Insights into MLC pathogenesis: GlialCAM is an MLC1 chaperone required for proper activation of volume-regulated anion currents

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Received May 11, 2013; Revised and Accepted June 18, 2013

Megalencephalic leukoencephalopathy with subcortical cysts (MLC) is a rare type of leukodystrophy caused by mutations in either MLC1 or GLIALCAM genes and is associated with myelin and astrocyte vacuolation. It has been suggested that MLC is caused by impaired cell volume regulation as a result of defective activation of astrocytic volume-regulated anion currents (VRAC). GlialCAM brings MLC1 and the ClC-2 Cl− channel to cell–cell junctions, even though the role of ClC-2 in MLC disease remains incompletely understood. To gain insights into the biological role of GlialCAM in the pathogenesis of MLC disease, here we analyzed the gain- and loss-of-function phenotypes of GlialCAM in Hela cells and primary astrocytes, focusing on its interaction with the MLC1 protein. Unexpectedly, GlialCAM ablation provoked intracellular accumulation and reduced expression of MLC1 at the plasma membrane. Conversely, over-expression of GlialCAM increased the cellular stability of mutant MLC1 variants. Reduction in GlialCAM expression resulted in defective activation of VRAC and augmented vacuolation, phenocopying MLC1 mutations. Importantly, over-expression of GlialCAM together with MLC1 containing MLC-related mutations was able to reactivate VRAC currents and to reverse the vacuolation caused in the presence of mutant MLC1. These results indicate a previously unrecognized role of GlialCAM in facilitating the biosynthetic maturation and cell surface expression of MLC1, and suggest that pharmacological strategies aimed to increase surface expression of MLC1 and/or VRAC activity may be beneficial for MLC patients.

INTRODUCTION

Megalencephalic leukoencephalopathy with subcortical cysts (MLC) is a rare type of leukodystrophy (genetic alteration of the white matter) that affects brain chloride and fluid homeostasis (1). MRI of patients shows diffuse signal abnormality, swelling of the cerebral white matter and the presence of subcortical cysts, mainly in the anterior temporal regions (2,3). Studies from an MLC patient brain biopsy revealed that the major phenotype of the disease was myelin vacuolation (4). These results, together with proton magnetic spectroscopic resonance data (5,6), suggested that MLC is caused by disturbed fluid homeostasis.

MLC1 was the first gene to be identified in MLC patients (7) and accounts for MLC in most patients. In mouse and human tissue, high MLC1 levels were present in astroglial–astroglial junctions and ependymal cells in blood–brain and CSF–brain barriers, apart from astrocytic junctions of Bergmann glia in the cerebellum (8–11). Thus, it has been proposed that MLC...
has an astrocytic origin (12), based on the fact that MLC1 is mostly expressed in astrocytes. In agreement with this hypothesis, depletion of MLC1 in astrocytes causes vacuolation, a phenotype that was also observed after re-examination of a MLC patient’s brain biopsy (13), which showed previously only myelin vacuoles. Interestingly, reduction in MLC1 in astrocytes and in lymphoblast cell lines from MLC patients was found to be associated with a reduced activity of volume-regulated anion currents (VRAC) by an unknown mechanism (14). In parallel, the rate of regulatory volume decrease after cell swelling was reduced in MLC1-depleted cells (14). It has also been suggested that MLC1 could interact functionally with the β-subunit of the Na⁺/K⁺ ATPase (15) and with the TRPV4 ion channel (16). However, the role of MLC1 is still completely unknown, and whether these interactions are related to the effects of MLC1 depletion on VRAC activity is unknown.

GLIALCAM was recently identified as a second gene mutated in MLC (17). It was demonstrated that GlialCAM functions as an escort molecule necessary to target MLC1 to cell–cell junctions (18). Moreover, the changed localization of the MLC1/GLIALCAM complexes seems to play an important role in the development of the disease, as most GLIALCAM mutations impair the correct trafficking of GlialCAM and MLC1 to astrocyte–astrocyte junctions (17,18).

