Megalencephalic leukoencephalopathy with subcortical cysts protein-1 modulates endosomal pH and protein trafficking in astrocytes: Relevance to MLC disease pathogenesis

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Abstract

Megalencephalic leukoencephalopathy with subcortical cysts (MLC) is a rare leukodystrophy caused by mutations in the gene encoding MLC1, a membrane protein mainly expressed in astrocytes in the central nervous system. Although MLC1 function is unknown, evidence is emerging that it may regulate ion fluxes. Using biochemical and proteomic approaches to identify MLC1 interactors and elucidate MLC1 function we found that MLC1 interacts with the vacuolar ATPase (V-ATPase), the proton pump that regulates endosomal acidity. Because we previously showed that in intracellular organelles MLC1 directly binds Na, K-ATPase, which controls endosomal pH, we studied MLC1 endosomal localization and trafficking and MLC1 effects on endosomal acidity and function using human astrocytoma cells overexpressing wild-type (WT) MLC1 or MLC1 carrying pathological mutations. We found that WT MLC1 is abundantly expressed in early (EEA1+, Rab5+) and recycling (Rab11+) endosomes and uses the latter compartment to traffic to the plasma membrane during hyposmotic stress. Although MLC disease-causing mutations differentially affect MLC1 localization and trafficking, all the mutated proteins fail to influence endosomal pH and protein recycling. This study demonstrates that MLC1 modulates endosomal pH and protein trafficking suggesting that alteration of these processes contributes to MLC pathogenesis.

Introduction

Megalencephalic leukoencephalopathy with subcortical cysts (MLC, OMIM 604004) is a rare congenital and incurable leukodystrophy characterised by early-onset macrocephaly, ataxia, seizures, degeneration of motor functions and mild cognitive decline. Magnetic resonance imaging (MRI) indicates diffuse signal abnormality, swollen appearance of the white matter and the presence of subcortical cysts. Megalencephalic leukoencephalopathy with subcortical cysts protein-1 (MLC1) is a membrane protein mainly expressed in astrocytes in the central nervous system. Although MLC1 function is unknown, evidence is emerging that it may regulate ion fluxes suggesting that alteration of these processes contributes to MLC pathogenesis.
alterations of the blood–brain barrier structure and astroglial activation
(Van der Knaap et al., 1995a,b, 1996). Enlarged intracellular vacuoles localized in the astrocyte end-feet contacting blood vessels have also been described (Duarrì et al., 2011).

Almost 75% of MLC patients carry different types of mutations in the MLC1 gene suggesting that functional alterations of the MLC1 gene product are the leading cause of this disease. However, to date no correlation between genotype and phenotype has been found (Leegwater et al., 2001, 2002; Patrono et al., 2003). Recently, mutations in the HEPACAM/GUILCAM gene encoding an adhesion-like molecule of unknown function have been found in a substantial fraction of MLC affected patients without MLC1 mutations, unveiling genetic heterogeneity of MLC disease (Boor et al., 2006; Jeworutzki et al., 2012; López-Hernández et al., 2011).

The MLC1 gene encodes an oligomeric and highly hydrophobic protein which shows low homology with some ion channels and transporters (Boor et al., 2005; Leegwater et al., 2001; Teijido et al., 2004). In the central nervous system (CNS), MLC1 is mainly expressed in perivascular and subpial astrocytes, particularly in astrocytic end-feet contacting blood vessels and meninges (glia limitans) and in astrocytic intracellular organelles (Ambrosini et al., 2008; Boor et al., 2007; Duarrì et al., 2011; Teijido et al., 2004). Bergmann glia and ependymal cells lining the ventricles also express MLC1 (Ambrosini et al., 2008; Boor et al., 2007; Duarrì et al., 2008; Teijido et al., 2004). Outside the CNS, MLC1 has been detected in monocytes and lymphocytes (Boor et al., 2005; Duarrì et al., 2008). Although myelin vacuolation is a typical feature of MLC disease, the myelin forming cells, oligodendrocytes, do not express MLC1 (Boor et al., 2005; Schmitt et al., 2003), suggesting that myelin degeneration may be secondary to astrocyte dysfunction. Indeed, the tissue distribution and structural features of MLC1 protein and MLC-associated brain damage suggest a possible role for MLC1 in the regulation of fluid and/or ion homeostasis, a function that in the CNS is mainly carried out by astrocytes (Parpura and Verkhratsky, 2012). Consistent with this hypothesis, we have shown recently that MLC1 is part of a macromolecular complex associated to the sodium, potassium-ATPase pump (Na, K-ATPase) which includes the inward rectifier potassium channel 4.1 (Kir4.1), the water channel aquaporin-4 (AQP4), the transient receptor potential cation channel subfamily V, member 4 (TRPV4), the cytoskeletal anchoring protein synaptophysin and the membrane raft-associated protein caveolin-1 (Brignone et al., 2011; Lanciotti et al., 2012). We have also provided evidence that MLC1 is involved in the astrocytic response to changes in the extracellular ion composition and cooperates with TRPV4 to activate intracellular calcium influx during hyposmotic stress (Lanciotti et al., 2012). Most importantly, we have found that these interactions and pathways are affected by MLC1 pathological mutations (Lanciotti et al., 2012). The TRPV4-mediated calcium influx is the first and essential step required for the activation of astrocytic regulatory volume decrease (RVD) which is needed to rescue the rapid and temporary cell swelling for the activation of astrocyte regulatory volume decrease (RVD) TRPV4-mediated calcium in.

Materials and methods

Cell cultures and treatments

Astrocyte-enriched cultures (about 95% purity) were generated from 1- or 2-day-old newborn rats and maintained in culture as previously described (Agresti et al., 1991). By using a retroviral bicistronic vector (pQCCXIN, Takara Bio Europe Clontech, France) and the packaging cell line (GP2, Hek293) retroviral particles carrying recombinant WT or mutated MLC1 (S246R, S280L, C125R) were generated as previously described (Lanciotti et al., 2012). By infection with recombinant retroviral particles, astrocytoma cell lines overexpressing WT and mutated MLC1 and a control cell line infected with the empty virus were generated, as previously described (Lanciotti et al., 2012). Cells were exposed to hypotonic solution, as described previously (Brignone et al., 2011). Cells stimulated with 100 nM bafilomycin A1 (Sigma-Aldrich, St. Louis, MO) in serum-free (SF) medium for 3, 6 and 48 hours (h) were used for immunofluorescence stainings and western blot analysis as described below. The procedures for human monocye isolation and culture are described in the Supplementary material.

