The Molecular Basis of Mucolipidosis Type IV

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Abstract: Mucolipidosis Type IV (MLIV) is a lysosomal storage disorder that is characterized by severe neurologic and ophthalmologic abnormalities. It is a progressive disease that usually presents during the first year of life with mental retardation, corneal opacities, and delayed motor milestones. First described in 1974, MLIV is a rare autosomal recessive disease and the majority of patients diagnosed to date are of Ashkenazi Jewish descent. MLIV was originally classified as a lysosomal storage disorder due to the abnormal accumulation of mucopolysaccharides and lipids. Extensive studies in MLIV cells, however, have shown that the abnormal storage is due to a defect in the late endocytic pathway. Positional cloning led to the recent discovery of a novel gene on human chromosome 19, *MCOLN1*, that is mutated in MLIV. To date 14 independent mutations have been reported in *MCOLN1*, with two mutations accounting for 95% of the Ashkenazi Jewish MLIV alleles. The identification of the MLIV gene has led to a simple tool for definitive diagnosis and will permit carrier screening in the Ashkenazi Jewish population. *MCOLN1* is a new member of the transient receptor potential (TRP) cation channel gene family. The protein encoded by *MCOLN1*, mucolipin-1, has six predicted transmembrane domains and a putative channel pore. The identification of mutations in *MCOLN1* represents the first example of a neurological disease caused by a TRP-related channel. While the function of mucolipin-1 is currently unknown, homology to the TRP superfamily and the recent description of the *C. elegans* mucolipin-1 homolog allow us to begin to speculate about the role of mucolipin-1 in diverse cellular processes.

Mucolipidosis Type IV (MLIV; MIM 252650) belongs to a group of inherited metabolic diseases known as the lysosomal storage disorders. In total more than 40 lysosomal storage disorders have been described, with a combined incidence of more than one in 5000 births [1]. This vast number of disorders is not surprising given that there are more than 70 known hydrolytic enzymes that participate in the turnover of macromolecules for reutilization in cellular synthesis. The majority of the lysosomal storage disorders are due to defects in glycan catabolism, however, MLIV and Niemann Pick type C result from defects in late endosomal transport and therefore represent a unique type of lysosomal storage disorder. MLIV was first described in 1974 as a new variant of the mucolipidoses in that it displayed corneal clouding and abnormal systemic storage bodies [2]. Interestingly, patients with MLIV do not show the dysmorphic features, skeletal changes or organomegaly of the other mucolipidoses. Prior to the discovery of the MLIV gene, definitive diagnosis was made by electron microscopic demonstration of inclusions in the lysosomes of different cell types in skin or conjunctival biopsies [3]. To date approximately 100 MLIV patients have been reported, the majority belonging to the Ashkenazi Jewish (AJ) population [4].

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CLINICAL FEATURES OF MLIV

MLIV is a progressive neurologic disease that usually presents during the first year of life with mental retardation, corneal opacities, strabismus, and delayed motor milestones [5]. Despite the fact that abnormal lysosomal storage has been demonstrated in all MLIV cells and tissues, patients with MLIV do not show mucopolysaccharide excretion, organomegaly or skeletal changes. MLIV can be distinguished from the other mucolipidoses by the absence of these features and the presence of corneal clouding, retinal degeneration and mental retardation. Visual impairment is often the first clinical sign of the disease and results from corneal clouding, retinal dystrophy and optic atrophy [6, 7]. Psychomotor retardation becomes noticeable in early childhood and appears to be non-progressive, with most patients reaching a maximum developmental level of only 15 months in language and motor function [8]. Interestingly, the visual impairment is typically progressive even in patients with static neurological disease. Patients show little deterioration for the first three decades of life, but the prognosis beyond this point is still unknown.