Histological studies in tissue indicated that GlialCAM is mostly localized in astrocyte–astrocyte junctions where it co-localizes with MLC1 (17), but also in oligodendrocyte processes (19,20). Proteomic studies searching for additional GlialCAM protein partners revealed that GlialCAM also acts as an auxiliary subunit of the CIC-2 chloride channel, targeting it to astrocytic cell junctions and modifying its functional properties (20). Similar to its effect on MLC1 targeting, GLIALCAM MLC-related mutations also impair the trafficking of CIC-2 to cell junctions. Strikingly, Clcn2 KO mice also displayed similar vacuolation to what is seen in MLC patients (21), suggesting its involvement in the pathogenesis of MLC. However, no CLCN2 mutations were detected in MLC patients (22) and no direct protein interaction was found between MLC1 and CIC-2 (13,20). Thus, the involvement of CIC-2 in MLC disease is unclear. However, very recently, it has been shown that CLCN2 mutations may cause certain forms of a human leukodystrophy characterized by white matter oedema (23).

Dominant mutations in GLIALCAM cause other type of disease called MLC2B characterized by transient features of MLC (24). Similar to the recessive GLIALCAM mutations, also dominant mutations cause MLC1 and CIC-2 trafficking defects (17,18,20). It is still unknown why dominant mutations and not recessive mutations show a trafficking dominant behavior. Furthermore, dominant mutations are also associated with different clinical phenotypes than MLC1 mutations, while the clinical and MRI phenotypes related to recessive MLC1 and GLIALCAM mutations are the same.

In the present work, we have analyzed biochemically and functionally what the consequences of a reduced GlialCAM expression are, and compared them with the effects of a reduced MLC1 expression. The effect of GLIALCAM and MLC1 mutations was also addressed. The results obtained indicate that GlialCAM is necessary for MLC1 protein expression, and its reduction affects the activity of VRAC, which may cause astrocyte vacuolation. Thus, this work extends the role of GlialCAM as a chaperone of MLC1 needed for proper VRAC activation. In addition, based on functional data obtained, we suggest that a therapeutic strategy aimed to recover the surface expression of MLC1 and/or the activation of VRAC currents can be envisaged as beneficial for all MLC patients.

RESULTS

Experimental approach to create an astrocyte cell model with reduced GlialCAM expression

To study the cellular effects of GlialCAM ablation, adenoviral vectors expressing two distinct shRNA (1 or 2) against rat GlialCAM with the fluorescent protein EmGFP, allowing visualization of transduced cells, were produced. An adenoviral vector expressing non-targeted or scrambled shRNA (SCR) was used as a control. RT-qPCR experiments performed 7 days after GlialCAM shRNA infection of astrocytes demonstrated that endogenous GlialCAM mRNA levels were profoundly depleted (Fig. 1A). Accordingly, western blot (WB) analysis indicated that the GlialCAM protein level was reduced by 80% when compared with the control SCR (Fig. 1B and Supplementary Material, Fig. S1). Immunofluorescence experiments confirmed no detectable GlialCAM protein in shRNA-treated cells (Fig. 1C). Both types of experiments (WB and immunofluorescence) also demonstrated the specificity of the GlialCAM antibodies used (17). As an additional control, to exclude side effects of shRNAs, we also complemented with an adenovector expressing human C-terminally flag-tagged GlialCAM, which was resistant to the rat-specific GlialCAM shRNAs (Fig. 1B and C).

Reduction in GlialCAM expression led to intracellular retention and reduced expression of MLC1 in rat astrocytes

We studied the consequences of reduced GlialCAM expression at the mRNA, protein and subcellular localization of MLC1 (Fig. 2). As a specificity control, we monitored the expression and subcellular localization of a subset of proteins associated with tight, adherent and gap junctions. Knockdown of GlialCAM led to a reduction (about 30%) of the MLC1 mRNA levels (not significant) (Fig. 2A). However, the MLC1 protein abundance was reduced to 60% of wild-type values (Fig. 2B and Supplementary Material, Fig. S1), which cannot be explained by the reduction in the mRNA levels. Protein levels of some junction proteins were not altered, excluding non-specific effects (Fig. 2B and data not shown).