Immunofluorescence and confocal microscopy analysis

Astrocytoma cells grown on polylysine-coated coverslips were incubated in isosmotic, hyposmotic or bafilomycin-containing solution for different time lengths, fixed for 10 minutes (min) with 4% paraformaldehyde and washed with PBS. After 1 h of incubation with blocking solution (5% BSA in PBS), cells were incubated for 1 h at room temperature (RT) with the following primary antibodies (Abs) diluted in PBS containing 0.025% Triton X100: affinity purified anti-MLC1 polyclonal Ab (pAb) (1:50, Atlas AB, AlbaNova University Center, Stockholm, Sweden), anti-V-ATPase B2 monoclonal Ab (mAb) (D11) (1:50, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-EEA1 mAb (1:50, BD Transduction Laboratories, Lexington, KY), anti-Rab5 mAb (D11) (1:25, Santa Cruz Biotechnology), anti-Rab11 mAb (clone47; 1:25, Millipore, Temecula, CA), anti-Lamp-2 mAb (1:100, Abcam, Cambridge, UK), anti-transferrin receptor (TfR) mAb (1:80, Abcam) and anti-TRPV4 (H-79) pAb (1:10, Santa Cruz Biotechnology). As secondary Abs, a biotinylated goat anti-rabbit IgG H + L Ab (4.3 μg/ml; Jackson Immunoresearch Laboratories, West Grove, PA) followed by streptavidin-TRITC (2 μg/ml; Jackson), and Alexa Fluor 488 goat anti-mouse IgG Ab (1:300, Invitrogen, Milan, Italy) were used. Coverslips were washed, sealed in Vectashield medium (Vector Lab, Burlingame, CA) and analyzed with a laser scanning confocal microscope (LSM 5 PASCAL, Carl Zeiss, Jena, Germany). In the colocalization experiments fluorescence intensity profiles or fluorochrome colocalization analysis based on Manders’ overlap coefficient (MOC, Manders et al., 1993) was evaluated with the profile analysis tool of the LSM 5 PASCAL or NIH ImageJ software. MOC value range is 0–1 (0: no colocalization, 1: all pixels colocalize).

Immunostaining of human brain tissue

Post-mortem brain tissue from a person without neurological disease was obtained from the UK MS Tissue Bank at Imperial College London, and stained as previously described (Brignone et al., 2011). Briefly, sections were incubated overnight (ON) at 4 °C with a mixture of rabbit anti-MLC1 pAb (1:250, ATLAS) and anti-V-ATPase B2 mAb (D11) (1:100, Santa Cruz Biotechnology) or anti-GAP mAb (1:20, Pharmingen BD Biosciences, Milan, Italy). After extensive washings, sections were incubated for 1 h at RT with a mixture of fluorescein-conjugated goat anti-rabbit and rhodamine-conjugated goat anti-rabbit Abs and images were analyzed with laser scanning confocal microscope (LSM 5 PASCAL).
Cytosolic and membrane protein extracts obtained from cultured rat astrocytes were subjected to pull-down assay using recombinant Histagged MLC1 bound to Ni-NTA resin and control empty resin, as previously described (Ambrosini et al., 2008; Brignone et al., 2011). Proteins pulled-down with recombinant MLC1 and control unspacificated resins eluted from control resin were separated by SDS-PAGE, stained with Coomassie Colloidal Blue (Invitrogen), and then were run by SDS-PAGE and visualized by an enhanced chemiluminescence reagent (Thermo Scientific), according to the manufacturer’s instructions, and exposed to X-ray films. Densitometric analyses of WB bands were performed using ImageJ software.

Endosomal pH measurement by FITC-dextran

Endosomal pH was measured by means of the video-imaging technique with the pH sensitive probe FITC-dextran. FITC-dextran (Sigma-Aldrich) loading was achieved by exposing astrocytoma cells grown on glass coverslips for 30 min to a hypotonic solution as previously described (Brignone et al., 2011), and then were run by SDS-PAGE and visualized by an enhanced chemiluminescence reagent (Thermo Scientific) in column, as indicated by the manufacturer. The NeutrAvidin® agarose bound biotinylated proteins were eluted from the column with 450 μl SDS-PAGE Sample Buffer (62.5 mM Tris–HCl, pH 6.8, 1% SDS, 10% glycerol, 50 mM DTT). Fractions containing eluted protein were analyzed by Western Blot, as described above.
following composition (mM): 120 KCl, 10 NaCl, 1 CaCl₂, 1 MgCl₂, 10 glucose and 10 HEPES buffer. The ratios of the fluorescence intensity values at 480 nm and 440 nm were converted to pH values by linear regression fitting to the line obtained with the calibration pH values 7.5 and 6.5. The pH value of the single FITC-dextran-containing endosomal vesicles was calculated from the average pH recorded in the extracellular solution. Recording and analysis of the data were made possible by the use of the Imaging Workbench software package (INDEC Systems, CA). For further data processing and presentation the Origin 7.5 software package (Microcal software, USA) was utilized. Data are shown as mean ± SEM values of pH values, and statistical significance was calculated using the Student's t-test.

**Transferrin (Tf) internalization and recycling assay**

Transferrin internalization and recycling assay was performed as previously described (Bacac et al., 2011), with minor modifications. Briefly, subconfluent U251 astrocytoma cells grown on polylysine-coated glass coverslips were washed twice with PBS and then starved for 2 h in internalization medium (IM) (DMEM, 0.01% BSA) to deplete endogenous transferrin. Where indicated, hypoxic cell treatment was performed as previously described (Brignone et al., 2011). Then, cells were cooled on ice for 5 min to block endocytosis, rinsed using ice-cold PBS, and incubated with ice-cold IM containing human transferrin conjugated to Alexa Fluor 488 (50 µg/ml, Molecular Probes, Invitrogen) for 1 h at 4 °C to allow cell-surface binding of transferrin. Cells were then washed on ice using cold PBS–BSA 0.5% to remove unbound transferrin, and the probe was chased at 37 °C for indicated time-periods in the presence of 100 × excess unlabeled iron saturated human holo-transferrin (Sigma-Aldrich) to prevent fluorescent transferrin re-internalization. At the end of each time-point, cells grown on coverslips were fixed on ice using 4% PFA and analyzed using a laser scanning confocal microscope (LSM 5 PASCAL).

**Results**

**MLC1 interacts with proteins involved in endosomal pH regulation**

A proteomic MS/MS analysis of MLC1 molecular interactors was performed on cytosolic and membrane protein fractions from rat primary astrocytes that were pulled down with recombinant His-tagged-MLC1 protein bound to NiNTA resin selectively linking histidine residues (see Materials and methods). Among the potential MLC1 interactors identified (manuscript in preparation), we found that 2 subunits (a1 and B2) of vacuolar ATPase (V-ATPase), the proton pump responsible for endosomal acidification (Forgac, 2007), were pulled-down by His-MLC1 in 2 out of 3 experiments (Table 1). In the same experiments we also detected the alpha and beta subunits of the Na, K-ATPase pump (Table 1), thus confirming the results obtained in rat astrocytes and human astrocytoma cells using the yeast two-hybrid system and biochemical assays (Brignone et al., 2011; Lanciotti et al., 2012). Because Na+, K-ATPase, besides controlling plasma-membrane potential and cell volume, is also involved in the regulation of endosomal pH (Fuchs et al., 1989), these findings suggested that MLC1 might play a role in this process.