MLIV patients have characteristic MRI findings, the most striking of which is the occurrence of a

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hypoplastic and dysgenic corpus callosum [8]. The thickness of the corpus callosum in MLIV patients is comparable with that of normal infants younger than 1 month of age and the splenium and rostrum are either dysplastic or absent. In addition, cerebellar atrophy is seen predominantly in older patients. Iron deficiency anemia in MLIV patients led to the discovery of markedly elevated blood gastrin levels apparently caused by constitutive achlorhydria, a finding that provides a simple biochemical marker to assist in diagnosis [4]. The majority of MLIV patients described to date are of Ashkenazi Jewish descent, and the carrier frequency in this population has recently been estimated to be 1/100 based on a population screen of 2000 subjects [9].

CELLULAR FEATURES OF MLIV

The storage materials in MLIV are heterogeneous, having been identified as sphingolipids, phospholipids, and acid mucopolysaccharides and accumulate in the lysosomes in cells from every tissue and organ of MLIV patients examined to date [10-12]. It has been shown, however, that the composition of the accumulating substances is different between somatic cells and those of the central nervous system [13]. The cytoplasmic inclusions appear as single-membrane bound granular inclusions and lamellar concentric bodies resembling those present in Tay-Sachs disease, and fibroblasts derived from MLIV patients have been shown to be auto-fluorescent [14]. Many lipid storage disorders are due to defective lysosomal hydrolases or activator proteins, but extensive studies have shown that the lysosomal hydrolases involved in the catabolism of the stored material in MLIV have normal activity and the storage products are normally catabolized and discharged. Experiments in cultured fibroblasts suggest that phospholipids and gangliosides accumulate in MLIV as the result of an abnormal endocytic process that leads to excessive transport of membranous components into the lysosomes [10]. In addition, examination of the movement of a lipid analogue along the lysosomal pathway has shown enhanced transport of membrane markers from late endosomes to lysosomes in MLIV cells [11]. A defect in the sorting and transport pathway, rather than a degradation defect, offers an explanation for the heterogeneous nature of the stored substances and the absence of the massive buildup and resulting organomegaly seen in many other lysosomal storage diseases.

MOLECULAR BASIS OF MLIV

In 1999 the MLIV gene was mapped to chromosome 19p13.2-13.3 following a genome-wide search for linkage utilizing 26 Ashkenazi Jewish families [15]. As expected, several genetic markers in the linked region displayed significant linkage disequilibrium, and haplotype analysis showed that there were five unique haplotypes present in the AJ population [16]. The major and minor AJ haplotypes are present on 72% and 23% of the chromosomes, respectively. Detailed haplotype analysis of these chromosomes allowed investigators to pinpoint the gene location, and in 2000 three independent groups demonstrated that mutations in a novel gene, *MCOLN1*, were responsible for MLIV [17-19].

The MLIV gene was identified using a classical positional cloning strategy. The candidate interval was narrowed using genetic information, and genes that mapped within the interval were systematically screened for expression differences and mutations in MLIV patients. *MCOLN1* was first implicated in the pathogenesis of the disease because some AJ MLIV patients showed a deficiency of the *MCOLN1* message by northern blot analysis [18]. Subsequent examination of both patient RNA and DNA led to the discovery of several independent mutations.

To date, 14 independent *MCOLN1* mutations have been reported in MLIV patients (Table 1) [9, 17-19]. The major Ashkenazi Jewish mutation, present on 72% of the AJ MLIV alleles, is an A – G transition at the 3' acceptor site of intron 3 (#1, Table 1). Examination of mRNA from several patients shows that this mutation results in deletion of exon 4 and various partial deletions of exon 5. This A – G transition creates a KpnI restriction site that permits simple detection of the mutation. The minor AJ mutation, found on 23% of the AJ MLIV alleles, is a 6434 bp genomic deletion that spans exons 1-6 and the first 12 bp of exon 7 (#2, Table 1). Simple assays for detection of these two mutations, which together account for 95% of MLIV alleles in the AJ population, have recently been described by several groups [9, 17-20]. Haplotype analysis in the AJ families suggested that there were three other rare mutations present in the AJ population, each of which was present in a single patient [16]. To date, two of these mutations have been identified. The first (#3, Table 1), found in a patient heterozygous for the minor AJ deletion mutation, is a single base pair insertion that results in a frameshift and truncated protein. The second rare AJ mutation (#4, Table 1) is a single amino acid deletion that is present in a patient heterozygous for the major splicing mutation. Interestingly, this amino acid deletion is present in a patient previously described to have an extremely mild clinical picture [21]. The third AJ patient that carries a unique haplotype is heterozygous for the common splice mutation, and despite screening, the second mutation in this patient has yet to be identified [16,18].

