"SAPIENZA"
UNIVERSITÀ di ROMA
Facoltà di Scienze Matematiche Fisiche e Naturali

"Pathophysiological role of MLC1, a protein involved in megalencephalic leukoencephalopathy with subcortical cysts"

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Contents

Abstract

Megalencephalic leukoencephalopathy with subcortical cysts (MLC), is a rare congenital and incurable leukodystrophy characterized by macrocephaly, subcortical fluid cysts and myelin vacuolation. The majority of MLC patients carry mutations in the *MLC1* gene encoding a membrane protein named MLC1 that is highly expressed in brain astrocytes contacting blood vessels, ependyma and meninges. Although the neuropathological features of MLC disease, the molecular structure and the cellular localization of MLC1 suggest a possible involvement of this protein in astrocyte-mediated osmoregulatory processes, the function of MLC1 is still unknown. Understanding the function of MLC1 protein whose mutations are the main cause of MLC is an essential step toward identification of disease mechanisms and development of effective therapies.

During the course of this thesis project we generated new data on MLC1 expression, distribution and functional associated pathways in astrocytes that are deregulated by pathological mutations, paving the way for the identification of the specific MLC1 function. We found that: i) endogenous MLC1 protein is expressed in cultured astrocytes, particularly in the plasma membrane where it interacts with caveolin-1 and proteins of the dystrophin/dystroglycan complex (DCG), and also in intracellular organelles and endoplasmic reticulum (Lanciotti et al., 2010); ii) MLC1 undergoes endolysosomal trafficking and, most of the missense mutations found in patients hamper MLC1 intracellular trafficking and localization at the plasma membrane (Lanciotti et al., 2010, 2012); iii) MLC1 directly binds the beta-1 subunit of the Na, K-ATPase enzyme and is part of a multiprotein complex that includes the inward rectifying potassium channel 4.1 (Kir4.1), caveolin-1 and syntrophin, and is involved in astrocyte response to hyposmotic stress (Brignone et al., 2011). Moreover, we generated a human pathological model based on astrocytoma cell lines overexpressing wild-type (WT) MLC1 or MLC1 carrying pathological mutations. Using this new MLC disease model we found that WT, but not mutated MLC1, functionally interacts with the transient receptor potential cation channel-4 (TRPV4) to activate swelling-induced calcium influx in astrocytes during hyposmotic stress (Lanciotti et al, 2012). These findings, together with a recent study showing defects in a chloride current in patient-derived lymphoblast cell lines subjected to hyposmosis (Ridder et al, 2011), represent

the first evidence that the MLC1 protein is involved in the molecular pathways regulating astrocyte response to osmotic changes.

Introduction

1. Leukoencephalopathies

The term leukoencephalopathy is a broad term used to classify the heterogeneous group of rare genetic diseases that cause childhood-onset cerebral white matter disorders, with either primary or secondary changes in myelin development or maintenance (Blumkin et al., 2009). Clinically, the typical child-disease with white matter abnormalities is characterized by the onset of symptoms within the first year of life, most often presenting as general developmental psychomotor delay and hypotonia. Later-appearing signs are spasticity and ataxia and serious ophthalmological abnormalities, as a rule severe learning and motor disabilities. However, these symptoms are not general and some variations of them, such as age at onset, severity of mental retardation and motor impairment, as well as the extension of white matter alterations, are also noted within this heterogeneous group of disorders (Kristjansdottir et al., 2000).

The diagnostics of cerebral white matter disorders was revolutionized by the advent of magnetic resonance imaging (MRI) in the late 1980s. MRI is very sensitive in showing macroscopic structural changes in the white matter: first, it can be used to show gross morphology and the involvement of different brain structures or tissues; second, to monitor the progression of the disease or the response to therapy; finally, the pattern of abnormal MRI findings, which can be typical for a particular disease type, can aid in the diagnosis, as summarized in Table I (adapted from Di Rocco et al., 2004). In fact, by the identification of specific "MRI pattern recognition" (van der Knaap et al., 1995), some new genetic diseases, such as megancephalic leukoencephalopathy with subcortical cysts (MLC) and vanishing white matter/childhood ataxia with central hypomyelination (CACH), have recently been identified (Di Rocco et al., 2004). So, MRI has provided a powerful tool for investigating the cerebral white matter in living individuals. Its increased use for the diagnosis of children with neurological impairment has expanded our knowledge about genetic disorders affecting white matter, contributing to the identification, localization and characterization of underlying white matter abnormalities in affected patients.

1.1 Leukoencephalopathies' classification.

The term leukoencephalopathy is applied to all white matter diseases of known and unknown origin. Although magnetic resonance imaging (MRI) has greatly improved the diagnosis of leukoencephalopathies, most of these diseases are still difficult to be recognised and almost 50% of them are of unknown cause. The white matter abnormality may appear similar in different forms of leukoencephalopathy so that in most cases, further investigations such as magnetic resonance spectroscopy, tissue biopsies, enzyme studies and molecular DNA analyses are needed to pinpoint a more accurate diagnosis. A specific diagnosis allows the physician to give prognostic information, monitor for known complications and ultimately may allow disease-specific therapeutic strategies. Therefore a specific protocol for studying and categorizing these patients is crucial.