Using a mAb recognizing the a1 subunit of V-ATPase, the interaction between MLC1 and V-ATPase was confirmed by WB analysis of proteins pulled-down from rat primary astrocytes, particularly in the membrane protein fraction (Fig. 1a). V-ATPase also co-purified with His-tagged WT MLC1 over-expressed in a human astrocytoma cell line (Fig. 1b). In astrocytoma cell lines overexpressing 3 MLC1 missense mutations (S246R, S280L and C125R) (Lanciotti et al., 2012), the amount of V-ATPase associated with MLC1 was markedly lower than in WT MLC1 astrocytoma cells (Figs. 1b, c), indicating that each of these pathological mutations affects MLC1 molecular interactions. Double immunostaining for MLC1 and V-ATPase revealed that in WT MLC1 astrocytoma cells MLC1 and V-ATPase colocalized in perinuclear vesicles (Fig. 1d, arrows; colocalization analysis, as described in the Materials and methods section, is shown in Fig. S1). In post-mortem human brain tissue V-ATPase immunoreactivity was detected in cytoplasmic vesicles of GFAP⁺ astrocytes (Fig. 1e, arrows in magnification) and in astrocytic end-feet surrounding blood vessels where it colocalized with MLC1 (Fig. 1f, arrows in magnification). Altogether, these findings suggest that in astrocytes MLC1 might interact with V-ATPase in intracellular organelles.

**MLC1 localization in specific endosomal compartments and effects of pathological mutations**

We (Lanciotti et al., 2012) and others (Duarri et al., 2008) have previously shown that the 3 MLC1 missense mutations analyzed here differently affect MLC1 intracellular localization. Similarly to WT MLC1, the S246R mutated protein reaches the plasma membrane, while the S280L and C125R mutations cause intracellular retention of MLC1 protein (Lanciotti et al., 2012) (see also Figs. 1a–d). To understand whether pathological mutations could affect MLC1 localization and molecular interactions in the cytoplasmic vesicular compartment, astrocytoma cell lines overexpressing WT or mutated MLC1 were immunostained with anti-MLC1 Ab in combination with Abs specific for endosomal markers. Double immunostainings for MLC1 and EA1 or Rab5, two proteins expressed in organelles of the early endosomal compartment (Jovic et al., 2010), revealed that WT MLC1 colocalized with both markers (Figs. 2a and e, respectively). When using Abs to proteins expressed in the recycling (Rab11) and late endosomal-lysosomal (Lamp-2) compartments, we observed that WT MLC1 immunoreactivity largely colocalized with Rab11⁺ recycling endosomes (Fig. 2i) and to a much lesser extent with Lamp-2⁺ organelles (Fig. 2m). While the distribution of S246R mutant protein in intracellular organelles was similar to that of WT MLC1 (Figs. 2b, f, j, n), lower amounts of S280L and C125R mutants were found in EA1, Rab5⁺ and Rab11⁺ organelles when compared to WT MLC1. Conversely, the same mutant proteins were more abundantly localized in Lamp-2⁺ organelles relative to the WT protein (Manders’ colocalization coefficient and statistical analysis is shown in Fig. S2).

To summarize, this set of experiments allowed us to conclude that intracellular MLC1 is expressed mainly in the early endosomal and Rab11⁺ recycling compartments and that pathological mutations can affect both MLC1 membrane localization and MLC1 distribution in specific endosomal compartments.

**Table 1**

MLC1 protein interactors identified by mass spectrometry in the membrane protein extracts derived from rat astrocytes.

| Protein name | Short name | Probability | Seq Cov % | MW (kDa) | Accession n | Peptides n |
|--------------|------------|-------------|-----------|----------|-------------|------------|
| V-type proton ATPase 116 kDa subunit a isoform 1 | VPP1 | 1.64E−06 | 4 | 96 | P25286 | 2 |
| V-type proton ATPase subunit B, brain isoform | VATB2 | 1.21E−06 | 11 | 57 | P62815 | 3 |
| Sodium/potassium-transporting ATPase subunit alpha | ATPa1 | 2.10E−11 | 19 | 113 | P06685 | 6 |
| Sodium/potassium-transporting ATPase subunit beta | ATPB3 | 2.48E−09 | 17 | 32 | Q53377 | 3 |

Probability corresponds to the Sequest protein score defined as the probability that the peptide assignment is a random match to the spectral data: the value is reported as = −10 − log (probability). % Seq Cov indicates the percentage of the protein sequence covered by matching peptides based on the number of matching amino acids over the total number of amino acids in the protein. MW is for Molecular Weight and is expressed in kDa. In the last column the number of peptides mapped for each identified protein is reported.
To exclude that MLC1 distribution in early and recycling organelles and the effects of MLC1 mutations observed in astrocytoma cells were due to tumor cell-specific trafficking pathways and/or overexpression of recombinant proteins we analyzed the distribution of endogenous MLC1 in blood monocyte-derived macrophages (Boor et al., 2005; Durarri et al., 2008; Petrini et al., 2013) that were obtained from healthy donors and MLC patients. In macrophages from healthy donors MLC1 immunoreactivity was present in the cell membrane, the endoplasmic reticulum cisternae and intracellular vesicles where it colocalized with Rab11 and EEA1 (Figs. S3a,d). In macrophages from two MLC patients, each carrying a different MLC1 mutation (see Materials and methods), MLC1 immunoreactivity was almost undetectable in the plasma membrane, as previously reported (Petrini et al., 2013), and showed reduced colocalization with cytoplasmic organelle markers, particularly with Rab11 (Figs. S3b,c,e,f).

**MLC1 traffics through the Rab11⁺ perinuclear recycling compartment**

The data presented above indicate that MLC1 is abundantly distributed in perinuclear vesicles, mainly in Rab11⁺ organelles. Since Rab11⁺ perinuclear organelles are part of the perinuclear (or pericentrosomal) recycling compartment (PNRC) which is important for slow recycling of proteins and storage of membrane proteins, including ion and water channels (Mukherjee et al., 1997; Sheff et al.,...
1999; Ullrich et al., 1996), we investigated trafficking of MLC1 through this compartment. In WT MLC1 astrocytoma cells MLC1 localization in PNRC was confirmed by the finding that MLC1 immunoreactivity overlapped with that of the transferrin receptor (TfR), which is specifically expressed in Rab11+ organelle (Presley et al., 1997; Ullrich et al., 1996) (Fig. 3, colocalization analysis is shown in Fig. S4).