Ten mutations have been reported in non-Jewish (NJ) patients or AJ patients with mixed heritage, and in each case the unique mutations were carried by the NJ parent. With the exception of mutations #5 and #13, each of these has been described in a single patient. Two of these mutations, a 2 bp

Table 1. MCOLN1 Mutations.

Mutation Number	Nucleotide Change[a]	Mutation Type	AA Change	Mutation Ancestry	Patient Genotype[b]	Reference
1	g.5534A>G	splice	-	AJ		[17-19]
2	g.511-6944del	6434-bp deletion	-	AJ		[17-19]
3	c.1209-1210insT	frameshift	-	AJ	2, 3	[17, 18]
4	c.1221-1223delCTT	aa del	F408del	AJ	1, 4	[17, 18]
5	c.1406A>G	splice	454-469del	NJ	5, 5	[18]
6	c.1336G>T	missense	V446L	NJ	6, 6	[18]
7	c.304C>T	nonsense	R102X	NJ	7, 8	[18]
8	c.1084G>T	missense	D362Y	NJ	-	[18]
9	c.473-474delCC	frameshift	-	NJ	2, 9	[18]
10	c.514C>T	nonsense	R172X	NJ	5, 10	[18]
11	c.694A>C	missense	T232P	NJ	2, 11	[9]
12	c.1395C>G	missense	F465L	NJ	1, 12	[9]
13	c.964C>T	nonsense	R322X	NJ	1, 13	[9]
14	c.1444insGCCCTGCTGCG	frameshift	-	NJ	2, 14	[9]

[a] g. indicates mutations numbered using genomic sequence (AF287270) c. indicates mutations numbered with using cDNA sequence, bp 1 = the first base of the start codon.

[b] indicates, using mutation numbers, the genotype of the patient carrying the mutation.

deletion and a 11 bp insertion, result in frameshifts that predict truncated proteins (#9 and #14, Table 1). A single base substitution at bp 47 of exon 12 creates a new preferred splice acceptor site and results also in a frameshift (#5, Table 1). This mutation was found in two patients that shared a common haplotype. Three mutations result in the creation of stop codons in exons 3 (#7, Table 1), 4 (#10, Table 1), and 8 (#13, Table 1). This last mutation may represent a mutational hot spot, since it has also been seen in an Arab Druze patient with a reportedly unrelated haplotype [9]. Interestingly, four mutations (#6, 8, 11, and 12, Table 1) are missense mutations that result in single amino acid substitutions in the protein encoded by *MCOLN1*.

MCOLN1 mRNA expression levels were evaluated for several AJ and NJ patients using Northern blot analysis on cultured fibroblasts or lymphoblasts [18]. Expression of *MCOLN1* was absent in patients homozygous or heterozygous for only the major and minor AJ mutations. The AJ patient carrying mutation #3 showed no expression, while the patient carrying mutation #4 showed apparently normal expression. Of the five NJ patients evaluated for *MCOLN1* expression (patients carrying mutations # 5, 6, 7, 8, 9, and 10), all showed normal expression except the patient carrying mutation #9 in combination with the minor AJ deletion mutation. The fact that several patients carry missense mutations and show apparently normal levels of *MCOLN1* mRNA, yet display a similarly severe clinical phenotype to those with drastic mutations and no *MCOLN1* expression, will provide important clues to the function of the protein product, mucolipin-1.