Different ways to classify leukoencephalopathies are available, based on either pathological, biochemical, genetic data, or combined clinical and histological/biochemical criteria.

The well-defined leukoencephalopathies can be divided into the following categories on the basis of the myelin damage typology: (1) dysmyelinating, showing abnormally formed myelin; (2) hypomyelinating, with decreased myelin production; and (3) spongiform, with cystic degeneration of myelin (Kaye, 2001).

A classification based on the biochemical defect observed can also be used to classify leukoencephalopathies and it encompasses (1) lipid disorders, (2) myelin protein disorders, (3) organic acids disorders, (4) defects of energy metabolism (associated with lactic acidosis), (5) other causes, and (6) unknown causes (Table II) (Kaye, 2001).

By using an integrated MRI and pathophysiological approach, as proposed by van der Knaap (2001), pediatric white matter disorders can be distinguished into well-defined leukoencephalopathies, and undefined leukoencephalopathies.

The second category, that represents up to 50% of leukoencephalopathies in childhood, requires a multidisciplinar approach (clinical, neuroradiological and electrophysiological analysis) in order to define novel homogeneous subgroups of patients for identifying their molecular or biochemical defects (Di Rocco et al., 2004; Blumkin et al., 2009). These classifications will certainly change as the molecular mechanisms for more leukoencephalopathies become elucidated.

Table I	**Classification of the most diffuseleucoencephalopathy** (*adapted from Di Rocco et al., 2004).*

Disorder	Classification	Genetic defect	MRI findings
Pelizaeus–Merzbacher disease	**Hypomyelinating**	Mutations in the proteolipid protein 1 (*PLP1)* gene	Arrested myelination (however normal for that stage); high signal intensity on T2-weighted images within unmyelinated WM; reduced WM volume, enlarged ventricular system, thin corpus callosum; brain atrophy mainly in the brainstem and cerebellum
Metachromatic leukodystrophy	**Dysmyelinating**	Mutations in the arylsulfatase A (*ARSA)* gene	Symmetrical periventricular WM abnormalities; early sparing of the arcuate fibers; involvement of the corpus callosum; brain atrophy in the advanced stages of disease
X-linked adrenoleukodystrophy	**Dysmyelinating**	Mutations in the ATP-binding cassette transporters (*ABCD1)* gene	Extensive WM lesions in the occipital regions with involvement of the splenium of the corpus callosum and sparing of the occipital arcuate fibers; early involvement of the pyramidal and occipito-parieto-temporo-pontine tracts are involved early
Globoid cell leukodystrophy	**Dysmyelinating**	Mutations in the galactosylceramidase (GALC) gene	Demyelination of the deep WM, progressively involving the subcortical white matter. Calcifications within the thalami, basal ganglia, and corona adiate shown by CT scan
Alexander disease	**Spongiform with cystic degeneration**	Mutations in the glial fibrillary acid proteic (*GFAP*) gene	Extensively abnormalWM with swollen appearance and cavitations, namely in the frontal lobes; abnormalities of basal ganglia, thalami, and brainstem; positive enhancement
Canavan disease	**Spongiform**	Mutations in the aspartoacylase (*ASPA)* gene	Subcortical cerebral and cerebellar WM are predominately involved with a swollen appearance; later involvement of the central WM; bilateral involvement of the globus pallidus and of the thalamus with sparing of the caudate nucleus and putamen; cerebral and cerebellar atrophy; marked increased NAA peak on MR spectroscopy
Megalencephalic leukoencepahlopathy with subcortical cysts	**Spongiform**	Mutations in the *MLC1 or HEPACAM* gene*	Diffusely abnormal, swollen cerebral WM with subcortical cysts in the anterior-temporal regions; sparing of the central WM; normal gray matter structures
Vanishing white matter (VWM or CACH**)	**Spongiform**	Mutations in the Eukariotic Initiation Factor 2 (*EIF-2B)* gene	Symmetric, diffuse involvement of the cerebral hemispheric WM, including the central tegmental tracts; cystic degeneration of WM that is replaced by CSF; cerebellar atrophy

WM, white matter.

