Molecular pathogenesis of megalencephalic leukoencephalopathy with subcortical cysts: mutations in MLC1 cause folding defects

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Megalencephalic leukoencephalopathy with subcortical cysts (MLC) is a rare type of leukodystrophy, most often caused by mutations in the MLC1 gene. MLC1 is an oligomeric plasma membrane (PM) protein of unknown function expressed mainly in glial cells and neurons. Most disease-causing missense mutations dramatically reduced the total and PM MLC1 expression levels in Xenopus oocytes and mammalian cells. The impaired expression of the mutants was verified in primary cultures of rat astrocytes, as well as human monocytes, cell types that endogenously express MLC1, demonstrating the relevance of the tissue culture models. Using a combination of biochemical, pharmacological and imaging methods, we also demonstrated that increased endoplasmatic reticulum-associated degradation and endo-lysosomal-associated degradation can contribute to the cell surface expression defect of the mutants. Based on these results, we suggest that MLC1 mutations reduce protein levels in vivo. Since the expression defect of the mutants could be rescued by exposing the mutant-protein expressing cells to low temperature and glycerol, a chemical chaperone, we propose that MLC belongs to the class of conformational diseases. Therefore, we suggest the use of pharmacological strategies that improve MLC1 expression to treat MLC patients.

INTRODUCTION

Leukodystrophies encompass a broad spectrum of inherited neurodegenerative disorders that affect brain white matter (1,2). Megalencephalic leukoencephalopathy with subcortical cysts (MLC) is a rare new leukodystrophy (3–6). Recognition of MLC was made possible only 13 years ago by means of magnetic resonance imaging (5,7). Its clinical characteristics are macrocephaly noted in the first years of life, motor disability and mild cognitive decline (5,8,9). In contrast to many other leukodystrophies, MLC progression is very slow (5,10,11).
Mutations in the MLC1 gene are the main cause of disease (12–14), although other unknown genes are also involved (15,16). In addition, there is a high intrafamilial variability, which may indicate the influence of modifier genes or environmental factors in the disease phenotype (13).

MLC1 is an oligomeric membrane protein with eight predicted transmembrane segments (17). Its homology to carrier proteins and its confinement to the plasma membrane (PM) suggest that it may mediate substrate translocation across the cell surface (12,18). Unfortunately, its precise role in the cellular physiology has not been identified yet (17,19).

Expression studies with RNA and antibody probes indicated that MLC1 is located in two neural populations: glial cells (17,20–23) and neurons (17,20). Specifically, in glial cells, it is concentrated in membrane contact regions, being enriched in distal glial processes and Bergman cerebellar glia. At present, it is still uncertain whether MLC1 is localized in membrane contact regions between endothelial cells and glial cells (i.e. forming part of the dystrophin glycoprotein complex) or in membrane contact regions between different glial cells (20,23,24). Although the exact localization has to be defined, the closer relationship with brain barriers suggest that MLC1 could participate in transport processes across the blood-brain and brain-cerebrospinal fluid barriers.

To study the molecular basis of the disease, in a previous work, we described a biochemical method to measure the levels of MLC1 protein at the PM in heterologous systems (17). Two studies with eight different MLC1 mutations (17,25) showed that mutations led to a reduced protein dosage. Here, we studied most of the MLC1 missense mutations (including one in-frame deletion) identified to date in heterologous systems as well as in primary rat astrocytes, a cell system with endogenous expression of MLC1 (26). Our results clarify the degradation pathways that follow these mutants. Moreover, using a new generated antibody, we showed for the first time that mutations also dramatically decrease the levels of MLC1 in cells from MLC patients.

The data presented here provide insights for understanding the relationship between the clinical phenotypes and the molecular defects of the protein. This work suggests that a common therapy used to improve MLC1 protein expression may be useful for the treatment of MLC patients.

RESULTS

Reduced PM expression of most MLC1 mutants

MLC1 gene encodes a membrane protein with a low degree of homology to the Kv1.1 potassium channel (13,18). We and others have failed to detect ion channel activity after its expression in several heterologous systems (17,19). As an alternative approach, we described a biochemical method to quantify the PM levels of MLC1 (17). To study the effect of most of the missense mutations including a deletion in a conserved poly-leucine stretch (26,27) (Fig. 1A), we introduced each of these mutations in human MLC1 containing two HA epitope tags and assayed the PM expression in the Xenopus oocyte system (Fig. 1B). We also measured protein expression levels by western blot analysis of total protein extracts. The same parameters were analysed in selected mutations in HeLa cells (Fig. 1C). Differences in surface expression for different mutants were found between these two systems, probably because the Xenopus oocyte is more permissive to folding mutants (28), due to its lower incubation temperature (18°C).