To study MLC1 recycling through the PNRC WT MLC1 astrocytoma cells were treated with bafilomycin, a specific inhibitor of V-ATPase...
which blocks endosomal acidification and leads to inhibition of TIR recycling to the cell membrane through PNRC without affecting TIR endocytosis and transport to the perinuclear compartments (Baravalle et al., 2005; Presley et al., 1993, 1997). Incubation of astrocytoma cells with 100 nM bafilomycin for 3, 6 and 48 h induced a progressive disappearance of WT MLC1 from the plasma membrane and its accumulation in Rab11+ vacuolar structures, as revealed by double immunostaining with anti-MLC1 and anti-Rab11 Abs (Fig. 4a). After 48-h stimulation all the membrane-associated MLC1 were recruited, along with TIR, to Rab11+ cytoplasmic organelles (Fig. 4b). These structures appeared morphologically swollen due to defective acidification, as already described in other bafilomycin-treated cells (Bacac et al., 2011; Baravalle et al., 2005).

When MLC1 mutant expressing astrocytoma cells were analyzed after bafilomycin treatment we found that, similarly to WT MLC1, the plasma membrane-associated S246R protein accumulated in Rab11+ intracellular organelles (data not shown). Conversely, no accumulation of the mutant S280L and C125R proteins, which fail to reach the plasma membrane, was observed in TIR+ or Rab11+ organelles (Figs. S5a,b). Using WB to monitor MLC1 protein levels we found that 48-h treatment with bafilomycin caused accumulation of the membrane-associated dimeric form of WT MLC1 and S246R proteins and a decrease of S280L and C125R mutants (Fig. 5a). Since bafilomycin did not increase MLC1 mRNA in WT MLC1 astrocytoma cells (Fig. S6b), accumulation of WT MLC1 and S246R mutant is likely caused by blockade of protein degradation consequent to bafilomycin-induced arrest of lysosomal function, as previously reported (Baravalle et al., 2005). In contrast, since S280L and C125R mutants are mainly retained in the endoplasmic reticulum (ER), their decrease may be caused by ER associated protein degradation (ERAD) (Duarrì et al., 2008), which is unaffected by bafilomycin treatment (Ishida et al., 2009). To summarize, these data indicate that, similarly to some water and ion channels (McEwen et al., 2007; Takata, 2006; Takata et al., 2008; Van de Graaf et al., 2006, 2008), in astrocytoma cells MLC1 traffics between the plasma membrane and Rab11+ organelles of the PNRC.

**Hyposmosis stimulates MLC1 recycling to the plasma membrane**

MLC1 recycling and the effect of pathological MLC1 mutations on this process were analyzed in hyposmotic conditions that in primary rat astrocytes favor MLC1 transport to the plasma membrane (Brignone et al., 2011; Lanciotti et al., 2012). To this end, MLC1 distribution in the recycling endosomal compartment was analyzed by immunostaining of astrocytoma cells expressing WT or mutated MLC1 with anti-MLC1 and anti-Rab11 Abs in basal conditions and after incubation in hyposmotic solution. A progressive increase in membrane expression of WT MLC1 was observed between 5 and 30 min after exposure to hyposmotic solution, leading to strong labeling of membranes in astrocytic end-feet and thin filopodia and at astrocyte–astrocyte contacts in the majority of MLC1+ cells (Figs. 5a,b). Hyposmotic treatment also induced intracellular redistribution of Rab11+ vesicles from their typical perinuclear localization throughout the cell body and cytoplasmic extensions where they colocalized with MLC1 (Fig. 5b). This result was confirmed by analysis of the fluorescence intensity profile of double stained MLC1+/Rab11+ cells (Figs. 5c,d) and by WB quantitation of hyposmosis-induced translocation of WT MLC1 to the plasma membrane using enriched plasma membrane protein fractions obtained after selective biotinylation of cell surface proteins (Fig. 5e). Interestingly, in the same experiments we found that only the dimeric form of MLC1 (60 kDa) is expressed at the plasma membrane after hyposmotic stress while the monomeric MLC1 component (36 kDa) is not detectable anymore (Fig. 5e), confirming that MLC1 dimerization is important for MLC1 functionality (Brignone et al., 2011).

In contrast, hyposmotic treatment did not induce plasma membrane translocation of the S280L MLC1 mutant protein, representative of MLC1 mutants not expressed in the plasma membrane, as revealed by both immunofluorescence staining and WB analysis of biotinylated proteins (Figs. S7a,b). Immunofluorescence staining of S246R MCL1 astrocytoma cells showed that, similar to WT MLC1, this mutant protein is expressed at the plasma membrane already in control conditions but that its membrane localization does not increase after 30-min incubation in hyposmotic solution (Fig. 5b). Since hyposmosis did not alter MLC1 mRNA levels (Fig. S9) these results demonstrate that the increase in plasma membrane expression of MLC1 WT induced by hyposmotic stress is mainly due to mobilization from intracellular organelles, primarily belonging to the Rab11+ PRNC. The hyposmosis-induced recycling process is hampered by pathological MLC1 mutations, independently of whether the mutated MLC1 protein localizes in the plasma membrane in isosmotic conditions.

**MLC1 is involved in the regulation of early endosome pH**

MLC1 interaction with proteins involved in the regulation of organelle acidification, like V-ATPase and Na, K-ATPase, and the high levels of expression of MLC1 in early and recycling endosomal compartments support the idea of a possible involvement of MLC1 in endosomal function and modulation of organelle acidity. To assess organelle pH in astrocytoma cell lines, we took advantage of the pH-sensitivity of FITC fluorescence emission in dynamic imaging experiments. After 30-min incubation of astrocytoma cells in the presence of FITC-conjugated dextran (see Materials and methods), endocytic vesicles containing the dye could be observed as fluorescence emitting dots. These were mainly localized around the cell nucleus, which was visualized with the chromatin selective dye Hoechst 33258 (Figs. 6a,b), and often clustered on one side of the nucleus.