The 1740 bp open reading frame of *MCOLN1* is composed of 14 exons and spans 13270 bp on human chromosome 19p13.3 (Genbank accession numbers: cDNA AF287269, genomic AF287270). It encodes a 580 amino acid protein with a predicted molecular weight of 65kDa that has been named mucolipin-1. Structural analysis of the amino acid sequence predicts that the protein has six transmembrane domains, with both the N- and C-termini residing in the cytoplasm. Comparison of the protein sequence against known protein motifs identified a transient receptor potential (TRP) cation channel domain at amino acids 331-521 and an internal channel pore at amino acids 496-521. This TRP domain spans transmembrane domains 3-6, with the pore-forming loop between the 5th and 6th domains. The predicted structure of mucolipin-1 is shown in figure **1**. PROSITE analysis predicts the presence of two proline-rich regions (amino acids 28-36 and 197-205), close to the cytoplasmic N-terminus and in the large extracellular loop between the first and second transmembrane domains. A lipase serine active site at amino acids 104-114 and a bipartite nuclear localization signal (amino acids 43-60) are also present, suggesting that this protein may have some lipid-related enzymatic function. The

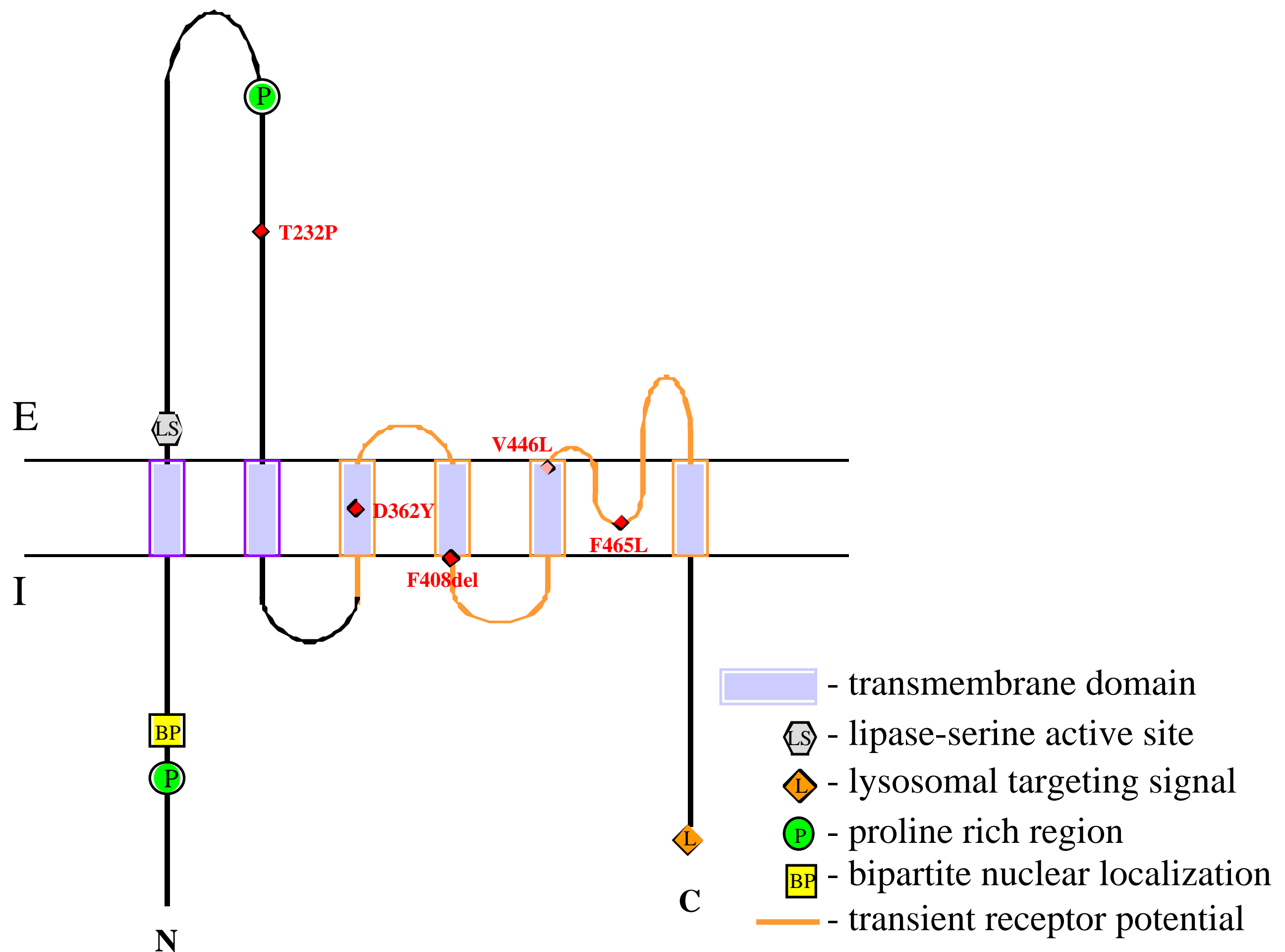

Figure 1. Putative structure of mucolipin-1. The protein has six predicted transmembrane domains, with the N- and C-termini residing in the cytoplasm. E = external, I = internal. The amino acid substitution mutations and the single amino acid deletion mutation are shown. Key to symbols is shown above. Protein motifs and patterns were determined using ProfileScan.

putative di-leucine motif (L-L-X-X) located at the c-terminus may serve as a late endosomal/lysosomal targeting motif [17-19].

