Molecular pathogenesis of megalencephalic leukoencephalopathy with subcortical cysts: mutations in \textit{MLC1} cause folding defects

Anna Duarri\textsuperscript{1,2,4,5,†}, Oscar Teijido\textsuperscript{8,†}, Tania López-Hernández\textsuperscript{2,†}, Gert C. Scheper\textsuperscript{10}, Herve Barriere\textsuperscript{11}, Ilja Boor\textsuperscript{10}, Fernando Aguado\textsuperscript{9}, Antonio Zorzano\textsuperscript{7,8}, Manuel Palacín\textsuperscript{5,6}, Albert Martínez\textsuperscript{9}, Gergely L. Lukacs\textsuperscript{11}, Marjo S. van der Knaap\textsuperscript{10}, Virginia Nunes\textsuperscript{1,3,4,†} and Raúl Estévez\textsuperscript{2,5,8,*,†}

\textsuperscript{1}CGMM-IDIBELL Gran Via s/n Km. 2.7, L’Hospitalet de Llobregat, Barcelona 08907, Spain, \textsuperscript{2}Sección de Fisiología and \textsuperscript{3}Sección de Genética, Departamento de Ciencias Fisiológicas II, IDIBELL-Universidad de Barcelona, Feixa Llarga s/n, L’Hospitalet de Llobregat, Barcelona 08907, Spain, \textsuperscript{4}CIBER de enfermedades raras (CIBERER), U-730, ISCIII, \textsuperscript{5}CIBER de enfermedades raras (CIBERER), U-750, ISCIII, \textsuperscript{6}CIBER de enfermedades raras (CIBERER), U-731, ISCIII, \textsuperscript{7}CIBERDEM, ISCIII, Barcelona, Spain, \textsuperscript{8}Department of Biochemistry and Molecular Biology and \textsuperscript{9}Department of Cell Biology, Faculty of Biology, Institute for Research in Biomedicine (IRB), Josep Samitier 1-5, Barcelona E-08028, Spain, \textsuperscript{10}Department of Pediatrics/Child Neurology, VU University Medical Center, Amsterdam, The Netherlands and \textsuperscript{11}Department of Physiology, McGill University, Montreal, Quebec, Canada H3G 1Y6

Received July 24, 2008; Revised and Accepted August 28, 2008

Megalencephalic leukoencephalopathy with subcortical cysts (MLC) is a rare type of leukodystrophy, most often caused by mutations in the \textit{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 \textit{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 \textit{MLC1} mutations reduce protein levels \textit{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).

\textsuperscript{†}These authors contributed equally to this study.

\textsuperscript{*}To whom correspondence should be addressed. restevez@ub.edu

\© The Author 2008. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oxfordjournals.org
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 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
ER-resident form. 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).
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 two different lysosomal inhibitors (ammonium chloride and pepstatin plus leupeptin) (30) (Fig. 4A). These inhibitors increased protein levels with different efficiencies (Fig. 4A). The house-keeping \( \beta \)-actin protein was used as a loading control. These experiments suggested that both ERAD and ELAD contribute to their degradation.

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). We measured the remaining protein of selected mutants after incubation with the protein synthesis inhibitor CHX 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.

Figure 2. Decreased stability of MLC1 mutants. HeLa cells transfected with wt MLC1 or the G59E, V210D, S246R and C326R mutants containing HA tags were incubated with the protein synthesis inhibitor CHX (100 \( \mu \)g/ml) for the times indicated (0, 3 and 6 hours). Cells were harvested, solubilized and processed by western blot against the HA epitope. No signal was detected in non-transfected cells. Exposure times varied for the mutants in order to clearly show the decrease in steady-state protein levels. Here, we also show that expression levels of the proteins without CHX were similar at all times indicated. On the right, a quantification of this experiment with adequate exposition times, using ImageJ software, is shown. Ponceau staining was used as a loading control. Two independent experiments gave similar results. Mutant colour refers to the classification explained in Figure 1.