Importantly, while MLC1 was predominantly confined to an intracellular perinuclear compartment (Fig. 2D, arrowheads) in astrocytes depleted of GlialCAM, MLC1 remained associated with cell–cell junctions in astrocytes transduced with control shRNA (Fig. 2C). Furthermore, the junctional localization of MLC1 was recovered after complementation of the astrocytes with flag-tagged human GlialCAM cDNA (Fig. 2E), which was resistant to this shRNA (Fig. 1 and Supplementary Material, Fig. S1). Therefore, altered processing and decreased stability of the MLC1 may account for the cellular phenotype of MLC1 rather than non-specific effects of the shRNA. Additional control experiments also showed that localization of some junction proteins was not changed in GlialCAM-depleted astrocytes (Supplementary Material, Fig. S2 and data not shown).
GlialCAM improves the protein stability and surface expression of MLC1 variants containing MLC-related mutations

The above results suggest that GlialCAM may function as a chaperone needed for correct MLC1 folding and expression. If this is the case, GlialCAM over-expression may correct the localization of MLC1 variants containing MLC-related mutations, which primarily affect MLC1 protein folding (25). To test this hypothesis, we constructed adenoviruses expressing a HA-tagged human MLC1 variant containing the MLC-related mutations Pro92Ser (P92S) or Ser246Arg (S246R), and they were used to transduce rat astrocytes with or without GlialCAM. The immunostaining of the mutant MLC1-P92S showed a perinuclear intracellular distribution in astrocytes (Fig. 3A), similar to the endoplasmatic reticulum (ER) (25). However, co-expression with GlialCAM led to a re-distribution of the P92S variant to astrocyte–astrocyte junctions (Fig. 3B left, arrowheads), co-localizing with GlialCAM (Fig. 3B, right, arrowheads). Similar results were obtained with the MLC1-S246R mutant (Supplementary Material, Fig. S3A). We next asked whether MLC1 intracellular retention could be attenuated in HeLa cells expressed heterologously. GlialCAM was indeed able to rescue the intracellular accumulation of all MLC1 mutants studied (G59E, C85W, L311Ins and N141K) and re-locate them to the cell junction [Supplementary Material, Fig. S3B–E and (18)].

Using a chemiluminescence-based cell surface ELISA assay, the MLC1 cell surface density was determined as a function of GlialCAM expression, taking advantage of the extracellular HA epitope. The MLC1-P92S has an 80% reduced cell surface expression relative to its wild-type counterpart (Fig. 3C). GlialCAM over-expression increased the relative surface-expression levels of the MLC1-P92S in rat astrocytes (Fig. 3D).
Knockdown of GlialCAM affects MLC1 protein expression also in HeLa cells

The above results indicate that GlialCAM has two important functions. On the one hand, as reported previously, it is necessary to target MLC1 to cell–cell junctions (18,20). On the other hand, GlialCAM is necessary to stabilize MLC1 and this stabilization is important for translocation to the plasma membrane. This second role was unexpected, as MLC1 localization at the plasma membrane was detected without GlialCAM expression in HeLa cells. To explain this controversy, we hypothesized that GlialCAM is endogenously expressed in HeLa cells at low levels. Importantly, GlialCAM protein could be detected at the plasma membrane, using a monoclonal antibody directed against extracellular GlialCAM by a chemiluminescence-based ELISA assay (Fig. 4A). Transfection of two independent shRNAs against GlialCAM reduced the antibody signal, confirming that the plasma membrane signal detected by the antibody was due to GlialCAM (Fig. 4A).

To address whether the stabilization effect of GlialCAM in MLC1 could be also observed in HeLa cells, we transfected HeLa cells with wild-type MLC1 or MLC-related mutant variants P92S (PS) or C326R (CR) together with and unrelated shRNA as a control or shRNAs against GlialCAM, and assayed the cellular and surface MLC1 expression by immunoblotting and cell surface ELISA, respectively. Knockdown of GlialCAM reduced total (Fig. 4B) and cell surface (Fig. 4C) expression of all MLC1 variants, including the wild-type MLC1, as observed in rat astrocytes. Considering that GlialCAM and MLC1 physically interact [revealed by split-TEV or FRET studies (18)], these data strongly suggest that GlialCAM exerts a stabilizing effect on MLC1, which is independent of the cell system studied.