Most mutations dramatically reduced PM levels (Fig. 1). Western-blot analyses of cell extracts showed that steady-state protein values were also reduced in parallel (data not shown). We classified the mutations in three classes on the basis of their effect on PM expression levels: severe mutations (in black) reduced PM levels to less than 35% of wild-type (wt) MLC1 value in oocytes, intermediate mutations with PM levels >35% of wt MLC1 value in oocytes and PM levels <10% of wt MLC1 value in HeLa cells (in grey) and mild mutations with PM levels >10% of wt MLC1 value in HeLa cells (in white). P294L mutant was considered as severe (not shown) on the basis of its total protein levels. Of the 21 mutants studied, only three (N141K, S246R and the deletion in frame of leucines 304 and 305) had slightly reduced or similar PM levels compared with wt MLC1.

Reduced stability of MLC1 mutants

We subsequently studied the protein stability of selected mutants of each class by measuring the remaining protein after incubation with the protein synthesis inhibitor cycloheximide (CHX) (Fig. 2). For reasons of clarity, in western blot experiments, we only focused on the band corresponding to the monomeric form of MLC1, because it is the most abundant. Severe mutants (C326R and V210D) were less stable than intermediate (G59E) or mild mutants (S246R), suggesting that reduced PM protein levels mirror protein stability values (Fig. 2).

Intracellular retention of MLC1 mutants

The reduced stability of the mutants was suggestive of altered intracellular processing. It is feasible to study the intracellular processing of glycoproteins, such as CFTR (29), because by monitoring their glycosylation state by western blot one can distinguish between the endoplasmatic reticulum (ER) resident protein and the fully glycosylated mature protein. Unfortunately, although MLC1 has a putative glycosylation site between the putative transmembrane domains 3 and 4, it is not glycosylated (17) (Fig. 3B). We constructed a chimeric protein with glycosylation sites in this predicted extracellular loop (MLC1GLYC, Fig. 3A) as a way to follow its intracellular processing. Whereas wt MLC1 size was not altered after treatment with Endoglycosidase F (EndoF) (Fig. 3B), which removes most of the glycans, the size of the heavy molecular weight bands of MLC1GLYC was reduced, indicating that it was, in fact, glycosylated (Fig. 3B, bracket). A minor proportion did not change its size after EndoF treatment, probably indicating a non-glycosylated protein fraction (Fig. 3B, arrow).

We next addressed the sensitivity of MLC1GLYC to Endoglycosidase H (EndoH), because it indicates the presence of proteins that have not yet been processed beyond the ER. Most of the glycosylated protein was insensitive to EndoH (Fig. 3C, bracket), indicating that it was fully processed, and only a minor proportion of glycosylated protein (Fig. 3C, asterisk) was EndoH sensitive, probably indicating the
In agreement with being fully processed, we were able to detect MLC1GLYC at the surface by luminescence-based experiments (data not shown). We subsequently introduced selected mutations into the reporter MLC1GLYC and addressed the post-translational modifications in their glycosylation (Fig. 3C). Whereas a mild mutant as MLC1GLYC S246R was very similar to MLC1GLYC protein, severe mutants as MLC1GLYC S280L or MLC1GLYC C326R did not acquire a fully glycosylated state. In these severe mutants, the glycosylated band was sensitive to EndoH treatment, indicating that these mutants were not able to reach the medial Golgi compartment (Fig. 3C).

**Figure 1.** Reduced PM expression of most MLC1 mutants in Xenopus oocytes and HeLa cells. (A) A predicted 2D model of the MLC1 protein, showing the location of the mutations studied and the introduced HA epitope tags. (B) Oocytes were injected with 10 ng of each cRNA construct, and the PM levels were measured using a luminescence-based method. The signal of non-injected oocytes was always below 5% the signal of wild-type (wt) MLC1-injected oocytes. Surface expression was normalized with the expression of wt MLC1. Data correspond to the summary of three experiments with \( n \geq 30 \) oocytes per construct. The inset shows an overview of the method. The total protein steady-state levels were reduced in parallel with their surface expression level (not shown). The dotted line indicated 35% in PM versus wt MLC1, a criteria used to classify the mutants. (C) PM analysis using a luminescence-based method in HeLa cells transfected with wt MLC1 or the selected mutants. Surface expression was normalized with the expression of wt MLC1. Data correspond to two or three experiments with \( n \geq 8 \) data points per construct. The inset shows an overview of the method. Total protein levels of each mutant were analysed at least twice in western blot studies. The dotted line indicated 10% in PM versus wt MLC1, a criteria used to classify the mutants. Mutations were classified in three classes on the basis of their effect on PM expression levels: severe (in black), intermediate (in grey) and mild (in white).
Lysosomal targeting and degradation of MLC1 mutants

Taken together, these experiments suggested that most mutants are retained in the ER, where they will probably be targeted for degradation by the proteasome. However, we found that some mutants can partially reach the PM (Fig. 1). We speculated that these mutants could be degraded in lysosomes. To address what were the degradation pathways, we measured the remaining protein of selected mutants after incubation with the protein synthesis inhibitor CHX in the presence of a proteasomal inhibitor (MG132), or in the presence of a proteasomal inhibitor (CHX) (30) (Fig. 4A). These inhibitors increased protein levels with different efficiencies (Fig. 4A). The house-keeping α-actin protein was used as a loading control. Two independent experiments gave similar results. Mutant colour refers to the classification explained in Figure 1.