The TRP superfamily consists of a diverse group of Ca^{2+} permeable non-selective cation channels that bear structural similarities to the *Drosophila melanogaster* TRP gene. These proteins have diverse functions and can be divided into six subfamilies, with mucolipin-1 being the founding member of the TRPML subfamily (for a recent review see [22]). The TRPML subfamily is comprised of three members, *MCOLN1*, *MCOLN2*, and *MCOLN3* (17-19 and unpublished data). *MCOLN2* and *MCOLN3* map adjacent to one another on human chromosome 1 and show 48.4 % and 57.2% amino acid identity to *MCOLN1*, respectively, and 59.5% identity to each other. These three proteins are approximately 70% identical across the TRP domain.

Mucolipin-1 shows significant structural similarity to polycystin-2, the product of the *PKD2* gene that is mutated in some cases of autosomal dominant polycystic kidney disease, ADPKD [17-19]. Unlike the other members of the TRP superfamily, both proteins have large extracellular loops between the first and second transmembrane domains. It has been shown recently that basolateral trafficking of proteins and lipids is impaired in polarized ADPKD cells, which suggests that mucolipin-1 and polycystin-2 may share a similar function in intracellular transport.

Comparison of the amino acid sequences of the mucolipin proteins to GenBank using BLAST (http://www.ncbi.nlm.nih.gov/blast) identified the *Drosophila* orthologue, CG8743, which shows 38% amino acid identity overall to mucolipin-1 and striking conservation across the TRP channel domain and the channel pore region (58% identity). The degree of similarity between the *Drosophila* protein and mucolipin-1, 2, and 3 is approximately the same. Recently the *Caenorhabditis elegans* mucolipin-1 homolog, *cup-5*, was identified during a screen for mutants defective in endocytosis [23]. Loss-of-function mutations in *cup-5* result in an enhanced rate of uptake of fluid-phase markers, decreased

degradation of endocytosed protein and accumulation of large vacuoles. Overexpression of *cup-5* caused the opposite phenotype, indicating that this gene controls aspects of endocytosis. The cellular features in the *cup-5* mutants mimic the defect seen in MLIV, providing an excellent model in which to study the structure and function of mucolipin-1.