Effect of GlialCAM MLC-related variants on MLC1 expression

GlialCAM variants containing MLC-related mutations affect GlialCAM and MLC1 trafficking to cell junctions, without substantially interfering with GlialCAM expression or the biochemical interaction between GlialCAM/MLC1 (17,18,20). We thus addressed whether GlialCAM mutations can compromise MLC1 stabilization in astrocytes. To this aim, we first depleted endogenous GlialCAM in astrocytes by adenovirus-mediated RNA interference, and then complemented with wild-type GlialCAM wild-type or two mutant variants, Arg92Gln (R92Q), found in MLC2A patients, or Arg92Trp (R92W), found in MLC2B patients. As a control for protein expression, we complemented with wild-type GlialCAM. Astrocytes were analyzed by WB or immunofluorescence. Localization of endogenous MLC1 was detected in astrocyte–astrocyte junctions. Scale bar: 20 μm.

Figure 2. Expression of MLC1 in GlialCAM-depleted astrocytes. (A) RT-qPCR experiments using specific primers to detect rat MLC1 mRNA. Data represent the mean ± SEM of four different astrocyte preparations either treated at MOI 10 with SCR (scrambled) or with a shRNAs against GlialCAM. Both shRNA Glial1 and Glial2 gave similar results. Ns, not significative; *P < 0.01. (B) Astrocytes (untreated or transduced at MOI 10 with the shRNA indicated during 7 days) were analyzed by WB using antibodies against the indicated proteins. β-Actin was used as a loading control. Different experiments gave similar results (n = 3–5). (C) Localization of MLC1 in astrocyte–astrocyte junction in cells transduced with the adenovirus expressing a control shRNA, which were visualized by the positive GFP signal. (D) Localization of MLC1 in astrocytes with reduced expression of GlialCAM was detected mostly intracellular around the nucleus (arrowheads). (E) Astrocytes were co-transduced with a shRNA against GlialCAM and flag-tagged human GlialCAM (resistant to shRNA) and processed by immunofluorescence. Localization of endogenous MLC1 was detected in astrocyte–astrocyte junctions. Scale bar: 20 μm.

Figure 3. Expression of MLC1-P92S in GlialCAM-depleted astrocytes. (A) Knockdown of GlialCAM reduced both GlialCAM and MLC1 expression (Fig. 5A and Supplementary Material, Fig. S1) and cell surface ELISA, respectively. Knockdown of GlialCAM reduced total (Fig. 4B) and cell surface (Fig. 4C) expression of all MLC1 variants, including the wild-type MLC1, as observed in rat astrocytes. Considering that GlialCAM and MLC1 physically interact [revealed by split-TEV or FRET studies (18)], these data strongly suggest that GlialCAM exerts a stabilizing effect on MLC1, which is independent of the cell system studied.

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the junctional localization of endogenous MLC1 was not corrected by the different mutated variants (Fig. 5D for R92Q and Fig. 5E for R92W).

Volume-regulated chloride currents are affected in MLC1- and GlialCAM-depleted astrocytes

Lack of MLC1 in rat astrocytes or in lymphoblast cell lines obtained from MLC patients have been related with a defective activation of VRAC (14), whose molecular identity is still unknown (27). We next addressed whether lack of GlialCAM may also affect VRAC currents.

Previous electrophysiological studies addressing VRAC currents were performed in mild trypsin-treated astrocytes (14). As this assays needed an enzymatic treatment to round astrocytes potentially leading to other effects related with the trypsin treatment (28), we switched to dB-cAMP-treated astrocytes that are already rounded due to the elongation of processes (29). We focused only in changes in VRAC activity, taking advantage of the fact that not all astrocytes express ClC-2 mRNA in isotonic conditions (30), but most express VRAC currents in hypotonic conditions. In both cases, reduction in MLC1 or GlialCAM led to a dramatic decrease of VRAC activity of 80%, although it was still detectable (Fig. 6).

Lack of GlialCAM and MLC1 causes astrocyte vacuolation

Lack of MLC1 in astrocytes and in MLC patients caused the appearance of vacuoles in the cytoplasm of astrocytes (13) and compare Fig. 7A with Fig. 7B). Similarly, knockdown of
GlialCAM also led to astrocyte vacuolation (Fig. 7C). Vacuoles appearing in MLC1 or GlialCAM-depleted astrocytes were also of endosomal origin, as revealed by staining with the endosomal protein marker EEA1 (early-endosomal antigen 1) (Fig. 7B and C).