* MLC1: Megalencephalic leukoencepahlopathy with subcortical cysts; HEPACAM: hepatic and glial cell adhesion molecule,

** CACH: Childhood ataxia with central nervous system hypomyelination

Table II. Biochemical classification of the leukoencephalopathies (*adapted from Kaye, 2001)*	
Lipid	Adrenoleukodystrophy Globoid cell leukodystrophy Metachromatic leukodystrophy Sjo¨gren-Larsson syndrome Cerebrotendinous xanthomatoses
Myelin Proteins	Pelizaeus-Merzbacher disease Myelin basicprotein deficiency
Organic Acid Disorders	Canavan's disease
Energy Defects	Progressive cavitating leukoencephalopathy Type IV glycogenstorage disease questa
Other Causes	CADASIL(Cerebral Autosomal Dominant arteriopathy with subcortical infarcts and leukoencephalopathy) Merosin deficiency
Unknown biochemical mechanism	Alexander's disease CACH (vanishing white matter) VLE (vacuolating leukoencephalopathy) Aicardi-Goutieres syndrome MLC (Megalencephalic leukoencepahlopathy with subcortical cysts)

1.2 White matter and Myelin: pathological targets in leukoencephalopathies

The central nervous system is composed of two different types of tissue: the grey matter and the white matter. The grey matter, which has a pinkish-grey color in the living brain, contains the cell bodies, dendrites and axon terminals of neurons; while the white matter appears pinkish-white to the naked eye because myelin is composed largely of lipid tissue veined with capillaries. Its white color is due to its usual preservation in formaldehyde. The white tissue is mainly composed of nerve cell processes, or axons, which connect various grey matter areas of the brain and carry nerve impulses and are insulated with the layer of proteic and fatty substance named myelin. On a dry weight basis, 40-50% of white matter is myelin, which gives white matter its color and is responsible for the fast nerve conduction (Baumann and Pham-Dinh, 2001). The white matter tissue is the main target for the brain damages in leukoencephalopathies whose genetic defects can lead to delayed and disturbed myelin formation, progressive demyelination or myelin structural alterations, like cystic degeneration, either with myelin loss or without myelin loss.

In the well-defined leukoencephalopathies pathological damages can be due directly to genetic alterations of myelin specific structural proteins or also secondary due to dysfunction of neurons or of *non neuronal* types of cells named glial cells.

Indeed all neural tissues, both white and grey matter, contain two major classes of cells: nerve cells (neurons) and glial cells (Figure 1.1). Glial cells constitute the large majority of cells in the nervous system and are necessary for neuronal development and myelin formation and for the correct functionality of mature neurons. Moreover they account for the maintenance of the general brain homeostasis conditions and for immune response. Glial cells can be divided into two major classes: *macroglia* which encompass ependymal cells, astrocytes and oligodendrocytes and *microglia* which represent the brain resident macrophages.

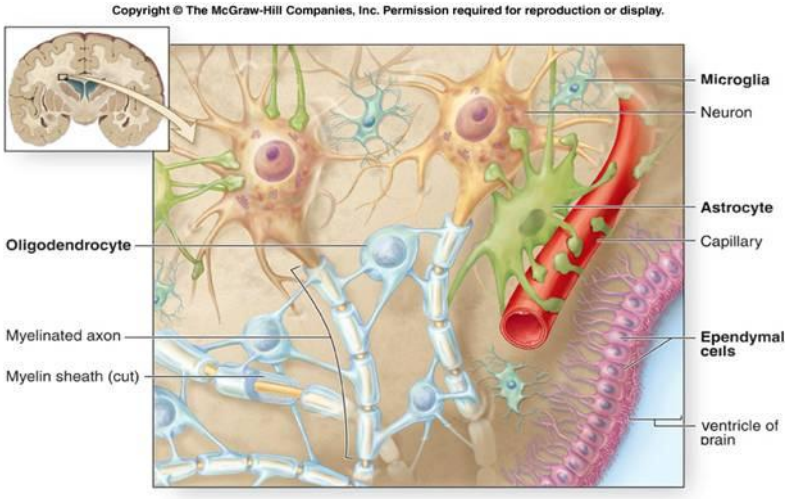

Fig.1.1 Cellular Organization in Neural Tissue. Neural tissue contains two distinct cell types: nerve cells, or neurons, and supporting cells, or neuroglia. Neurons are responsible for the transfer and processing of information in the nervous system, while neuroglia for isolating the neurons, for trophic and structural support to neurons and for immune response in the brain.

1.2.1 The myelin forming cells and myelin structure

The myelin sheath is crucial to propagate electrical signals more efficiently (Kandel E.R. et al., 2000). In the CNS, myelin is formed by differentiation of the plasma membrane of a glial cell component: the oligodendrocytes. Oligodendrocyte damages and alteration of their functionality are often found as primary pathogenic causes for leukoencephalopathies. Moreover, the other glial component astrocytes and many soluble factors (Di Rocco et al., 2004) are essential for the correct myelin development and maintenance. Recently mutations in astrocyte specific proteins and astrocyte dysfunctions have been recognised as the leading cause of some cystic leukodystrophies (Brenner et al., 2001) among which MLC disease (van der Knaap et al., 2012). For these reasons the main structural and functional features of these two types of glial cells will be discussed in the next sections.