As expected, membrane permeabilization induced by the ionophore nigericin revealed the pH sensitivity of most FITC-dextran loaded vesicles. When the pH of FITC-dextran loaded vesicles was tested in
Fig. 4. Effect of baflomycin on WT MLC1 protein localization and expression. a. Double immunofluorescence stainings for MLC1 (red) and Rab11 (green) were performed in WT MLC1 astrocytoma cells that were grown in basal culture conditions (CTR) or treated with 100 nM baflomycin (BAF) for 3, 6 and 48 h. Note the progressive disappearance of MLC1 from the plasma membrane and concomitant accumulation of the protein in Rab11+ intracellular vacuolar structures. b. Double immunostaining for MLC1 (red) and transferrin receptor (TfR, green) shows that after 48-h treatment with 100 nM baflomycin, all MLC1 proteins colocalize with TfR in intracellular vacuolar structures. Scale bars: 10 μm.
control astrocytoma cells infected with an empty vector and WT MLC1 overexpressing astrocytoma cells, large variations in pH values were calculated within a single cell (data not shown), suggesting heterogeneity of endocytic vesicles. Interestingly, endocytic vesicles of WT MLC1 astrocytoma cells had a more basic pH (6.76 ± 0.05) while those of cells expressing the S280L and C125R mutants had a more acidic pH (6.37 ± 0.04 and 6.36 ± 0.05, respectively) compared to mock-infected control cells (6.49 ± 0.03). The pH value of endocytic vesicles of S246R mutant astrocytoma cells (6.50 ± 0.02) was undistinguishable from that of control cells (Figs. 6c,d). These findings indicate that WT MLC1 overexpression increases endosomal pH and that this effect is not reproduced by overexpression of mutated MLC1.

To understand whether a specific endosomal compartment is affected by MLC1-induced changes in vesicular pH, WT MLC1 astrocytoma cells pre-loaded for 30 min with FITC-dextran (as described above) were stained with anti-MLC1 Ab and Abs to specific organelle markers (Rab5, EEA1, Rab11, Lamp-2). We observed that FITC-dextran mainly localized in MLC1+ vesicles, in EEA1+ early endosomes (Figs. 7a,b, arrows) and, to a lesser extent, in Rab5+ organelles (Fig. 7c, arrows). In the same cells no colocalization was found between FITC-dextran and Rab11 or Lamp-2 immunoreactivities (Figs. 7d and e, respectively), indicating that the changes in organelle pH measured by FITC-dextran affect predominantly the EEA1+/Rab5+ early endosomal compartment.

In support of these findings, electron microscopy analysis of cytosolic extracts derived from rat primary astrocytes (see Materials and methods) confirmed that MLC1 is abundantly expressed in the membrane of EEA1+ organelles, in EAA1+ intraganelle compartments and in the membranes of Rab5+ vesicles (Fig. S10), a localization compatible with a functional role of endogenous MLC1 in the early endosomal compartment.

**MLC1 overexpression increases transferrin and TRPV4 channel recycling**

Because control of the luminal ionic composition of endolysosomal vesicles is an essential regulatory step for protein sorting to the degradative or recycling compartments (Grant and Donaldson, 2009; Luzio et al., 2007) we next investigated whether MLC1 over-expression, by altering endosomal pH, could influence protein trafficking. Since WT MLC1 overexpression induced a minor acidification of early endosomes (see above), a condition favoring protein sorting toward the recycling pathway (Grant and Donaldson, 2009; Jovic et al., 2010; Yamashiro and Maxfield, 1987 and reference therein) we monitored protein recycling in MLC1 overexpressing astrocytoma cells using a classical assay based on the recycling properties of transferrin (TF). This experiment was performed in basal conditions and after hyposmotic stress which favors MLC1 recycling to the plasma membrane, as shown above. Astrocytoma cell lines expressing WT MLC1, S246R or S280L mutated proteins were incubated with Alexa Fluor 488-conjugated TF at 4 °C to allow binding to surface TfR. After washing out the excess of fluorochrome-labeled TF (T0), we further incubated the cells at 37 °C for 20 min and overnight in the presence of excess unlabeled TF to monitor TF trafficking. By comparing TF-associated fluorescence in WT and mutant MLC1 astrocytoma cells at these different time points we did not find any appreciable difference in TF trafficking among cells expressing WT or mutated MLC1 in control conditions (data not shown). However, when MLC1 expressing astrocytoma cells were pre-incubated in hyposomotic medium for 30 min to activate MLC1 recycling before incubation with Alexa Fluor 488-conjugated TF, we observed that compared to T0, TF-associated fluorescence in WT MLC1 positive cells started to decrease after 20 min (not shown) and disappeared almost completely after overnight incubation, indicating extracellular release of TF molecules, as previously reported (Bacac et al., 2011; Takahashi et al., 2012) (Figs. 8a,d). In contrast, a considerable amount of TF-associated fluorescence was detectable in discrete intracellular aggregates in S246R and S280L MLC1 astrocytoma cells after overnight incubation, suggesting that WT but not mutant MLC1 accelerated TF recycling (Figs. 8b,c,e,f). These results were confirmed by fluorescence intensity analysis with confocal microscope (Fig. 8g).

The above findings prompted us to investigate whether MLC1 could also influence the recycling of known MLC1-interacting proteins. We focused our attention on TRPV4 because, among the MLC1 interactors identified so far, it is the only one for which a functional interaction with MLC1 has been demonstrated (Lanciotti et al., 2012). Preliminary experiments indicated that in WT MLC1 astrocytoma cells TRPV4 is localized in Rab11+ perinuclear vesicles (Fig. S11). Using the TF recycling assay to analyze TRPV4 distribution in astrocytoma cells during recycling we found that at T0 in WT MLC1 cells and in cells carrying S280 MLC1 mutation TRPV4 had a similar distribution, mainly in the cytoplasmic compartments where it partially colocalized with Alexa Fluor 488-conjugated TF (Figs. 9a,c). After 20 min of TF internalization (data not shown) and particularly after overnight incubation (Figs. 9b,d), TRPV4-TF colocalization almost disappeared in WT-MLC1 astrocytoma cells but not in cells expressing the S280L mutant, while TRPV4 still colocalized with TF in discrete vesicles in the cell body and cytoplasmic processes (Fig. 9d, arrows). These experiments suggest that WT and mutated MLC1 can differently influence TRPV4 intracellular localization and recycling. In order to verify whether TRPV4 recycling to the plasma membrane was modulated by MLC1 expression we performed photobleaching assays and WB analysis of cell surface proteins derived from WT MLC1 and S280L overexpressing astrocytoma cells, in control conditions and after hyposmotic stress. These experiments showed that in WT MLC1 cells low levels of TRPV4 were expressed in the plasma membrane in control conditions and that hyposmosis consistently increased TRPV4 translocation to the plasma membrane. In contrast, in mutant expressing astrocytoma cells TRPV4 was never found among biotinylated surface proteins, neither in control nor after hyposmotic stimulation (Fig. 9e). Overall, these results concur to demonstrate that in astrocytoma cells WT, but not mutant MLC1, influences TRPV4 trafficking and plasma membrane expression.