We next aimed to correlate if vacuolation was directly linked to an increase in intracellular water. Treatment of astrocytes for 24 h with a hypoosmotic medium induced the appearance of water vacuoles of endosomal origin [(15) and Fig. 7D]. To test if a defective VRAC activity causally can contribute to the vacuolation phenotype, we inhibited VRAC activity, indirectly by attenuating the chloride gradient or directly. This was accomplished by incubating astrocytes with Bumetanide (Fig. 7E), which blocks sodium/potassium/chloride co-transporters (31–34), or by DCPIB (Fig. 7F), the most specific VRAC inhibitor known (35). Both treatments led to the appearance of vacuoles of endosomal origin. In order to correlate the biochemical and electrophysiological data with the vacuolation phenotype, we quantified the number of cells containing vacuoles for all experimental groups (see Materials and Methods). Quantification of the vacuolation phenotype in cells treated with Bumetanide or DCPIB revealed a similar degree of defect as cells treated with the shRNAs against MLC1 or GlialCAM (Fig. 8 and Supplementary Material, Fig. S4) [Bumetanide, 15.4 ± 2.3% (4 independent exp, 1033 cells); DCPIB, 18.8 ± 2.9% (4 independent exp, 1226 cells)].

Recovery of MLC1 in junctions rescued the vacuolation defect and VRAC activation
The astrocyte vacuolation caused by the lack of GlialCAM was rescued with complementation with human GlialCAM (Fig. 8A and Supplementary Material, Fig. S4). However,
Figure 6. VRAC activity is reduced in astrocytes with reduced expression of MLC1 and GlialCAM. Representative family of whole-cell chloride currents in dbcAMP-treated cultured neocortical astrocytes transduced with shRNA scrambled or shRNA against MLC1 or GlialCAM, elicited by the pulse protocol shown in the inset. Astrocytes that did not show CIC-2-like currents in isotonic conditions were selected. They correspond to about 60% of recorded cells. In about 85% of these cells do not having CIC-2 current in isotonic conditions, application of a hypotonic solution activated VRAC current. As expected, these currents were blocked by DCPIB, but not with iodide replacement (data not shown). The plot shows average steady-state current–voltage relationship of dbcAMP-treated astrocytes transduced with shRNA SCR (circles), shRNA MLC1 (filled circles) or shRNA GlialCAM (triangles) in isotonic and hypotonic conditions. Results corresponds to $n = 15$ for shRNA SCR, $n = 14$ for shRNA MLC1 and $n = 14$ for shRNA GlialCAM. $^*P < 0.05$ t-test versus scramble for both MLC1 and GlialCAM groups.

Figure 7. Similarities between the vacuolation induced by the lack of GlialCAM and the inhibition of VRAC activity. (A–C) Primary cultures of astrocytes were transduced with adenovectors expressing shRNA SCR, shRNA against MLC1 or shRNA against GlialCAM. After 7 days, cells were fixed and immunostained using antibodies against early-endosomal antigen 1 (EEA1). In (D–F), cells were transduced with adenovector expressing shRNA SCR and incubated with 50 μM bumetanide during 7 days (D) or 10 μM DCPIB during 7 days (E) or with a hypo-osmotic medium during 24 h (F). Cells were then fixed and immunostained using antibodies against EEA1. Green signal corresponds to GFP which is expressed together with the shRNA. Scale bar: 20 μm.
complementation with GlialCAM containing the MLC-related mutations R92Q or R92W, or further expression of MLC1 did not rescue the vacuolation defect (Fig. 8A). In agreement with these cellular defects, localization of over-expressed human MLC1 in GlialCAM-depleted astrocytes was detected mostly intracellularly (Supplementary Material, Fig. S5).

Similarly, vacuolation caused by the lack of MLC1 was rescued by expressing MLC1 or GlialCAM together with MLC1 (Fig. 8B). However, further expression of GlialCAM did not rescue the vacuolation phenotype. Rescue of MLC1 expression by MLC1 containing MLC-related mutations also failed to counteract the vacuolation phenotype (Fig. 8B), probably due to its intracellular localization (Fig. 3). However, restoring the localization of these proteins in junctions (Fig. 3) after co-expressing with GlialCAM successfully suppressed the vacuolation (Fig. 8B).

We next addressed whether the vacuolation could be correlated with changes in VRAC activity. Over-expression of MLC1 in dB-cAMP-treated astrocytes increased VRAC activity [Fig. 8C and (14)]. However, over-expression of GlialCAM alone or MLC1 containing the MLC-related mutation S246R did not increase VRAC activity [Fig. 8C and (14)]. In agreement with the rescue of the vacuolation defect, over-expression of MLC1 S246R together with GlialCAM also increased VRAC activity (Fig. 8C). Therefore, we suggest that defective VRAC activity may be directly linked to astrocyte vacuolation.