1.2.2 Oligodendrocytes

The term oligodendroglia was introduced by Rio Hortega to describe those neuroglial cells that show few processes in material stained by metallic impregnation techniques. Oligodendrocytes are cells highly specialized to myelinate central nervous system axons. An oligodendrocyte extends many processes, each of which contacts and repeatedly envelopes a stretch of axon and forms a double membrane structure called the mesaxon, which elongates and spirals around the axon in concentric layers

(Baumann and Pham-Dinh, 2001), with subsequent condensation of this multispiral membrane-forming myelin. On the same axon, adjacent myelin segments are formed by different oligodendrocytes. The number of processes that form myelin sheaths from a single oligodendrocyte varies according to the area of the CNS and possibly the species, from 40 in the optic nerve of the rat to 1 in the spinal cord of the cat (Baumann and Pham-Dinh, 2001 and references therein). Myelination by oligodendrocytes begins when axons to be myelinated acquire a diameter of approximately 1 μm (Morell P., 1984). A small amount of myelin is formed in humans before birth, but most myelin develops after birth, during the first two years of life. Myelination of the cerebral hemispheric white matter continues until adolescence (van der Knaap M. S. and Valk J., 2005). Its unique composition and its unique segmental structure responsible for the saltatory conduction of nerve impulses allows the myelin sheath to support the fast nerve conduction in the thin fibers in the vertebrate system. It is present both in the central and in the peripheral nervous system (PNS), but the myelin-forming cells and composition of myelin differ between these two systems. Schwann cells are the supporting cells of the peripherical nervous system; like oligodendrocytes Schwann cells wrap themselves around nerve axons, but the difference is that a single Schwann cell makes up a single segment of an axon's myelin sheath. The oligodendrocyte is not alone a myelin forming cell, but there are also satellite oligodendrocytes that may not be directly connected to the myelin sheath. Satellite oligodendrocytes are perineuronal and may serve to regulate the microenvironment around neurons (Ludwin, 1997).

1.2.3 Myelin: structure and composition

The insulating properties of the myelin sheath, which favours rapid nerve conduction velocity, are largely due to its structure, its thickness, its low water content, and its richness in lipids (Baumann and Pham-Dinh, 2001).

Each myelin sheath is composed of multiple segments of myelin, which are modified extensions of oligodendroglial cell process, surrouding a portion of an axon in a spiral fashion (Figure 1.2) (Sherman and Brophy, 2005).

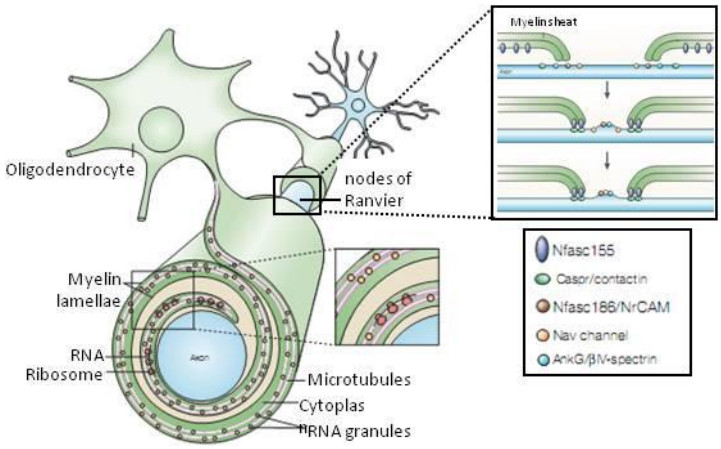

Fig.1.2 The relationship between an oligodendrocyte and myelinated nerve fiber in the CNS. An oligodendrocyte extends and surrounds neighbouring axons and the myelinating process are composed in a spiral fashion. At node of Ranvier myelination causes clustering of voltage-gated sodium channels (adapted from Sherman and Brophy, 2005).

A structural feature that characterizes myelin sheath is its periodic structure, with alternating concentric electrondense and light layers: the major dense line (dark layer) is formed by the close apposition of the internal faces followed by the extrusion of the cytoplasm of the expanding myelinating processes of the oligodendrocyte; the apposition of the external faces of the membrane of the myelinating cell forms the intraperiodic lines (or minor dense lines) (Figure 1.3). The concentric layers that forms a sheath around the axon are called lamellae. The several sections of myelin sheaths are separated from each other by small unmyelinated segments in which the bare axon is exposed to the interstitial space. These segments, called nodes of Ranvier, contain a cluster of voltage-gated sodium channels (Figure 1.2). The periodicity of the lamellae is 12 nm and each myelin sheath segment or internode appears to be 150–200 μm in length. When the axon membrane is excitated, the electrical impulse cannot flow through the high resistance myelin sheath, but flows out through and depolarizes the axonal membrane at the next node. This salutatory conduction allows the fast nerve conduction at a lower energy cost that is crucial for functional integration of the CNS.