**Discussion**

Understanding the functional role of MLC1 protein in astrocytes and the effects of MLC1 mutations leading to MLC disease is an essential step toward the identification of disease mechanisms and the development of effective therapies for patients affected by this rare childhood-onset leukodystrophy. Using human astrocytoma cell lines stably overexpressing WT or mutated MLC1 proteins to study the localization and function of MLC1 in endosomal organelles, we have uncovered a role for MLC1 in pH regulation and protein trafficking in the endocytic compartment and described the impact of pathological MLC1 mutations on this pathway. Although of tumoral origin, this is a useful and reliable experimental model to study the pathophysiological role of MLC1 as the results obtained so far in human astrocytoma cells have been reproduced in rat primary astrocytes and MLC patient-derived blood cells (Lanciotti et al., 2010; and this study).

*Intracellular MLC1 localizes in early and recycling endosomes and traffics along the Rab11+ perinuclear recycling compartment*

We have shown that WT MLC1, but not mutated MLC1 proteins (C125R, S280L), which show defective plasma membrane localization, is abundantly expressed in early endosomal organelles identified by EEA1 and Rab5 markers (reviewed by Jovic et al., 2010; Maxfield and McGraw, 2004). Of interest, Rab5 also labels primary early endosomal vesicles, both clathrin-coated vesicles (Zerial and McBride, 2001) and caveolin-positive vesicles. The latter are responsible for caveolar-dependent endocytosis (Aoki et al., 2007; Hagiwara et al., 2009) which represents the main endocytosis route for MLC1 and its associated proteins in astrocytes (Lanciotti et al., 2010) before their sorting to EEA1+ endosomes (Fig. 10). In line with the present findings, colocalization of MLC1 and EEA1 was detected in human brain tissue.
The present results indicate that MLC1 also accumulates in perinuclear Rab11+ endosomal vesicles. The latter define a specific intracellular compartment known as perinuclear (or pericentrosomal) recycling compartment (PNRC) where many proteins, including ion and water channels and receptors, are stored and from where these molecules can be mobilized and recruited to the plasma membrane in physiological conditions (Innamorati et al., 2001; Mukherjee et al., 1997; Parent et al., 2009; Sheff et al., 2010).
component is not detectable anymore. One representative experiment out of three performed is shown. Molecular weight markers are indicated on the left (kDa).

Amount of the dimeric (60 kDa) membrane-associated MLC1 component (CTR versus HYPO in Eluate lanes). Note that in the surface protein fraction the monomeric (36 kDa) MLC1 under hyposmotic stress, the

immuno

accumulation (Lanciotti et al., 2010). Because microtubule disruption affects the perinuclear localization of Rab11 + vesicles and of membrane proteins that undergo PNRC-mediated storage and recycling (Baravalle et al., 2005; Vossenkämper et al., 2007), these findings suggest that also in primary astrocytes endogenous MLC1 accumulates in this compartment. In different cell types the presence of perinuclear pools of membrane proteins has been reported to exert an important role in the replenishment of the constitutively internalized proteins and for the maintenance of steady-state surface levels of receptor and transporter proteins like transferrin (Ullrich et al., 1996), thromboxane and dopamine receptors (Li et al., 2012; Thériault et al., 2004), glucose transporters (Ishiki and Klip, 2005; Widmer et al., 2005) and claudin-1 (Dukes et al., 2011). By showing that MLC1 is recycled through this pathway and that its recycling

Fig. 5. Effects of hyposmotic stress on WT MLC1 intracellular traffic. a,b. Astrocytoma cell lines overexpressing WT MLC1 were incubated in control (CTR) or hyposmotic (HYPO) solution for 30 min and then stained with Abs to MLC1 (red) and Rab11 (green). In control cells (a) MLC1 immunoreactivity is found in the cell membrane and in the perinuclear cytoplasm, where it colocalizes with Rab11 + vesicles (merge). Hyposmotic stress (b) induces a marked increase in MLC1 immunoreactivity throughout the cell body and processes, in the plasma cell membrane and at astrocyte-astrocyte contacts (asterisks), and the redistribution of Rab11 + vesicles along the astrocyte cell body and cytoplasmic extensions (arrowheads). Scale bars: 10 μm. c,d. Immunofluorescence pixel intensity along the white dotted arrows drawn in representative cells in a and b was obtained using the profile analysis tool of the LSM 5 PASCAL. After hyposmotic stress, the fluorescence intensity peaks of MLC1 and Rab11 re-distribute from the central perinuclear area, typically observed in control conditions (arrow in c), to a more peripheral cytoplasmic localization and toward the plasma membrane (arrows in d). A strong increase in MLC1 fluorescence intensity is observed after hyposmotic stress (compare red lines in c and d).

e. WB of total cell proteins (Input) and of enriched surface proteins after biotinylation experiments (Eluate) from WT MLC1 astrocytoma cells maintained in control (CTR) and (HYPO) hyposmotic conditions. Although hyposmotic stress does not affect the total amount of WT MLC1 protein (CTR versus HYPO in Input lanes), it strongly increases the amount of the dimeric (60 kDa) membrane-associated MLC1 component (CTR versus HYPO in Eluate lanes). Note that in the surface protein fraction the monomeric (36 kDa) MLC1 component is not detectable anymore. One representative experiment out of three performed is shown. Molecular weight markers are indicated on the left (kDa).

Fig. 6. WT and mutated MLC1 differently regulate endosomal pH. a,b. Fluorescence images of astrocytoma cells pre-loaded with FITC-dextran (green) and the chromatin selective dye Hoechst 33258 (blue), to depict endosomes and nuclei, respectively. b. Higher magnification of the area selected in (a), depicting the polarized localization of FITC-dextran loaded endosomes. Scale bar: 23 μm. c. The bar graph shows the mean ± SEM pH values in control astrocytoma cells infected with empty vector (CTR) and astrocytoma cells overexpressing WT and mutated MLC1; the number of recorded cells for each cell line ranged between 43 and 83. Significant differences between CTR and MLC1 overexpressing cells were calculated using Student’s t test; *P < 0.05. d. The graph shows the time-course of pH changes in labeled endosomes of WT and S280L MLC1 astrocytoma cells recorded in a representative experiment. Note that the pH recorded in the time lag before the application of calibration solutions is more basic in WT compared to S280L MLC1 astrocytoma cells.