Figure 8. Correlation between VRAC activity and the vacuolation phenotype of astrocytes. (A and B) Quantification of the number of cells showing vacuolation after transduction with the shRNA-indicated and complemented or not with adenoviruses expressing the indicated constructs. In (A), data correspond to four to five independent experiments with the number of cells analyzed for each group as follows: shRNA SCR ($n = 1483$), shRNA Glial1 ($n = 1646$), shRNA Glial1 + Glial ($n = 1502$), shRNA Glial1 + Glial R92W ($n = 1776$), shRNA Glial1 + Glial R92Q ($n = 1917$), shRNA Glial1 + MLC1 ($n = 1606$). **$P < 0.01$ compared with shRNA Glial1. The background of cells with vacuoles of shRNA SCR (MOI 10) is 13.81 ± 1.98%. In (B), data correspond to four to five independent experiments with the number of cells analyzed for each group as follows: shRNA SCR ($n = 1009$), shRNA MLC1 ($n = 1064$), shRNA MLC1 + MLC1 ($n = 1094$), shRNA MLC1 + Glial ($n = 1164$), shRNA MLC1 + MLC1 P92S ($n = 1582$), shRNA MLC1 + MLC1 P92S + Glial ($n = 1379$), shRNA MLC1 + MLC1 S246R ($n = 1248$), shRNA MLC1 + MLC1 S246R + Glial ($n = 1203$). ***$P < 0.001$ compared with shRNA MLC1. The background of cells with vacuoles of shRNA SCR (MOI 5) is 5.38 ± 1.23%. (C) Representative traces at +80 mV of whole-cell chloride currents in isotonic (black) or hypotonic conditions (grey) elicited by the pulse protocol showed in the inset, in dBcAMP-treated cultured neocortical astrocytes transduced with shRNA scrambled or adenoviruses over-expressing MLC1, GlialCAM, MLC1 S246R or GlialCAM + MLC1 S246R. Astrocytes that did not show ClC-2 currents in isotonic conditions were selected. The plot shows average conductance values at +80 mV in hypotonic conditions. Results correspond to $n = 9$ for shRNA SCR, $n = 8$ for MLC1, $n = 5$ for GlialCAM, $n = 9$ for MLC1 S246R and $n = 9$ for GlialCAM + MLC1 S246R. *$P < 0.05$ t-test versus Scramble for all groups. Differences in the kinetics of inactivation were observed between groups in an unrelated manner, even in the same experimental group, and probably are related with differences in ionic strength between cells.
DISCUSSION

In this work, we have studied the effects of reducing GlialCAM expression in astrocytes, a recently identified MLC1 and CIC-2 subunit (18,20). Taking into account these new results presented here, we conclude that whereas GlialCAM is necessary for the targeting to astrocyte–astrocyte junctions of both MLC1 and CIC-2 (18,20), it has an additional role as an MLC1-chaperone. Based on the fact that the stabilization is observed in HeLa cells, that GlialCAM interacts directly with MLC1 (18) and that GlialCAM stabilizes also ER retained folding mutant variants of MLC1 (18), we propose that GlialCAM may protect MLC1 from ER-associated degradation. We hypothesize that this ER-protective effect of GlialCAM was not previously recognized due to the presence of endogenous GlialCAM expression in all the cell lines studied. Thus, it can be seen how expression of MLC1 in astrocytes without GlialCAM resulted in no plasma membrane staining of MLC1. As expression of MLC1 in astrocytes alone is able to arrive to the plasma membrane, it suggests that astrocytes should have an excess of endogenous GlialCAM compared with MLC1.

It has been shown that MLC-related mutations in GlialCAM do not substantially affect the interaction with MLC1 (18). Here, we also show that MLC-related GlialCAM variants are able to stabilize MLC1 protein, although they are not able to direct MLC1 to astrocyte junctions.