While the function of mucolipin-1 is unknown, we do know that loss of this protein results in a severe defect in endocytosis in humans. It is also likely, based on homology, that mucolipin-1 functions as a cation channel, perhaps as a homo- or heterotetramer reminiscent in structure to that proposed for other TRP channel proteins [24]. It is interesting that four patients have been found to carry amino acid substitution mutations and yet have a similar severe clinical phenotype to those patients completely lacking mucolipin-1 protein. Since these point mutations appear to be sufficient to knock out the function of mucolipin-1, they provide insight into the function of the protein, particularly with respect to its putative role as a cation channel. The predicted location of these mutations based on the protein structure is shown in figure 1. Three of these mutations, #12 (F465L), #8 (D362Y), and #11 (T232P) are present in patients heterozygous for deleterious truncating mutations. The F465L substitution is located in the channel pore and D362Y is located in the third transmembrane domain, and each of these may interfere with the channel function of the protein. The patient with mutation #6 (V446L) is from a consanguineous family and is homozygous for this amino acid substitution. This single amino acid substitution is predicted to be at the border of the 5th transmembrane domain and the fact that this patient is clinically indistinguishable from the others demonstrates the potential vulnerability of the mucolipin-1 protein. Conversely, mutation #4, which results in the deletion of a single phenylalanine near the intracellular border of the 4th transmembrane domain, is present in a mildly affected patient heterozygous for the major AJ splice mutation. This patient has extremely mild neurological involvement yet is typically achlorhydric with severe visual impairment, perhaps suggesting some residual mucolipin-1 function in this patient. While the number of MLIV patients is too few to draw any conclusive genotype-phenotype correlations, the study of these mutations in model systems will shed important light on the normal function of the protein.

Studies are now underway to create a mouse model of MLIV, the *C. elegans* model has already been described, and the *Drosophila* gene has been identified. Future studies will likely first address the channel function of mucolipin-1 and will determine the conductance and ion selectivity of the channel. We will have to determine the mode of activation of the channel, and more importantly, determine the intracellular localization of mucolipin-1. Is this integral membrane protein present in the plasma membrane or does it reside in the endosomes and lysosomes, as might be predicted by the cellular defects seen in MLIV? The constitutive achlorhydria in MLIV patients and the observed cellular vacuolation suggests that mucolipin-1 is critical for cellular secretion. Cellular trafficking studies in MLIV cells clearly show an abnormality in lipid transport, and mucolipin-1 must play a major role in the development of white matter tracts and in the maintenance of neurons and retinal cells. Polycystin-2 exhibits different subcellular localizations and presumably different roles in the various tissues in which it is expressed, and the different phenotypes seen in MLIV cell types suggests that the same will be true for mucolipin-1.

MLIV is a rare disorder, however it is likely that there are a significant number of MLIV patients who remain undiagnosed. The identification of the MLIV gene, *MCOLN1*, now provides a simple tool for clinical diagnosis as well as carrier screening and prenatal diagnosis in the Ashkenazi Jewish population. *MCOLN1* is the first TRP gene implicated in neurological disease, and together with *PKD2* it demonstrates the important role that this cation channel superfamily plays in human disease. It is too early to tell if MLIV might prove amenable to therapy because we do not yet understand the function of mucolipin-1, but the challenges are many given that MLIV is a neurologic disease. The study of mucolipin-1 in MLIV patients as well as in model organisms will advance our understanding of the fundamental process of endocytosis, and hopefully result in the development of potential therapies for Mucolipidosis Type IV.

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LIST OF ABBREVIATIONS

MLIV = Mucolipidosis Type IV

TRP = Transient receptor potential

AJ = Ashkenazi Jewish

NJ = Non-Jewish

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