Common in the PNS, but rare in the CNS are the Schmidt-Lanterman clefts (also called Schmidt-Lanterman incisures), regions within compact myelin in which the cytoplasmic faces of the enveloping myelin sheath are not tightly juxtaposed to form the major dense line, and include cytoplasm from the cell responsible for making the myelin. Myelin is less compacted at the inner and outer end of the spiral, forming inner and outer loops that retain small amounts of oligodendrocyte cytoplasm near the node of Ranvier. These little expanded loops are arranged on a roughly regular and symmetric pattern on each

side of the node, forming a sequence of loops, termed the paranodal region or paranode, which makes contact with the axon.

The importance of myelin in human development is highlighted by its involvement in an array of different neurological diseases such as leukodystrophies and multiple sclerosis (MS) in the CNS and peripheral neuropathies in the PNS (Sherman and Brophy, 2005).

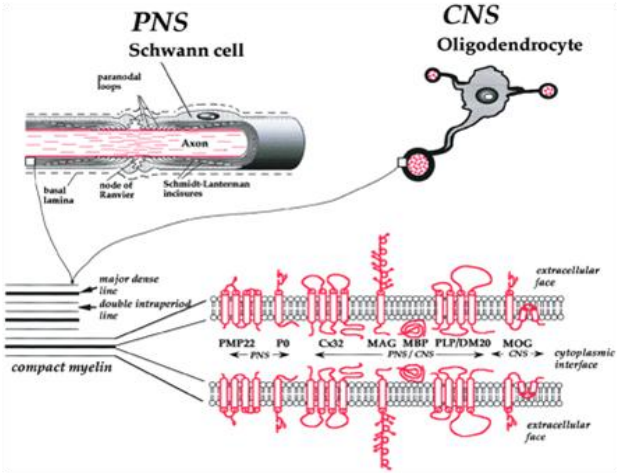

Fig.1.3 Myelinating glial cells, myelin structure, and composition in the PNS and in the CNS. In the PNS, the myelinating Schwann cell myelinates only one segment of axon, whereas in the CNS the oligodendrocyte is able to myelinate several axons. The compact myelin is formed by the apposition of the external faces of the membrane of the myelinating cell, forming the "double intraperiodic line"; the apposition of the internal faces followed by the extrusion of the cytoplasm, form the "major dense line" (adapted from Baumann and Pham-Dinh, 2001).

Compared with other membranes, the composition of myelin is unique, with low water content (40%) and a very high percentage of lipids compared with the plasma membranes of most eukaryotic cells. On a dry weight basis, myelin contains 70% lipids and 30% proteins, all formed in the oligodendrocytes. This lipid-to-protein ratio is very peculiar to the myelin membrane, while it is generally the reverse in other cellular membranes (Baumann and Pham-Dinh, 2001).

The lipid layers are composed of cholesterol, phospholipids, glycolipids, and hydrocarbon chains. Glycolipid (galactocerebroside and sulfatide) and cholesterol are in the outer layer of the membrane, exposed to the extracellular space. Phospholipids are

hydrophobic and are located in the inner (cytoplasm) side of membrane. The area between outer and inner membrane layers is composed primarily of hydrocarbon chains (long chain fatty acids).

Most myelin proteins are unique to myelin. Each protein is localized at a specific site in the myelin membrane, reflecting its function. The two major structural proteins of myelin are proteolipid protein (PLP) and myelin basic protein (MBP), accounting for 50% and 30% of the total protein, respectively. Both proteins play a role in maintaining stability and periodic structure of myelin: MBP is necessary for myelin compaction, adhering the cytoplasmic leaflets of the myelin membrane, while PLP (and its isoform DM20) stabilizes the intraperiod line of the membrane (Campagnoni and Skoff, 2001). However the pathobiology of myelin mutants reveal novel biological functions of the MBP and PLP genes The non-classic MBP gene products appear to be components of transcriptional complexes in the nucleus, and they also may be involved in signaling pathways in T-cells and in neural cells. The non-classic PLP/DM20 gene products appear to be components of intracellular transport vesicles in oligodendrocytes. There is evidence for other functions of the classic PLP/DM20 proteins, including a role in neural cell death mechanisms, autocrine and paracrine regulation of oligodendrocytes and neurons, intracellular transport and oligodendrocyte migration (Campagnoni and Skoff, 2001). In addition, a few other myelin proteins have been identified: myelin-associated glycoprotein (MAG), which is localized in the periaxonal myelin membrane and is the mediator of the axonal-glial contacts, essential for the initiation of myelination; myelin-oligodendrocyte glycoprotein (MOG), which is localized on the outside surface of the myelin sheaths and it is thought to transmit signals from the extracellular environment and to be an important target in autoimmune demyelinating diseases (Quarles, 2002.); and the 2,3-cyclic nucleotide 3-phosphodiesterase (CNP), only found in non compact parts of myelin (e.g. in paranodal loops), that seems to be not involved in myelin assembly but essential for axonal survival (Lappe-Siefke et al., 2003; Di Rocco et al., 2004).