1999; Takata et al., 2008; Ullrich et al., 1996). The finding that hyposmosis increases the mobilization of Rab11 + /MLC1 + vesicles from perinuclear areas toward astrocytic plasma membrane and end-feet suggests the presence of an intracellular pool of MLC1 that is capable of recycling back to the cell surface in stress conditions. We also observed accumulation of MLC1 in Rab11 + and TR + organelles after astrocytoma cell treatment with the V-ATPase inhibitor bafilomycin that selectively inhibits protein trafficking along the degradative-lysosomal pathway but not the transport of TR to PNRC (Baravalle et al., 2005). This finding confirms Rab11 + vesicle-mediated bidirectional trafficking of MLC1 from the plasma membrane to the PNRC and vice versa. Interestingly, in a previous study we observed that also in rat primary astrocytes endogenous MLC1 was localized in perinuclear vesicles and that treatment with nocodazole, a drug which disrupts microtubule organization, abolished MLC1 perinuclear accumulation (Lanciotti et al., 2010). Because microtubule disruption

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proteins that undergo PNRC-mediated storage and recycling (Baravalle et al., 2005; Vossenkämper et al., 2007), these findings suggest that also in primary astrocytes endogenous MLC1 accumulates in this compartment. In different cell types the presence of perinuclear pools of membrane proteins has been reported to exert an important role in the replenishment of the constitutively internalized proteins and for the maintenance of steady-state surface levels of receptor and transporter proteins like transferrin (Ullrich et al., 1996), thromboxane and dopamine receptors (Li et al., 2012; Thériault et al., 2004), glucose transporters (Ishiki and Klip, 2005; Widmer et al., 2005) ion and water channels like Kv1.5 (McEwen et al., 2007), AQP2 (Nedvetsky et al., 2007; Takata, 2006), TRPV5-6 (Van de Graaf et al., 2006, 2008) and junctional proteins, like E-cadherin (Balzac et al., 2005) and claudin-1 (Dukes et al., 2011). By showing that MLC1 is recycled through this pathway and that its recycling
toward the plasma membrane is stimulated by hyposmosis, this study unveils the importance of endosomal recycling in the regulation of MLC1 function and impairment of this process by pathological MLC1 mutations.

Localization of endogenous MLC1 in EEA1+ and Rab11+ organelles and the effects of pathological mutations on MLC1 intracellular distribution were also observed in monocyte-derived macrophages from healthy donors and MLC1 mutated patients. These findings allow us to

Fig. 7. Characterization of FITC-dextran positive vesicles in WT MLC1 astrocytoma cells. WT MLC1 astrocytoma cells were pre-loaded with FITC-dextran for 30 min to identify endosomes in which pH changes have been recorded (see Fig. 6) and then labeled with anti-MLC1 Ab or Abs specific for endosomal organelles. a,b. MLC1 (red, a) and EEA1 (red, b) immunoreactivities are found in FITC-dextran positive vesicles (green) (arrows). c. Immunostaining for Rab5 (red) reveals a slightly lower degree of localization in the FITC-dextran positive vesicles (green) (arrows). d, e. No overlap is found between FITC-dextran (green) and Rab11 (red, d) or Lamp-2 (red, e) immunoreactivities. Scale bars: 10 μm.
exclude that the intracellular distribution and trafficking properties of WT MLC1 as well as the defects induced by MLC1 mutations in astrocytoma cells might be due to MLC1 overexpression or to the tumoral nature of the cells.

**MLC1 is involved in the regulation of early endosome acidity**

The identification of V-ATPase and Na, K-ATPase, also known to regulate early endosome pH, as proteins interacting with MLC1 (this study and Brignone et al., 2011, respectively) led us to hypothesize a role for MLC1 in the control of organelle pH. FITC-dextran measurement coupled with vesicle immunostainings revealed a decrease in the acidification of early endosomes (EEA1+ and Rab5+) in WT MLC1 astrocytoma cells compared to mock infected control cells. Importantly, overexpression of all 3 pathological MLC1 mutants (S246R, S280L, C125R) did not increase endosomal pH, indicating altered regulation of this pathway in MLC disease. Because the S246R mutation does not affect membrane expression of MLC1, these data suggest that reduced MLC1 localization in the plasma membrane is not the sole indicator of a pathological phenotype. Altogether, these data confirm the hypothesis that MLC1 is involved in the regulation of organelle acidity, possibly by limiting vesicle acidification. A similar behavior has been described for the Na, K-ATPase which exerts an essential role in the maintenance of the slightly acidic pH typical of early endosomes in which it is localized (Cain and Murphy, 1988; Cain et al., 1989; Feldmann et al., 2007; Grabe and Oster, 2001) and whose direct interaction with MLC1 has been demonstrated by our group (Brignone et al., 2011). Future experiments will aim to clarify the exact mechanisms through which MLC1 can influence endosomal pH and the molecular and functional relationships between MLC1 and V-ATPase. Because it is known that abnormal acidification can lead to endosome enlargement (Bacac et al., 2011; Forgac, 2007; Martina et al., 2009), these data lead to the hypothesis that the formation of intracellular vacuoles observed in MLC1 silenced rat astrocytes and in astrocytes in the brain of MLC patients (Duarri et al., 2011) might be due to endosome swelling caused by dysregulation of organelle pH.

The mildly acidic pH of the endocytic pathway is strictly regulated by a variety of ion channels, transporters and exchangers present in the endosomal membranes (Scott and Gruenberg, 2011) where also endogenous MLC1 is localized (Fig. S10). Along with V-ATPase other proteins

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**Fig. 8.** Transferrin recycling assay in WT and mutant MLC1 astrocytoma cell lines after hyposmotic stress. Astrocytoma cell lines expressing WT MLC1, S246R or S280L mutated protein were pre-incubated with Alexa Fluor 488-conjugated transferrin (Tf) at 4 °C to allow binding to surface TfR. After washing out labeled Tf (T0), cells were incubated at 37 °C overnight (ON) in the presence of excess unlabeled Tf and then stained with anti-MLC1 Ab (red). a,b,c. Alexa Fluor 488-conjugated Tf (green) shows comparable binding to the surface of WT and mutated MLC1 astrocytoma cells (T0). d,e,f. After overnight (ON) incubation with excess unlabeled Tf, Tf disappears almost completely from the surface of WT MLC1 astrocytoma cells (d), whereas it is still present in S246R and S280L mutant astrocytoma cells (e,f). Scale bars: 10 μm. g. The bar graph shows the mean ± SEM values of the Alexa Fluor 488-conjugated Tf fluorescence intensity in the different astrocytoma cell lines after ON incubation; 10 to 15 random fields (field area = 230 μm²) were analyzed. Significant differences between WT MCL1 and mutated (S246R, S280L) astrocytoma cells were calculated using Student’s t test; *P < 0.05. **P < 0.005. ***P < 0.0005.
can influence vesicle acidification, including different components of the transient receptor potential (TRP) type, of the two pore (TPC) type of calcium channels (Abe and Puertollano, 2011; Martina et al., 2009; Morgan et al., 2011) and of the chloride channels, that mainly control late/lysosomal compartment acidity (Edwards and Kahl, 2010; Faundez and Hartzell, 2004). We cannot exclude that, in addition to an effect on the activity of its molecular interactors (V-ATPase, Na,K-ATPase), MLC1 may function itself as an ion channel and directly influence organelle proton influx.