Figure 3. ER retention of MLC1 mutant proteins. (A) Schematic 2D model of the chimera MLC1GLYC reporter protein, indicating the highly glycosylated loop from the LAT4 transporter (44), added between the putative transmembrane domains 3 and 4, the presence of the two HA epitope tags and the mutations studied with this reporter protein. (B) Extracts from transiently transfected HeLa cells with wt MLC1 or MLC1GLYC containing HA tags were treated with Endoglycosidase F (EndoF), which cleaved glycans of both the high-mannose and the complex type linked through asparagine to the protein backbone. No change in the motility of wt MLC1 was observed after incubating with EndoF; in contrast, the motility of the heavy molecular weight bands from MLC1GLYC (bracket) was reduced, indicating that it was glycosylated. Probably, the lower molecular band of MLC1GLYC (arrow) corresponds to an unglycosylated protein form. The band around 78 kDa of wt MLC1 probably corresponds to the dimeric form. (C) Extracts from transiently transfected HeLa cells with MLC1GLYC alone or MLC1GLYC containing selected mutations were processed by incubation with Endoglycosidase H (EndoH), which is able to remove glycans only if they have not suffered modifications in the Golgi complex. A high molecular weight broad band (bracket) EndoH insensitive in MLC1GLYC and MLC1GLYC S246R was not present in MLC1GLYC S280L or MLC1GLYC C326R. Only a lower molecular weight minor band (asterisk), that was the only glycosylated protein form present in the mutants S280L and C326R was sensitive to EndoH. These results suggest that these mutants are mostly retained in the ER. The band with lower molecular weight (arrow) of MLC1GLYC probably corresponds to an unglycosylated protein form, because its motility does not change after EndoH or EndoF treatment. Another independent experiment gave similar results. Mutant colour refers to the classification explained in Figure 1.
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 PLC\(\delta\)1 (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: \(R_r = 0.8 \pm 0.02, n = 10\)).

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: \(R_r = 0.5 \pm 0.07\); A245P: \(R_r = 0.4 \pm 0.05\); S280L: \(R_r = 0.4 \pm 0.07, n = 10\)). Interestingly, although the mild mutant S246R was also detected at the PM, intracellular staining was greater than that of wt MLC1 (Fig. 5A), as measured in a reduced colocalization with PH-GFP (\(R_r = 0.6 \pm 0.04, n = 10\)).

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 5. Expression of MLC1 mutants in rat primary astrocyte cultures. (A) Astrocytes were co-transfected with PH-GFP (pleckstrin homology domain of PLCδ1 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 analysised 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 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. 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. Cells were fixed, and the level of wt MLC1 and the S246R mutant at the PM were measured using a luminescence-based method. The signal was normalized to the value at time 0 for each of the experimental group (wt MLC1 or S246R). Data correspond to a summary of three independent experiments (n = 12). Wt MLC1 surface levels were reduced to 59.3 ± 4.1% and S246R mutant to 35.1 ± 7.1%. (F) In a similar manner, infected astrocytes were incubated with CHX (100 μg/ml) together or not with the proteasome inhibitor MG132 (50 μM) 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 (ΔCt value, see Materials and Methods) amounts of mRNA of MLC1 than that in brain. Due to this negative result, we tried to detect protein expression in primary PBLs cells from human unaffected controls. Additionally, to improve the detection, we separated these PBLs into monocytes and lymphocytes. A specific band, with the same size as human MLC1 detected in brain or in transfected cells, was detected in PBLs and enriched in monocytes.

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.
MLC1 or the G59E mutant after incubation at 33°C. Two other independent experiments gave similar results. (B) PM analysis using a luminescence-based method in HeLa cells transfected with wt MLC1 or the G59E mutant after incubation at 33°C plus 10 mM glycerol. Data correspond to an independent experiment with four data points per construct, and are expressed in light arbitrary units (a.u.). Another independent experiment gave similar results. (C) Transfected HeLa cells with wt MLC1 or the mutants indicated containing HA tags were incubated or not 24 h after transfection with the protein synthesis inhibitor cycloheximide (CHX, 100 μg/ml) for 6 h plus the proteasomal inhibitor MG132 (50 μM) or the FDA-approved drug Velcade® (bortezomib) (Millennium Pharmaceuticals, Inc) at 2.6 and 5.4 μM. From three independent experiments, Velcade® (bortezomib) was able to recover protein expression about 25–40% for A157E and 5–15% for S280L mutants. β-Actin detection by western blot was used as a loading control (not shown). Mutant colour refers to the classification explained in Figure 1.