1.3 Astrocytes

Among the central nervous system (CNS) glial population, astrocytes are the most numerous components and account for a large portion of total brain volume (20-50%). Their name was coined by Michael von Lenhossek who called "astrocytes" the star-shaped cells found in the CNS. Generally astrocytes are divided into two types, fibrous and protoplasmic, similar in function but distinct in morphology and distribution (Kandel E.R. et al., 2000). The fibrous astrocytes contain many filaments and are found in the white matter areas. Protoplasmic astrocytes are found in gray matter and are characterized by many short, thick and highly branched processes associated with nerve cell bodies, dendrites and particularly synapses (Kandel E.R. et al., 2000). Both types of astrocytes form gap junctions between distal processes of neighboring astrocytes (Wanga and Bordey, 2008; Sofroniew and Vinters, 2010). Originally thought as "brain glue", it has become clear in recent years that astrocytes are important for virtually all brain functions (Ransom B. et al., 2003). Due to their strategic location and structural and biochemical features astrocytes are the cells optimized by the natural selection to sense their surroundings and dynamically respond to changes in the microenvironment. They have infact several critical functions including promoting neuronal maturation, synapse formation, neuronal survival during development, regulating angiogenesis, and maintaining a viable microenvironment for neurons (Wanga and Bordey, 2008). Either protoplasmic and fibrous astrocytes are reported to contact and have bidirectional relationships with blood-brain barrier (BBB) components with whom they participate to BBB formation and maintenance and where they regulate the exchanges between intraparenchimal milieu and the external environment. Astrocytes also contact meninges and ependymal layer lining the ventricles contributing to support brain cerebrospinal fluid barrier structures (Li et al., 2010; Mack AF and Wolburg H, 2012). Briefly, they control ion and fluid homeostasis (uptake functions), support neuron development and functions (trophic and metabolic functions), blood brain barrier formation and maintenance and react to different types of pathogenic insult in the brain. Moreover they also support oligodendrocyte functions and myelin formation. Due to their multitasking activities astrocytes are involved almost all the diseases of the CNS. In the following sections astrocyte functions will be described in detail (Figure 1.4)

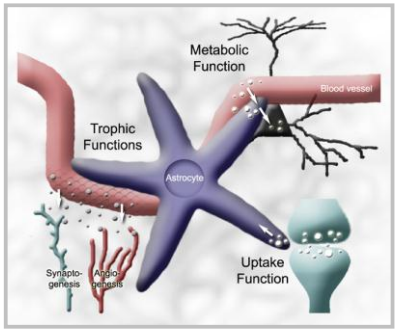

Fig.1.4.The well-established functions of astrocytes. Astrocytes have several homeostatic functions maintaining a viable nervous system environment for neurons. These functions include: (1) providing metabolic support for neurons, (2) taking up K+ and neurotransmitters (3) Synaptogenesis, angiogenesis and brain blood barrier formation and maintenance (adapted from Wanga and Bordey, 2008).

a) Metabolic function.

Astrocytes are uniquely poised to provide a local metabolic support for neurons and the role of astrocytes in the supply of energy substrates to neurons is one of their oldest known functions. Astrocytes are located at a strategic position between blood capillaries and neurons: astrocyte processes, on the one hand, project toward blood vessels that terminate into structures called endfeet, which almost entirely cover the blood vessel walls, and on the other hand contact neuronal perikarya, axons (at nodes of Ranvier), and synapses (Figure 1.1). Magistretti and colleagues proposed an important hypothesis termed the astrocyte-neuron lactate shuttle hypothesis (ANLSH) in which, in response to glutamatergic neuronal activity, glucose enters the CNS via the astrocytic processes and is there converted, by aerobic glycolysis, to glycogen and lactate, which then serve as the principal food for neurons (Magistretti et al.,1993; Pellerin and Magistretti, 1994; Voutsinos-Porche et al., 2003). This activity-dependent mechanism involves Na+-coupled glutamate uptake in astrocytes and activation of the Na+/K+ ATPase, which triggers glucose uptake from the blood and its glycolytic processing, resulting in the release of lactate from astrocytes (Figure 1.5a) (Giaume et al., 2010). Moreover astrocytes are the principal storage sites of glycogen granules in the CNS, which utilization can sustain neuronal activity during hypoglycaemia and periods of high neuronal activity. Even when glutamate spillover stimulates a distant astrocyte closed to a blood vessel (dark blue in Figure 1.5b), glucose and metabolites produced after glucose uptake from the blood permeate gap junction channels and reach adjacent astrocytes that are in contact with the active neurons providing them with energy substrates (light blue in Figure 1.5b).