Conceivably, mutants might reach the lysosomes from the PM. To explore this possibility, we first studied the turnover of a selected intermediate mutant (in order to have enough detection sensitivity) at the cell surface (P92S). The cell-surface half-life (T_{1/2}) of P92S mutant was about 10 times lower (T_{1/2}~4 h) than that of the wt MLC1, determined by the disappearance of the anti-HA antibody binding in the presence of CHX (Fig. 4B). Similar results were obtained for the severe mutant S280L (19).

Next, we determined the destination of internalized mutants by monitoring the vesicular pH of MLC1 containing vesicles (Fig. 4C). Transiently transfected COS cells were incubated with anti-HA antibody complexes with Fab fragments conjugated to the pH-sensitive fluorophore FITC. We used COS cells, because they have increased total and surface expression of the mutants. The antibody-MLC1 complex was internalized and chased at 37°C. The luminal pH of MLC1-positive intracellular vesicles was accomplished by fluorescence ratiometric video image analysis as described previously for a panel of internalized cargo (31,32). Internalized MLC1 was detected mainly in vesicles with luminal pH of 6.4 ± 0.02 (n = 468 vesicles) (Fig. 4C), typical of recycling endosomes. In contrast, intermediate mutants G59E and P92S or severe mutants V210D, S280L and C326R were mainly confined to vesicles with luminal pH ≤ 5.3 (n > 475 vesicles) (Fig. 4B), characteristic of late endosomes and lysosomes, a finding consistent with their increased cell surface turnover. Mild mutants S246R and N141K were also mainly present in recycling endosomes.
Biochemical investigation of MLC1 mutants in astrocytes

The rapid turnover of the mutants could be due to the heterologous systems used (oocytes, HeLa and COS cells), which do not express MLC1 endogenously. Experimental work by several groups has detected MLC1 mRNA and protein in brain astrocytes (17, 21–23). We, therefore, isolated primary rat cortical brain astrocytes and confirmed its expression (not shown). Initially, we co-transfected wt MLC1 and selected mutants together with the GFP-tagged pleckstrin homology domain of PLCδ1 (PH-GFP), a fluorescent protein probe that labels the PM (33). No HA antibody signal was detected in non-transfected astrocytes (Fig. 5A). MLC1 was detected at the PM, mainly colocalizing with PH-GFP (yellow staining) (Pearson’s correlation coefficient: Rr = 0.8 ± 0.02, n = 10).

In contrast, the intermediate mutant G59E or the severe mutants A245P and S280L were almost exclusively detected in intracellular compartments (Fig. 5A), showing less degree of colocalization with PH-GFP (G59E: Rr = 0.5 ± 0.07; A245P: Rr = 0.4 ± 0.05; S280L: Rr = 0.4 ± 0.07; n = 10). Interestingly, although the mild mutant S246R was also detected at the PM, intracellular staining was greater than that of wt MLC1, as measured in a reduced colocalization with PH-GFP (Rr = 0.6 ± 0.04, n = 10).

These experiments suggested that the measured severity of the mild mutant S246R was higher in the astrocyte model. To verify these results with another experimental approach, we decided to study this mutant in more detail, performing biochemical quantification experiments of protein levels, as described before. Viral systems allow a high efficiency of expression in primary cultures. We, therefore, constructed adenoviruses expressing wt MLC1 or the mutant S246R. Infecting with these adenoviruses at different MOI revealed that the expression of mutant S246R was reduced at all MOI studied.