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 an 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 characterized in the protein, as was neatly illustrated by the severity of each mutation depends on the cell system used to evidence that MLC1 protein levels will be reduced in all cases. This explanation on the basis of our new data, because we provided evidence that MLC1 protein levels will be reduced in all cases.

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 MLC 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 vitro 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...AQDEMP5A1N (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 recombination site sequences 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 recombined 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 cytopathic effect were observed (10–13 days post-transfection). Cells and medium were collected to obtain the crude viral lysate. The transducing units per milliliter of each cell lysate were determined by α-hexon staining-based method (45).

Rat astrocytes primary culture, tranfection 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...
described (46). 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 cm² 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: TQEPFREALAYDRMP), 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, the antiserum were affinity purified using the peptide covalently coupled to Sulpholink (Pierce). The polyclonal antibody was tested by immunoblotting, immunofluorescence and immunoprecipitation on transfected HeLa cells expressing human MLC1 and on non-transfected cells.

Transfected astrocytes were fixed for 15 min with 3% paraformaldehyde in PBS, washed in PBS, incubated 10 min in PBS containing 50 mM ammonium chloride, 10 min in PBS containing 20 mM glycine and blocked and permeabilized for 2 h at RT with 10% FBS in PBS 0.1% Triton X-100. Subsequently, coverslips were incubated with primary antibody [anti-HA (3F10) at 1:500 (Roche)] diluted in 10% FBS in PBS 0.1% Triton X-100] overnight at 4°C. After three washings, they were incubated with TRITC-conjugated donkey anti-rat (Jackson) in PBS 0.1% Triton X-100 at RT for 2 h. Finally, they were washed in PBS prior to mounting in Vectashield medium (Vector Laboratories) with DAPI (Sigma). Images were acquired using an Olympus DSU spinning disk confocal microscopy.

**Measurement of surface expression by luminescence**

HA 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 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

**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, 0.1 mM CaCl₂ and 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.
informative and showed linkage to the
mutations in this patient are not known, the family was
purposes (35); frozen tissue was used for the study. Although
matter was obtained from a MLC patient (EL18) for diagnostic
A brain biopsy containing neocortex and subcortical white
samples by centrifugation on a Ficoll-Hypaque (Seromed) gra-
vesicle population was calculated as the arithmetic mean of
bodies against the MLC1 protein and GFAP (21).
and white matter, were obtained at autopsy from patients
human control brain tissue specimens, consisting of neocortex
Mononuclear cells were isolated from peripheral blood
samples by centrifugation on a Ficoll-Hypaque (Seromed) gra-
dient. Lymphocytes and monocytes were isolated by overnight
attachment to a dish cell culture.

Human brain tissue and human monocytes
A brain biopsy containing neocortex and subcortical white
matter was obtained from an 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. Quantitat-
ive real-time PCR analysis confirmed decreased MLC1 expres-
sion in lymphoblasts of this patient (26). Frozen human
core 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 antibod-
ies against the MLC1 protein and GFAP (21).

FUNDING
This study was supported in part by FIS PI04/1680 (R.E.), FIS
PI04/1692 (V.N.), FIS PI04/2433 (A.M.), SAF2006-01631
(R.E.), Fundación Ramon Areces project (R.E.), ELA Foun-
G.C.S., I.B. and M.S.V.d.K. are supported by the Dutch Organ-
ization for Scientific Research ZonMW (program grant
903-42-097, TOP grant 9120.6002), the Hersenstichting
(grants 10F02(2),02, 13F05.04 and 15F07.30) and the
Optimix Foundation for Scientific Research. G.L.L. was sup-
ported by grants from the Canadian Institutes of Health
Research (CIHR), National Institutes of Health (NIH,
NIDDK) and the Canadian Foundation for Innovation.
G.L.L. is a holder of a Canada Research Chair. R.E. was a
researcher from the Programa Ramón y Cajal of the Spanish
Ministry of Science and Technology.

