distribution was distinct from that of cathepsin B, and it was found diffusely throughout the cytoplasm with apparent concentration at membranes of vesicular structures. More recent data have also indicated nuclear localization for CSTB (Riccio *et al*., 2001). Using several different cell types and polyclonal anti-CSTB antibodies, Riccio *et al*. (2001) demonstrated that CSTB was mainly localized in the nucleus in proliferating cells and both in the nucleus and the cytoplasm in differentiated cells. By comparison, cathepsin B was shown to be essentially cytoplasmic, and CSTB and cathepsin B were shown to co-localize only partially (Riccio *et al*., 2001). The separate compartmentalization of CSTB and cathepsin B as well as the localization of CSTB in both nucleus and cytoplasm could suggest that CSTB has functions in the cell other than cathepsin inhibition.

In order to identify proteins interacting with CSTB, Di Giaimo *et al*. (2002) used the yeast two-hybrid technique with rat CSTB cDNA as a bait in screening a cDNA library from developing rat cerebella. They identified five proteins interacting with CSTB. Three were known proteins: rat neurofilament light polypeptide (NF-L), rat activated protein kinase C receptor (RACK-1) and rat brain β-spectrin. One protein was related to human myotubularin, while the fifth was a novel protein of unknown function. Interestingly, although present in the cDNA library, no interaction of CSTB was detected with cathepsins B, H or L. The three previously known proteins identified in the screen were shown to interact with CSTB in GST pull-down assays as well as to partially co-localize with CSTB in immunofluorescence analysis of differentiated cultured primary cerebellar granule cells (Di Giaimo *et al*., 2002). Moreover, all three proteins were shown to co-immunoprecipitate in a specific manner with CSTB from rat cerebellar cell extracts, but not from cerebral hemispheres, suggesting *in vivo* interaction of the four proteins in the cerebellum. Confocal immunofluorescence analysis indicated that all four proteins were mostly expressed in the granule cells of developing rat cerebellum, and in Purkinje cells of adult rat cerebellum. Based on these data, the authors concluded that CSTB participates in the formation of a multiprotein complex that has a specific cerebellar function, possibly involved in cell growth and differentiation (Di Giaimo *et al*., 2002).

Cystatin B-deficient mouse model for Unverricht–Lundborg disease

Mice deficient for CSTB have been produced by targeted disruption of the mouse *Cstb* gene (Pennacchio *et al*., 1998). The mice develop a phenotype that resembles the human phenotype, with progressive ataxia and myoclonic seizures. However, in contrast to the human phenotype, no tonic–clonic seizures, photosensitivity or spike–wave complexes in EEGs, typical in human patients, have been observed in the mice. Initial analysis of the mice revealed apoptotic death of cerebellar granule cells, indicating that CSTB has a role in preventing cerebellar apoptosis and suggesting that EPM1 should be classified as a primary neurodegenerative disorder that selectively targets specific mammalian cells (Pennacchio *et al*., 1998). Further evidence supporting the hypothesis of an endogenous neuroprotective role for CSTB arises from studies in a rat kindling model of epilepsy (D'Amato *et al*., 2000), in which seizure activity has been shown to induce marked and widespread upregulation of CSTB mRNA and protein in rat forebrain neurons.

Later, additional neuropathology in *Cstb*-knockout mice was reported (Shannon *et al*., 2002). In addition to cerebellar granule cell apoptosis, less marked neuronal apoptosis was detected in the hippocampal formation and entorhinal cortex in 3- to 4-month-old mice. In older mice, gliosis was present in the hippocampal formation, entorhinal cortex, neocortex and striatum. Widespread gliosis was also present in the white matter, which may be a secondary phenomenon (Shannon *et al*., 2002). In addition to neuronal death by apoptosis, the superficial neurons of the prosubiculum in the cerebral cortex displayed prominent cellular atrophy. The observed neuropathology was similar in seizure-prone and seizure-resistant genetic backgrounds. The data indicate that neuronal atrophy is an important consequence of CSTB deficiency independent of seizure events, suggesting that

CSTB is important in maintaining normal neuronal architecture and size and that in addition to cellular death, cellular dysfunction may also be important in producing the phenotype (Shannon *et al*., 2002). Interestingly, the neuropathological changes were distributed unevenly in the mouse brain, suggesting differential neuronal sensitivity to CSTB deficiency.

Consistent with the observed pathology in the mice, mRNA expression profiling of CSTB-deficient mice shows increased expression of seven genes involved in proteolysis, apoptosis and glial activation, among them that for cathepsin S (Lieuallen *et al*., 2001).

Conclusion

Recent advances in the molecular understanding of PMEs have important implications. Pinpointing the molecular defects in individual diseases allows an aetiological diagnosis, which has already resulted in improved clinical practice and classification of these disorders. Moreover, identification of the primary defects underlying PMEs has provided a starting point towards understanding the molecular pathogenesis of these disorders. Towards this goal, important tools including animal and cellular models have been created. However, the physiological function of the PME proteins and the disease mechanisms are still largely unknown. Further research utilizing both classical methods and the modern tools provided by the genome project is needed to dissect the diseases at a molecular and cellular level and to compile this information into an understanding of the basic mechanisms underlying PMEs. This knowledge will be the basis for the development of rational methods for the prevention and treatment of these devastating disorders. Moreover, the research is expected to provide insights into molecular mechanisms involved in neuronal function and survival as well as seizure generation, thus providing insights into more common disorders that share underlying mechanisms.

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