Figure 4. Lysosomal degradation of MLC1 mutants. (A) Transfected HeLa cells with wt MLC1 or the indicated mutants containing HA tags were incubated or not 24 h after transfection with the protein synthesis inhibitor CHX (100 μg/ml) for 6 h plus the proteasomal inhibitor Z-Leu-Leu-Leu-al (MG132, 50 μM) or several lysosomal inhibitors [ammonium chloride (NH4Cl, 10 mM)] or pepstatin plus leupeptin (Pep + Leu, 5 μg/ml each)]. Cell extracts were obtained and processed by western blot. From three different experiments, for mutants G59E, A157E and V210D, respectively, densitometry studies indicated that MG132 increased mutant expression levels (in percentage) to 22, 31, 36; ammonium chloride to 21, 32, 33 and pepstatin plus leupeptin to 8, 13, 9. β-actin protein detection was used as a loading control. (B) Twenty-four hours after transfection, cells were incubated with CHX. At a range of time points, cells were washed and fixed. PM levels were measured using a luminescence-based method. The signal was normalized to the value at time 0 for each of the experimental groups (wt MLC1 or P92S). The result is a representative experiment of two experiments with similar results. (C) Transiently transfected COS cells were incubated at 37°C for 90 min with FITC-conjugated anti-mouse Fab fragments and anti-HA antibody and chased in the absence of antibodies for 30 min before live imaging. The pH of individual vesicles was measured by fluorescence ratiometric video-image analysis. The figure shows the vesicular pH distribution of endocytosed wt MLC1 and MLC1 mutants. These distributions were obtained from 468, 482, 567, 671, 604, 615 and 639 vesicles for wt MLC1, G59E, P92S, N141K, V210D, S246R, S280L and C326R, respectively. They were obtained in three independent experiments. The results show that severe and intermediate MLC1 mutants are mostly localized in lysosomes after internalization from the PM. A minor proportion of wt MLC1 protein is also targeted to lysosomes, probably as a consequence of the overexpression. Mild mutants N141K and S246R were similar to wt MLC1, although a three-Gaussian distribution was used to fit the average pH of each type of vesicle population (see Material and Methods).
Figure 5. Expression of MLC1 mutants in rat primary astrocyte cultures. (A) Astrocytes were co-transfected with PH-GFP (pleckstrin homology domain of PLC1 fused to green fluorescent protein), as a marker of PM, together with wt MLC1 or the indicated mutants (G59E, A245P, S246R and S280L) containing HA tags. Cells were fixed and permeabilized, and immunofluorescence was performed using 3F10 (against the HA tags) as a primary antibody. No signal due to the HA epitope was observed in cells transfected only with the PH-GFP plasmid. The bar line correspond to 20 μm. Nuclei were stained using DAPI. PH-GFP is shown in green, MLC1 in red, nuclei in blue and colocalization between the green channel and the red channel in yellow. The degree of colocalization between PH-GFP and MLC1 proteins was analysed using the Pearson’s correlation coefficient (Rr) obtained with an ImageJ software plugin, using 10 single plane images from different cells corresponding to two independent experiments. The values of Rr were 0.8 ± 0.02, 0.5 ± 0.07, 0.4 ± 0.05, 0.6 ± 0.04 and 0.4 ± 0.07 for wt MLC1, G59E, A245P, S246R and S280L, respectively. Mutant colour refers to the classification explained in Figure 1. (B) Astrocytes were infected or not with adenoviruses expressing wt MLC1 or S246R mutant containing two HA tags at different MOI. Forty-eight hours later, extracts were obtained and analysed by western blot. At equal MOI, the expression of the S246R mutant was always lower than wt MLC1. β-Actin detection by western blot was used as a loading control. (C) Astrocytes were infected with adenoviruses expressing wt MLC1 or the mutant S246R with HA tags at MOI = 2. PM levels of wt MLC1 and S246R mutant were measured using a luminescence-based method, as described in Materials and Methods. The luminescence signal of non-infected astrocytes was always lower than the signal from infected astrocytes with MLC1 proteins. Data correspond to an independent experiment with four data points per construct, and are expressed in light arbitrary units (a.u.). From six independent experiments (n = 24), the level of the S246R mutant in comparison with wt MLC1 was 26 ± 5%. The inset shows an overview of the method. (D) Astrocytes were infected or not with adenoviruses expressing wt MLC1 and S246R mutant with HA tags at MOI = 2. Thirty-six hours post-infection, cells were treated or not with cycloheximide (CHX, 100 μg/ml) at the times indicated. Cell extracts were obtained, and the remaining protein was analysed by western blot against the HA epitope. β-Actin detection by western blot was used as a loading control (not shown). The result is a representative experiment of three with similar results. On the right, a quantification of this experiment using ImageJ software is shown. (E) Similarly, 36 hours post-infection with the indicated adenoviruses, astrocytes were incubated or not with CHX (100 μg/ml) for 6 h. Cell extracts were processed by western blot. β-Actin detection by western blot was used as a loading control (not shown). The result is a representative experiment of three with similar results. Mutant colour refers to the classification explained in Figure 1.
MLC1 expression in human MLC patients

A major caveat regarding all the experiments described is that they may be artefacts caused by overexpression. To validate the relevance of these experiments in tissue culture models, we attempted to detect MLC1 protein levels by western blot in samples from MLC patients. We developed a new antibody against human MLC1 protein which was able to specifically detect human MLC1 expressed in HeLa cells and the endogenous expression in brain (Fig. 6A).