Reduction in both GlialCAM and MLC1 led to astrocyte vacuolation and a decreased VRAC activity (14). The function of VRAC currents which are heterogeneous is related with the efflux of water during the regulatory volume response (36). A decreased VRAC activity may cause an increase of intracellular water. We suggest that astrocyte vacuolation could be a consequence of increased water content, as blocking VRAC or the presence of a hypoosmotic medium (15) led to a similar type of vacuolation of endosomal origin. However, it is not clear how increased water content causes the appearance of vacuoles. It could be that astrocytes, which are cells evolutionary adapted to live in changing osmotic conditions, have gained the ability to keep the excess of intracellular water in the form of a vacuole, as happens in other organisms that form contractive vacuoles, and then they can survive in extracellular changing conditions (37–41).

How MLC1 and GlialCAM are related to VRAC activity? At this moment, it is very difficult to provide a clear answer to this question, as the function of MLC1 is unknown and the protein(s) responsible for VRAC activity still have to be discovered (27). Identification of the VRAC channel is a major and difficult task, as revealed by the reports of many potential candidates that later turned out to be wrong, such as ICln, p-Glycoprotein or the CIC-3 transporter (27,36). GlialCAM may affect VRAC activity due to the lack of MLC1, as GlialCAM over-expression by itself cannot compensate the vacuolation caused by the lack of MLC1. However, we believe that it is unlikely that MLC1 is directly responsible of VRAC, as VRAC activity is present in all the cells of the body (36), whereas MLC1 is mostly restricted to the brain and no other MLC1-homologous genes exists in mammals (7). In addition, VRAC activity is still present in astrocytes depleted of MLC1. MLC1 may affect VRAC activity indirectly, for instance, by modifying the adhesion strength of astrocytes or by affecting water or chloride handling in the brain. In this sense, aquaporin 4 depletion has also been shown to affect VRAC activity (42). It is worth to mention that MLC1 protein has also been found surrounding the endosomal membranes of astrocytes treated with a hypoosmotic solution (15) or with blockers such as DCPIB or Bumetanide (this work, data not shown). In these endosomal vesicles, MLC1 could contribute to the formation of water vacuoles by regulating water and/or chloride fluxes.

Interestingly, although GlialCAM proteins containing MLC-related mutations re-establish MLC1 protein expression, they do not direct MLC1 to junctions and are not able to rescue the vacuolation defect caused by the lack of MLC1. Therefore, we suggest that the correct localization of MLC1 is critical to perform its physiological function. In relation with putative roles suggested for MLC1 in ion transport or adhesion (10,14), its localization in junctions may be needed to allow transcellular fluxes of solutes or to detect easily changes in adhesion strength.

No treatment exists yet for MLC disease (1). Although the role of MLC1 is unknown, over-expression of GlialCAM rescues the vacuolation and the VRAC activity defect caused by some MLC1 mutants. Thus, the present data suggest that therapeutic strategies aimed at improving the surface expression of MLC1 or increasing the activity of VRAC may be envisaged as beneficial for MLC patients. This could be done by isolating pharmacologic chaperones based on increased MLC1 plasma membrane expression (43) or molecules aimed to increase VRAC activity, as has been done for other chloride channels (44).

MATERIALS AND METHODS

Primary culture, adenoviral transduction and vacuolation analysis

Rat primary quiescent astrocyte cultures were prepared as described previously from P0–P2 pups (13). Dibutyryl-cAMP differentiated rat astrocytes, which were used for electrophysiological measurements, were obtained as described (29). Adenoviruses expressing three copies of the flag epitope fused to human GlialCAM, either wild-type or containing the MLC-related mutations R92Q or R92W have been described (17). Similarly, adenoviruses expressing HA-tagged wild-type MLC1 or containing the MLC-related mutations S246R or P92S were also constructed. Transduction of astrocytes was performed as already described (25).

To study the vacuolation of astrocytes, we counted the cells with showed clear vacuoles. A cell was positive for vacuolation if have at least three vacuoles of a size bigger than 0.5 μm. The minor vacuolation observed in astrocytes transduced with the corresponding group expressing SCR shRNA instead of the specific shRNA was subtracted from all the experimental groups. This was done to compare directly MLC1 and GlialCAM depleted astrocytes, as the multiplicity of infection was different between both groups (MOI 5 for shRNA MLC1 and MOI 10 for shRNA GlialCAM) and the day of processing after the infection (5 days for shRNA MLC1 and 7 days for shRNA GlialCAM).