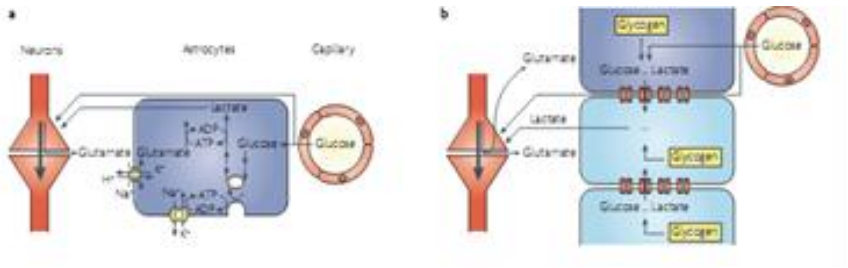

Fig.1.5 Astroglial metabolic networks sustain neuronal activity. a) The mechanism underlying glutamate-induced glycolysis during glutamatergic synaptic activity. b) The contribution of the astrocyte network to the glucose–lactate shuttle shown in a) (adapted from Giaume et al., 2010).

b) Uptake function: taking up potassium ion (K+) and neurotransmitters

Astrocytes are the main cellular element involved in the mainteinance of extracellular homeostasis in the CNS. Numerous transport systems expressed in astroglial membranes provide the control over the concentrations of ions, neurotransmitters, neuromodulators, metabolites and other active molecules in the interstitium.

During normal neuronal activity, neurotransmission leads to the build up of K+ ions into the extracellular space, and if not corrected, results in neuronal depolarization, hyperexcitability, and seizures. On the base of a mechanism termed spatial buffering, astrocytes take up the excess of extracellular K+ ions, distribute them through the gap junction-coupled astrocytic syncytium and extrude the ions at sites of low extracellular K+ level. To achieve spatial K+ buffering, astrocytes are poised with passive and active uptake capabilities, via channels (predominantly by inwardly rectifying K+ channels (Kir)) and co-transporters, and via Na+/K+ ATPases, respectively (Wanga and Bordey, 2008). Astrocyte processes at synapses also play essential roles in transmitter homeostasis by expressing high levels of transporters for neurotransmitters such as glutamate, GABA and glycine that serve to clear the neurotransmitters from the synaptic space. Glutamate is the major excitatory transmitter of the brain and one of the most important tasks of astrocytes is to maintain a low concentration of extracellular glutamate. Glutamate uptake by astrocytes terminates its eff ects as a neurotransmitter, prevents extracellular glutamate levels from reaching excitotoxic levels, and leads to futher utilization as a metabolic substrate. To this aim astrocytic processes surrounding synapses take up glutamate through transport proteins, particularly EAAT1 and EAAT2 (Huang et al., 2004). After the uptake into astrocytes, the transmitters are converted by

enzymes into precursors and recycled back to synapses for reconversion into active transmitters (Sofroniew and Vinters, 2010).

c) Astrocyte in the control of ion and water exchange and cell volume regulation

Astrocytes have long been recognized as the chief regulator of water homeostasis in the brain and contribute to a stable osmotic environment in several ways (Del Zoppo G. J. and Hallenbeck J. M., 2000). The next paragraph describes four examples of how these glial cells, via the detection of osmotic changes, are capable of initiating an osmo-regulatory process in the brain. The relative contribution of these mechanisms varies depending on a number of factors including the density, expression pattern and water fluxing capacity of the individual channels and co-transporters (Agre P. et al., 2004). The first osmo-regulatory process in which astrocytes are involved is the previously mentioned potassium spatial buffering that is primarily mediated by Kir4.1 channel. In this way astrocytes maintain extracellular potassium optimal for neuronal signaling. The net ion gain results in osmotic water uptake via AQP4 and causes a slight cell swelling and subsequent water clearance via astrocytic endfeet at the blood-brain barrier (Nagelhus E.A. et al., 2004). The second example is neurotransmitter recycling, which can also lead to local changes in ions and water. Glutamate transport is sodium-dependent and accompanied by net uptake of ions and water, again contributing to water clearance via astrocytic endfeet at the blood-brain barrier (Nagelhus E.A. et al., 2004). The two previously described examples concern activity-dependent water flux. This activity dependent water influx is superimposed on a constitutive efflux of a substantial amount of water derived from the metabolic breakdown of glucose, the main energy substrate of the brain (Amiry-Moghaddam M. and Ottersen O. P., 2003). The last osmo-regulatory process is called regulatory volume decrease (RVD). RVD is a corrective process leading to cell volume recovery after rapid cell swelling as a response to decreased external osmolarity. RVD is caused by an efflux of chloride, potassium and organic osmolytes, such as amino acids plus their derivates (taurine, proline, alanine) (Strange K. et al.,1996). The chloride channels activated by cell swelling are called volume-regulated anion channels (VRACs).