MLC1 mRNA was reported to be expressed in peripheral blood leukocytes (PBLs) (34). At first, we attempted to detect MLC1 protein in Epstein–Barr virus-immortalized B lymphoblasts from unaffected controls, because these cells are stable and can be propagated. Unfortunately, we were unable to detect MLC1 protein expression, which may be attributed to the very low levels of mRNA expression in lymphoblasts compared with brain tissue. In fact, quantitative real-time PCR of human lymphoblast showed that these cells expressed about 1000-fold lower (n = 24) of wt MLC1 values (Fig. 5C). Incubation with CHX revealed that S246R mutant showed reduced total (Fig. 5D) and PM stability levels (Fig. 5E). Possibly, this reduced stability was due to an increased proteasomal and lysosomal degradation, as incubation with the proteasomal inhibitor MG132 only partially raised S246R mutant protein levels after CHX treatment (Fig. 5F).

Figure 6. Analysis of MLC1 expression in human MLC patients. (A) Characterization of a new polyclonal antibody against human MLC1 protein. Left panel: affinity-purified rabbit antibody against the N-terminal region of human MLC1 recognize a ~34 kDa and a ~70 kDa band in lysates of HeLa cells transfected with human MLC1 cDNA (hMLC1T), probably corresponding to the monomeric and dimeric form, respectively. These bands were not visible in non-transfected (NT) cells or in transfected cells (hMLC1T) using the pre-immune serum (PI). Middle panel: this antibody recognizes bands of the same size in extracts from human brain tissue, without showing any unspecific band. Right panel: fresh human PBLs were obtained using a Ficol gradient. They were further fractionated to monocytes and lymphocytes on the basis of monocyte adherence to plastic dish. Extracts were obtained and processed for western blot analysis. A band showing the same mobility as the monomeric form of MLC1 detected in human brain was specifically enriched in control monocytes (CTRL1 and CTRL2). For reasons of clarity, we only show the protein band corresponding to monomeric human MLC1, because the intensity of the band corresponding to dimeric MLC1 was lower. Cells from different controls showed variation in expression level, but this was not investigated further. (B) Left panel: fresh monocytes from an unrelated control (CTRL3) and from three different MLC patients containing at least a missense mutation were isolated; extracts were obtained and processed by western blot. The band identified as MLC1 protein was absent in the EL18 patient. An unspecific band was visible in some monocyte extracts (asterisk). Right panel: fresh monocytes from another unrelated control (CTRL4) and from three different MLC patients harbours the mild mutation S246R in homozygous state (Fig. 6C). MLC1 protein expression was detectable in control cells, but not in patient cells. In all, these results suggested that a reduced presence of the MLC1 polypeptide may be a common phenomenon for MLC patients harbouring missense mutations.

To be sure that this detected band corresponded to MLC1, we studied in parallel blood monocytes and human brain from the only MLC patient (EL18) who had adequate tissue quality (35). Although the mutations in this patient are not known, it has reduced MLC1 mRNA levels in immortalized lymphoblasts (26). Looking at MLC1 protein expression in monocytes from this patient, no band with the same size as MLC1 was detected, compared to those from a healthy control (Fig. 6B, left). Similarly, no MLC1 immunostaining was detected in brain sections from this patient (Fig. 6B, right). Thus, we concluded that monocytes could be used to follow human MLC1 endogenous protein expression.

Then, we isolated monocytes from different MLC patients with at least a missense mutation. One of these patients harbours the mild mutation S246R in homozygous state (Fig. 6C). MLC1 protein expression was detectable in control cells, but not in patient cells. In all, these results suggested that a reduced presence of the MLC1 polypeptide may be a common phenomenon for MLC patients harbouring missense mutations.
Chemical strategies to improve expression of MLC1 mutants

Finally, we decided to explore several experimental approaches that could improve mutant expression. First, we consider the possibility that incubation at low temperature or with chemical chaperones such as glycerol could raise mutant protein levels, as has been described in typical folding mutants of other proteins (36,37). In transfected HeLa cells, all the mutations studied, including the mild mutant S246R, increased steady-state values after lowering the temperature to 33°C and addition of glycerol (Fig. 7A). In one of the intermediate mutants, we studied [G59E, (38)], incubation of G59E-transfected HeLa cells at low temperature and with glycerol increased PM values concomitantly, whereas wt MLC1 surface levels were not significantly altered with this treatment (Fig. 7B).

As another alternative approach, and because the expression of most mutants was increased after blocking proteasomal degradation (Fig. 4A), we explored the possibility of using FDA-approved proteasomal inhibitors like Velcade® (bortezomib) as a possible pharmacological approach in these patients (39). Our first aim was to check whether Velcade® (bortezomib) incubation could reduce mutant degradation after CHX treatment in transfected HeLa cells. As depicted in Figure 7C, Velcade® (bortezomib) partially reduced the degradation of the intermediate mutant A157E and the severe mutant S280L, with a higher efficiency for the intermediate mutant A157E. The house-keeping β-actin protein was used as a loading control.