MLC1 influences protein recycling

Early endosome organelles constitute the sorting station where the fate of internalized proteins and lipids is decided by a complex interplay of molecular and structural determinants mainly leading to changes in intra-organelle pH (Jovic et al., 2010). There is broad evidence that the acidification of endocytic vesicles is essential for the regulation of uncoupling of receptor–ligand complexes, intracellular membrane flow and protein sorting toward the recycling or degradative pathway (Jovic et al., 2010; Luzio et al., 2007) and that inhibition or overexpression of proteins involved in the regulation of endosomal pH affects protein trafficking (Bacac et al., 2011; Hara-Chikuma et al., 2005; Le Louvier and Puertollano, 2011; Martina et al., 2009; Smith and Lippiat, 2010). Our experiments indicate that WT but not mutated MLC1 favors transferrin and TRPV4 channel recycling. The present results are in agreement with a functional role of MLC1 in endosomal pH regulation, since recycling vesicles are characterised by a slightly less acidic pH compared to vesicles sorted toward the lysosomal degradative pathway (Grant and Donaldson, 2009; Scott and Gruenberg, 2011). The demonstration that S280L and S246R MLC1 mutations do not promote transferrin and TRPV4 channel recycling supports the hypothesis that pathogenic MLC1 mutations influence endosomal maturation and protein sorting decision.

We also show for the first time that TRPV4 is expressed in Rab11+ perinuclear vesicles and is subjected to recycling pathway, as previously reported for TRPV5 and TRPV6, two transient receptor cation channels belonging to the same family of vanilloid-type of transient receptors (Van de Graaf et al., 2006). The influence of MLC1 on the recycling rate of its molecular partner TRPV4 could explain the molecular mechanism underlying the functional effect of MLC1 on the TRPV4-mediated calcium influx that we have recently described (Lanciotti et al., 2012). Defective TRPV4 recycling in hyposmotic conditions in MLC1 mutant expressing astrocytoma cells could be responsible for a dysfunctional astrocyte response to osmotic stress. The analysis of proteins exposed to hypotonic stress in WT and S280L MLC1 astrocytoma cell lines is shown in Fig. 9. Astrocytoma cell lines expressing WT or mutated S280L MLC1 proteins were incubated with Alexa Fluor 488-conjugated Tf at 4 °C to allow binding to surface TIR. After washing out labeled Tf (T0), cells were incubated at 37 °C for 20 min and overnight (ON) in the presence of excess unlabeled Tf and then stained with anti-TRPV4 pAb. AC TRPV4 (red) and Alexa Fluor 488-conjugated Tf (green) partially colocalize in intracellular vesicles in WT MLC1 and S280L mutant astrocytoma cell plasma membranes at T0. b.d. After ON incubation with unlabeled Tf, TRPV4-Tf colocalization disappears in WT MLC1 astrocytoma cells (b) but not in S280L astrocytoma cells where TRPV4 still colocalizes with Tf in clustered intracytoplasmic vesicles (d, arrows). Scale Bars: 10 µm. e. WB of total cell proteins (input lanes) and of enriched surface proteins after biotinylation (eluates) reveals that 30-min incubation in hypotonic solution induces an increase in surface expression of TRPV4 in WT MLC1 astrocytoma cells but not in cells expressing S280L mutation. Molecular weight markers are indicated on the left (kDa).

Fig. 10. Schematic representation of MLC1 intracellular trafficking. A model of MLC1 intracellular trafficking is proposed on the basis of our previous results (Lanciotti et al., 2010) and the data presented in this paper. MLC1 is internalized via caveolae-mediated endocytosis and traffics through Rab5+ and EEA1+ early endosomes where it is sorted to the recycling or degradative pathway. Most of the intracellular MLC1 protein is stored in the perinuclear Rab11+ recycling vesicles from which it is recycled to plasma membrane in stress condition (Hyposmosis).
on the cell surface in basal and hypoxic conditions revealed a marked reduction in plasma membrane localization of TRPV4 in mutant MLC1 expressing astrocytoma cells. Interestingly, defects in TRPV4 function have been recently reported to be responsible for cyst formation in the autosomal recessive polycystic kidney disease (Zaika et al., 2012), suggesting that also in MLC disease cyst formation could be caused by TRPV4 functional alterations. To date, TRPV4 is the only identified MLC1 interactor whose activity is modulated by MLC1 in rat astrocytes and human astrocytoma cells (Lanciotti et al., 2012). Recently, mutations in the gene encoding Hepacam/Glialcam, an adhesion molecule of unknown function, have been found in a considerable percentage (>50%) of MLC patients without mutations in MLC1 and Hepacam/Glialcam protein has been reported to regulate specifically MLC1 expression at astrocyte–astrocyte junctions (Duarri et al., 2011). WB analysis and biochemical assays indicated that Hepacam/Glialcam is expressed in astrocytoma cell lines and binds MLC1, and that MLC1 mutations differently affect this interaction (Lanciotti et al., 2012). Further studies are needed to clarify MLC1–Hepacam/Glialcam interaction in the astrocytoma cell model.

**Conflict of interest statements**

The authors have no conflicting financial interests.

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**Appendix A. Supplementary data**

Supplementary data can be found online at http://dx.doi.org/10.1016/j.nbd.2014.02.003.

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Boor, J., Provero, P., Stamenkovic, I., 2011. Securin and separase modulate membrane trafficking following changes in brain homeostasis induces or amplifies brain damage in MLC patients carrying mutated MLC1 genes. It is known that some junction proteins are continuously being endocytosed and recycled back to the plasma membrane (Chalmers and Whiteley, 2012; Dukes et al., 2012) via caveolar-dependent internalization and Rab11 recycling pathway (Descoleaux et al., 2008; Dukes et al., 2011; Nottage and Bilslyker, 2012) and that their traffic can be modulated by the activity of ion channels responsible for endosomal acidification (Nottage and Bilslyker, 2012). Abnormal recycling of junction proteins can lead to the formation of intracellular vacuoles (Dukes et al., 2012) similar to those observed in the brain of MLC patients (Duarri et al., 2011). We hypothesize that MLC1-mediated recycling becomes relevant when MLC1 is recruited to the plasma membrane due to an increased functional demand of the astrocyte. In particular, regulation of MLC1 in the astrocyte membrane could be critical for the astrocyte response to changes in extracellular osmolarity.

Endocytosis and recycling are essential processes in the regulation of the expression of cell surface molecules that mediate glial cell differentiation and neuronal–glial interactions during brain development (Chen et al., 2011; Shilo and Schegert, 2011; Yap and Winkleler, 2012). Modification of these processes may result in brain oedema and disturbance of myelin formation, which are observed also in other leukodystrophies associated with specific defects in astrocyte maturation and function (Bugiani et al., 2011; Messing et al., 2012). By increasing our understanding of the molecular mechanisms that lead to brain damage in MLC patients and could be shared with other leukodystrophies, this study opens new perspectives for the development of therapeutic targets.

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