ACKNOWLEDGEMENTS
We thank Elon Pras for providing us with lymphoblast cell
lines from MLC patients; Ignacio Pascual-Castroviejo for
blood from MLC patients; Soledad Alcántara, Miguel
Morales and Xavier Gasull for help with primary astrocyte
cultures; Joan Gil, Mercé de Frias and Felix Rueda for PBLs
isolation; Artur Llobet for the gift of the PHGFP plasmid;
Manel Cascalló for help with adenoviral production;
Millennium Pharmaceuticals Inc. for the gift of Velcade®
(bortezomib) and Michael Maudsley for editorial support.

Conflict of Interest statement. The authors declare no conflict
of interest.

REFERENCES
Leukoencephalopathy, megalencephaly, and mild clinical course. A
recently individualized familial leukodystrophy. Report on five new cases.
J. Child Neurol., 11, 439–444.
Megalencephaly and leukodystrophy with mild clinical course: a report on
5. van der Knaap, M.S., Barth, P.G., Stroink, H., van Nieuwenhuizen, O.,
with swelling and a discrepancy mild clinical course in eight children.
Megalencephalic leukodystrophy in an Asian Indian ethnic group.
7. van der Knaap, M.S., Valk, J., Barth, P.G., Smit, L.M., van Engelen, B.G.
and Tortori Donati, P. (1995) Leukoencephalopathy with swelling in
children and adolescents: MRI patterns and differential diagnosis.
Megalencephalic leukoencephalopathy with subcortical cysts in two
siblings owing to two novel mutations: case reports and review of the
leukoencephalopathy with subcortical cysts. J. Child Neurol., 18,
646–652.
10. Pascual-Castroviejo, I., Van der Knaap, M.S., Prok, J.C., Garcia-Segura, J.M.,
megalencephalic leukoencephalopathy: 24 year follow-up of two siblings.
11. Sajo, H., Nakayama, H., Ezo, T., Araki, K., Sone, S., Hamaguchi, H.,
megalencephalic leukoencephalopathy with subcortical cysts (van der
Boor, P.K., Mejskjaer-Nosjuk, V., van der Maarel, S.M., Frants, R.R.,
Oudejans, C.B. et al. (2001) Mutations of MLC1 (KIAA0072), encoding a
putative membrane protein, cause megalencephalic leukoencephalopathy
13. Leegwater, P.A., Boor, P.K., Yuan, B.Q., van der Steen, J., Visser, A.,
Konst, A.A., Oudejans, C.B., Schutgens, R.B., Prok, J.C. and van der
responsible for megalencephalic leukoencephalopathy with subcortical
14. Topcu, M., Gartizou, C., Ribiere, F., Yalcinkaya, C., Tokus, E., Ortekin, N.,
megalencephalic leukoencephalopathy: 24 year follow-up of two siblings.
megalencephalic leukoencephalopathy with subcortical cysts (MLC).
16. Patrono, C., Di Giacinto, G., Eynard-Pierre, E., Santorelli, F.M.,
Rodriguez, D., De Stefano, N., Federico, A., Gatti, R., Benigno, V.,
Megabane, A. et al. (2003) Genetic heterogeneity of megalencephalic
leukoencephalopathy and subcortical cysts. Neurology, 61, 534–537.
17. Topcu, M., Martinez, A., Pasch, M., Zorrano, A., Soriano, E., Del Rio, J.A.,
the MLC1 protein involved in megalencephalic leukoencephalopathy with
18. Meyer, J., Huberth, A., Ortega, G., Syagailo, Y.V., Jatzke, S., Morsser, R.,
A nonsense mutation in a novel gene encoding a putative cation channel is