DISCUSSION

The present study extends our knowledge on the molecular basis of the defect for mutations that do not disrupt completely the synthesis of full-length MLC1 protein.

Our data indicate that most MLC1 mutants are mainly retained in an intracellular compartment, probably the ER, as evidenced by the studies with the chimeric protein containing glycosylation sites (MLC1GLYC). Although we do not know whether this chimera is functional, we believe that can be used to follow the intracellular trafficking of MLC1, because it is able to reach the PM and because the introduction of the mutations mirrors what happens in the protein without glycosylation sites. We hypothesized that the ER retention is a consequence of the misfolding caused by the mutations, as suggested by the recovery of protein expression by lowering the temperature or by adding chemical chaperones. Our previous studies indicated that MLC1 forms oligomers in vivo in the ER (17). Mutations could impede the correct oligomerization, which may be required for ER exit. Alternatively, mutations could simply disrupt protein structure.

This study also shows that mutants are also targeted to lysosomes after their internalization from the PM, as has been described for mutations in CFTR leading to cystic fibrosis (32). This observation supports the hypothesis that, apart from the ER quality control, there is a peripheral quality control mechanism regulating the conformation of PM proteins. We speculate that the proportion of degradation in proteasomes and lysosomes will vary depending on each particular mutation. A minor proportion of wt MLC1 protein is also targeted to lysosomes, probably as a consequence of the overexpression. In summary, we suggest that reduced PM levels are a consequence of increased protein degradation caused by an alteration of mutant protein trafficking.

We have classified the severity of the mutations on the basis of their impact on MLC1 PM protein levels. The lack of detailed clinical data for all the MLC patients limits possible correlations between this biochemical parameter and the phenotype of each patient. Possible genotype–phenotype correlations are also difficult to estimate due to the high intrafamilial phenotypic variability, the slow progression of the disease and the fact that the disease phenotype may worsen abruptly after minor head trauma or intercurrent infections (5). But, as a general impression, patients with truncated pro-
teins are not more severely affected than those patients with missense mutations. This phenotypic correlation can now be explained on the basis of our new data, because we provided evidence that MLC1 protein levels will be reduced in all cases.

Our study has also shown that the measured biochemical severity of each mutation depends on the cell system used to characterize the protein, as was neatly illustrated by the example of the S246R mutant. This mutant has roughly similar surface levels and protein stability to the wt MLC1 protein in *Xenopus* oocytes and transfected cell lines (Figs 1–4), although its protein levels are increased after reducing the temperature and adding glycerol (Fig. 7), which may indicate a minor folding problem. In contrast, surface expression and stability levels fell in primary cell culture of astrocytes (Fig. 5), and protein levels were not even detectable in monocytes from MLC patients harbouring this mutation (Fig. 6C). Possibly, the level of expression of the mutants in each cell system and the performance of the quality control machineries (ER and peripheral) determine the measured severity of each particular mutation.

Remarkably, most of the mutations studied led to attenuated cell surface expression of MLC1. There is an attractive parallelism in other genetic disorders, like cystinuria (40) or Bartter’s syndrome (41). In these heterogeneous disorders that affect transport processes through the membrane, mutations that affect the catalytic subunit may affect surface expression or reduce functional activity, whereas mutations that affect the chaperone subunit cause only a reduction in surface expression. Given the hypothesis that the molecular cause of MLC1 is a defect in ion transport, and since there are still other unidentified MLC-causing genes, we speculate that MLC1 may function as a chaperone-like molecule of other proteins with ion transport activity.

We assumed that lack of expression will be a common feature for MLC patients with mutations in *MLC1*, based on our *in vitro* and *in vivo* data. In fact, in order to finally prove that this is a common defect, we should extend the *in vivo* study to more MLC patients with mutations in the *MLC1* gene. It will also be interesting to study MLC1 protein levels in MLC patients without linkage to the *MLC1* gene (15,16). Meanwhile, we suggest that this simple biochemical assay (Western blot from monocytes) could be used as an additional diagnosis tool.

Although the physiological pathway in which MLC1 operates is still unknown, the observation of a common defect for all the mutants indicates that therapeutic approaches directed at increasing the expression or stability of MLC1 could be beneficial in MLC patients with associated *MLC1* missense mutations. We have shown that the reduction of temperature, treatment with glycerol (this study) and curcumin (17) increased MLC1 expression levels. Furthermore, here we have addressed the effect of the first FDA approved drug that works as a proteasomal inhibitor (Velcade® (bortezomib)) (39), showing positive results in a transfected cell line model. If the mutants retain totally or partially their putative transport or chaperone function, a chemical based-therapy (42,43) could be used to treat MLC, although these results are very preliminary and further work with mouse models containing *MLC1* mutations is needed.