RNA interference and RT-qPCR

RNAi entry-clone (Gateway, Invitrogen) vectors were prepared using the Block-it PolIII miRNA EmGFP expression vector kit following manufacturer’s instructions. Entry clones were
recombinated using LR clonase into the vector pAdVD EST-CMV/V5. Adenoviruses were produced and titrated using fluorescence microscopy detecting EmGFP which is expressed together with the shRNA. The adenoviruses expressing a negative control shRNA (shRNA SCR, scrambled) or expressing an shRNA directed against rat MLC1 (shRNA 905) were described previously (13). The sequence of the oligos used to knockdown rat GlialCAM expression was: shRNA Glial1 (shRNA 1392); 5′-aagataagctctccacagtgttgccacgtctacatcgactgtttt-3′ and shRNA Glial2 (shRNA 908): 5′-tatagagccagctaggctggctgtatt-3′.

Total RNA was prepared from a 10 cm culture plate using NucleoSpin RNA/Prot (Macherey–Nagel) as indicated by the manufacturer. RNA concentration was determined in a Nanodrop spectrophotometer. All samples had an A260/A280 ratio >1.8. RNA was stored at −80°C until further use. Eight hundred to 1000 ng of total RNA were reverse-transcribed with Transcriptor First Strand cDNA synthesis kit (Roche) following manufacturer’s instructions at 25°C for 10 min followed by 50°C for 60 min, using random hexamers as primers. cDNA was stored at −80°C. qPCR was performed with Lightcycler 480 probes master kit (Roche) following manufacturer’s instructions in a final volume of 10 μl, with cDNA at 1:10 dilution. The primers and UPL probes used were designed with the ProbeFinder version 2.45 (Roche) by the on-line application (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?Id=UP030000) considering exon spanning and covering all splicing species and have been previously indicated (13). qPCR was run in a LightCycler 480 II on 384 well plates with the settings previously indicated. Efficiency curves were used for each of the analyzed genes and relative gene expression for each sample was calculated using ACTB (actin) as reference after testing its suitability for the experimental setup. All amplifications were inside the linear range. Data came from six different astrocyte preparations. Means of triplicates were used for each analysis. The standard deviation of the triplicates was 0.2 or lower. Data analysis was made with Lightcycler software (Fujifilm) or the freely available Image J program (http://rsbweb.nih.gov/ij/) with similar results.

For immunofluorescence staining, cells were fixed and permeabilized (in mM): 144 NMDG-Cl, 2 CaCl2, 2 MgCl2, 5 HEPES, 5 glucose at pH 7.3 and 308 mOsm/kg. Hypotonic extracellular solution (in mM): 144 NMDG-Cl, 2 CaCl2, 2 MgCl2, 5 HEPES, 5 glucose at pH 7.4 and 310 mOsm/kg. Hypotonic extracellular solution (−25%) was prepared by decreasing NMDG-Cl concentration to 105 mM (229 ± 2 mOsm/kg). All solution osmolarities were adjusted with sorbitol. An Ag/AgCl ground electrode
mounted in a 3 m KCl agar bridge was used. Membrane currents were recorded in the whole-cell patch clamp configuration, filtered at 2 kHz, digitized at 10 kHz and acquired with pClamp 9 software (Molecular Devices). Data were analyzed with Clampfit 9 (Molecular Devices) and Prism 4 (GraphPad Software, Inc., La Jolla, CA, USA). Whole-cell capacitance and series resistance were compensated with the amplifier circuitry. Series resistance was always kept below 10 MΩ and compensated at 70–80%. All recordings were performed at room temperature (22–23°C). Currents were evoked with 4 s pulses from +80 to −120 mV from a holding potential of 0 mV.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS
We thank Michael Pusch, Thomas Jentsch and Herbert Schwarz for helpful comments about the manuscript.

Conflict of Interest statement. None declared.

FUNDING
This study was supported in part by SAF 2009-07014 (R.E.) and SAF2012-31486 (R.E.), PS09/02672-ERARE to R.E., ELA Foundation 2009-017C4 project (R.E. and V.N.), 2009 SGR01490 (V.N.) and Canadian Institute of Health Research 12/0034/0003 (X.G.), SAF 2009-12606-C02-02 (V.N.), 2009 SGR SAF2012-31486 (R.E.), PS09/02672-ERARE to R.E., ELA FOUNDING.

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