**MATERIALS AND METHODS**

**Molecular biology**

Constructs were made using recombinant PCR and were then sequenced. The construction of MLC1 with 2 HA tags has been described previously (17). To construct an MLC1 glycosylation reporter (MLC1GLYC), we introduced some amino acids from the extracellular loop of the highly glycosylated amino acid transporter LAT-4 (44) between the putative transmembrane domain 3 and 4 (before the MLC1 non-conserved intracellular loop). The amino acid sequence in this region was **CKLVNMLKSE...AQDEMPSAIN** (flanking residues of the LAT-4 inserted sequence in bold, residues 39 to 87).

**Adenovirus production**

Each wild-type or mutant (S246R) *MLC1* cDNA containing two HA epitope tags was amplified with two primers bearing attB1 and attB2 gateway recombination sites using *Pfu* Polymerase (Stratagene). PCR products were gel-purified and cloned into the pDONR221 vector using BP clonase (Invitrogen). Correct entry clones were identified by restriction analysis and fully sequenced. Each was recombinated using LR clonase (Invitrogen) with the vector pAd/CMV/V5-DEST to construct a packageable vector plasmid. Adenoviruses were obtained according to the ViraPower Adenoviral Expression System (Invitrogen) protocol. Briefly, constructs were digested with *PacI* (New England Biolabs) and purified to expose the ITRs. HEK293 cells were transfected with Lipofectamine 2000 (Invitrogen). Twelve hours later, the medium was removed and replaced with fresh DMEM (Biological Industries). Forty-eight hours post-transfection, cells were trypsinized and transferred to a sterile 10 cm tissue culture plate until 80% of visible regions with cytotoxic effect were observed (10–13 days post-transfection). Cells and medium were collected to obtain the crude viral lysate. The transducing units per millilitre of each cell lysate were determined by α-hexon staining-based method (45).

**Rat astrocytes primary culture, transfection and transduction**

Purified rat astrocyte cultures were prepared from 1 to 3 days old Sprague Dawley rats (Charles River), as previously described with some modifications (46). Briefly, cerebral cortices were dissected and the meninges were carefully removed in cold sterile PBS 1x+0.3% BSA+0.6% glucose. The tissue was trypsinized for 10 min at 37°C and mechanically dissociated in complete DMEM medium (Dulbecco’s Modified Eagle’s Medium with 10% heat-inactivated foetal bovine serum (Biological Industries), 1% penicillin/streptomycin (Invitrogen) and 1% glutamine (Invitrogen) plus 40 U/ml DNase I (Sigma)) 10 times through a small bore fire-polished Pasteur pipette. The cell suspension was pelleted and resuspended in fresh complete DMEM, filtered through a 100 μm nylon membrane (BD Falcon) and plated into 75 cm² cell culture flasks (TPP). When the mixed glial cells reached confluence, contaminating microglia, oligodendrocytes and precursor cells were dislodged by mechanical agitation and removed as previously

**Materials and Methods**

**Molecular biology**

Constructs were made using recombinant PCR and were then sequenced. The construction of MLC1 with 2 HA tags has been described previously (17). To construct an MLC1 glycosylation reporter (MLC1GLYC), we introduced some amino acids from the extracellular loop of the highly glycosylated amino acid transporter LAT-4 (44) between the putative transmembrane domain 3 and 4 (before the MLC1 non-conserved intracellular loop). The amino acid sequence in this region was **CKLVNMLKSE...AQDEMPSAIN** (flanking residues of the LAT-4 inserted sequence in bold, residues 39 to 87).

**Adenovirus production**

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Astrocytes were then trypsinized, plated in poly-D-lysine-coated (Sigma) glass coverslipped in 24-well plates at about 80 000 cells/cm² and cultured for another 3–7 days. Cultured astrocytes were identified by their positive GFAP staining (a typical astrocyte marker, Dako). Their negative staining to class III β-tubulin (TUJ1, a neuronal marker (47), Covance) indicated the absence of neurons. These astrocytes expressed MLC1, as detected by western blot and immunofluorescence staining (data not shown).

Astrocytes were co-transfected with pCDNA3-wt MLC1 and mutants (G59E, A245P, S246R and S280L) together with PH-GFP, to visualize membrane staining (33). Astrocytes were plated on poly-D-lysine-coated (Sigma) glass coverslipped in 24-well plates (TPP) at 60% of confluence. The next day, astrocytes were transfected with lipofectin (Invitrogen) at 1:2 ratio (DNA:lipofectin) in Opti-MEM medium (as described in manufacturer’s instructions) and after 3 h, medium was replaced with complete DMEM and incubated 48 h at 37°C. To infect astrocytes with adenovirus, they were then trypsinized and plated at 5·10⁵ cells on 35 cm plates. The next day, wt MLC1 or S246R mutant adenoviruses were added at a MOI of 2, and kept overnight at 37°C. Medium was then removed, new fresh medium was added and cells were incubated at 37°C until they were processed (24–48 h).

Immunological procedures

Immune sera against one human MLC1 synthetic peptide, corresponding to the first amino acids of the N terminal region of the protein (peptide: TQEPREELAYDRM), were raised in rabbits using the services provided by Eurogentec. The peptide was coupled to keyhole limpet haemocyanin via a cysteine residue that has been added to the C-terminal region, mimicking its N-terminal position. After three boosts of immunization, the antisera were affinity purified using the peptide. The region, mimicking its N-terminal position. After three boosts of immunization, the antisera were affinity purified using the peptide.

Antibodies (3F10, Roche) and chemiluminescence techniques were used to measure surface expression in MLC1-injected Xenopus oocytes, MLC1-transfected HeLa cells and MLC1-infected astrocytes, as previously described (19). The mutant P294L (Fig. 1A) was studied only by measuring total protein expression levels, because this mutation disrupted the extracellular HA tag (see Materials and Methods).

Real-time PCR

MLC1 expression in one brain sample was compared to three lymphoblast cell lines (VU0046, VU0047 and VU0048). Duplicate samples were analysed. The quantitative PCR experiments were performed using an ABI PRISM 7700 sequence detector (Applied Biosystems). Transcript-specific primers were generated with Primer Express software (Applied Biosystems) and designed to bind exon–exon boundaries to prevent genomic DNA amplification. The PCR reaction was carried out in a volume of 10 µl, using SYBR green PCR mix, 3.0 µM primers and 0.1 µg cDNA. The PCR program was applied following manufacturer’s guidelines.

The cycle of threshold value (Ct) was used to calculate the relative expression level of MLC1 and was normalized to the transcripts for the housekeeping genes GADPH or β-actin. The Ct value is defined as the cycle number at which fluorescent emission reaches a fixed threshold during the exponential phase of amplification. The Ct value is inversely correlated with the original amount of mRNA. The resulting gene expression is given in ΔCt, i.e. the mean Ct value corrected for a housekeeping gene.

Vesicular pH measurement of MLC1-containing endocytic organelles

The pH of endocytic vesicles containing MLC1 was measured by fluorescence ratio imaging of internalized anti-HA antibody (Covance) complexed with FITC-conjugated goat anti-mouse Fab antibody (Jackson ImmunoResearch Laboratories). Cells were incubated with the primary and secondary antibodies in tissue culture medium at 37°C for 1–3 h, antibodies were chased for 30 min in antibody free medium and then washed with NaKH medium (140 mM NaCl, 5 mM KCl, 20 mM Hepes, 10 mM glucose, 1 mM MgCl₂, pH 7.3), and imaged on a microscope (Axiovert 100; Carl Zeiss Microimaging, Inc.) at 35°C, equipped with a Hamamatsu ORCA-ER 1394 (Hamamatsu) cooled CCD camera and a 63 × NA 1.4 Planachromat objective. Image acquisition and analysis were performed with MetaFluor® software (Molecular Devices). Images were acquired at 490 ± 5 nm, and 440 ± 10 nm excitation wavelengths, using a 535 ± 25 nm emission filter. In situ calibration curves were obtained by clamping the vesicular pH between 4.5 and 7.0 in K⁺-rich medium (135 mM KCl, 10 mM NaCl, 20 mM Hepes or 20 mM MES, 1 mM MgCl₂, and 0.1 mM CaCl₂, containing 10 µM nigericin, 10 µM monensin, and 5 µM carbonyl cyanide p-chlorophenylhydrazon) and recording the fluorescence ratio of cells loaded FITC-Fab and anti-HA antibody in MLC1 expressers. The fluorescence ratios as a function of extracellular pH provided the standard curve for the pH determination of MLC1-containing vesicles. In addition, one point calibration was done on each coverslip by clamping the vesicular pH to 6.5. Mono- and multi-peak Gaussian fits for vesicular pH were performed with Origin 7.0 software.
Human brain tissue and human monocytes

A brain biopsy containing neocortex and subcortical white matter was obtained from a MLC patient (EL18) for diagnostic purposes (35); frozen tissue was used for the study. Although the mutations in this patient are not known, the family was informative and showed linkage to the MLC1 locus. Quantitative real-time PCR analysis confirmed decreased MLC1 expression in lymphoblasts of this patient (26). Frozen human control brain tissue specimens, consisting of neocortex and white matter, were obtained at autopsy from patients without neurological disease and neuropathologic abnormalities. During life, the patients or their next of kin had given consent for autopsy and the use of brain tissue for research purposes. Immunohistochemistry was performed with antibodies against the MLC1 protein and GFAP (21).

Mononuclear cells were isolated from peripheral blood samples by centrifugation on a Ficoll-Hypaque (Seromed) gradient. Lymphocytes and monocytes were isolated by overnight attachment to a dish cell culture.

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