d) Synaptic support

Recent findings have shown that astrocytes are required for synaptogenesis and for the structural maintenance and proper functioning of synapses. Indeed one of the most

important role of astrocytes is to optimize the functionality of synapses by maintaining the interstitial space homeostasis by a tight control of fluid, ion, pH and transmitter homeostasis at the synaptic clefts as described above (Kimelberg and Nedergaard 2011). Clear evidence has accumulated indicating that the CNS synapse is composed of three elements: the presynapse, the postsynaptic membrane and the nearby astrocyte that, with its processes, make intimate contacts with the neuronal synaptic structures. The existence of a bidirectional communication between astrocytes and neurons was termed "tripartite synapse" (Faissner et al., 2010). Inded astrocytes release factors including extracellular matrix (ECM) proteins, cytokines, metabolites such as cholesterol influencing synaptic development nd functioning and interact with neurons via cell–cell and cell–ECM based adhesion mechanisms. Increasing experimental evidence demonstrated that astrocytes actively participate in modulating synaptic activity also by releasing neurotransmitters (so-called gliotransmitters). Several mechanisms of transmitter release from astrocytes have been proposed. Astrocytes react to synaptically released neurotransmitters with intracellular calcium ([Ca(2+)]) elevations, which in turn generates the regulated secretion of astrocyte-derived gliotransmitters, mainly glutamate and ATP, but also GABA and D-serine , mainly through a process of Ca dependent vesicular release (review by Zorec et al., 2012).

Other soluble factors produced by astrocytes that can influence synaptic activity are neurosteroids, growth factors and cytochines that can be released by astrocytes in different areas of the brain or in pathological conditions.

Then, the concept of the tripartite synapse proposes that astrocytes constitute an integral part of the synapse, as cellular elements involved in the processing, transfer and storage of information by the nervous system.

1.3.1 *Astrocytes relationship with oligodendrocyte and myelin*

The astrocyte-mediated growth factor production, which promote neuronal survival in normal (Dreyfus et al., 1999) and injured brain (Schwartz et al., 1996), is also essential for survival of oligodendrocytes, the myelin forming cells. The importance of astrocytes as modulators of oligodendrocyte proliferation and differentiation in vitro was first recognized more than 20 years ago when astrocytes were found a major source of proliferating and differentiating factors in particular PDGFα and FGF2 for

oligodendrocyte progenitors (Bögler et al., 1990; Noble and Murray, 1984; Noble et al., 1988; Richardson et al., 1988).

Additional contribute to myelin maintenance is given by astrocytes through gap junction communication as demonstrated by studies on mice double-deficient for connexins forming heterotypic interactions between astrocytes and oligodendrocytes which show extensive white matter vacuolation and myelin defects (Tress et al., 2011).

Moreover via gap junctions astrocytes are structurally and functionally interconnected into a highly organized network which allows them to communicate and coordinate their activities like a syncitium (a multinucleated mass of cytoplasm resulting from the fusion of cells) by the transfer of calcium waves, small molecules including second messengers and ions. Into a lesser extent astrocytes through gap junctional communication can also modulate the activity of adjacent cells like oligodendrocytes and neurons (Thesis M and Giaume C, 2012).

1.3.2 *Astrocytes and brain pathology*

Considering the key role played by astrocytes in the maintenance of brain homeostasis, the strong metabolic cooperations between astrocytes and neurons and their direct and indirect influence on myelin formation and oligodendrocyte survival it is conceivable that astrocytic dysfunctions and incorrect neuron-astrocyte or oligodendrocyte-astrocyte interactions may be involved in CNS and neuronal damaging.

According with these observations it is not surprisingly that the number of CNS inflammatory diseases in which the contribute of astrocytes to neurodegeneration became pathological important is continuously updating. Moreover due to astrocytic influence on oligodendrocytes and myelin formation astrocyte dysfunction can participate to demyelinating disease.

Evidence is emerging that alterations in astrocyte functionality plays a crucial role in the pathogenesis of different types of brain disorders including neurodegenerative diseases as demonstrated by the genetic leukodystrophy, Alexander disease. in which myelin degeneration is caused by mutations in the gene encoding the astrocytic specific glial fibrillary acidic protein GFAP. Along with Alexander disease, Megalencephalic leukoencephalopathy with subcortical cysts (MLC) and Vanishing white matter disease (VWM/CACH) are other two examples of diseases in which myelin degeneration occurs as a secondary defect following a primary astrocyte dysfunction.

1.4 Ependymal cells

Other glial cells potentially involved in brain damages observed in some leukoencephalopathies are the ependymal cells (Ambrosini, 2008). Ependymal cells are the epithelial cells that line the central canal system of the brain and spinal cord. Recently, several functions for these cells have been suggested, including a barrier for filtration of brain molecules, protection of the brain from potentially harmful substances from the cerebrospinal fluid (CSF), movement of cellular debris, and optimization of the dispersion of neural messengers in the CSF (Spassky N. et al., 2005). Further investigation will be needed to understand the role of these cells in the pathogenesis of leukoencephalopathies.

1.5 Microglia

The involvement of inflammatory processes and immune response in some leukoencephalopthies have been recently demonstrated (Luzi P et al., 2009) and activation of CNS resident macrophages has been found.

The CNS resident macrophages are microglial cells. These cells are a collection of phagocytic cells that arise from macrophages outside mettere bene formattazionethe nervous system and are physiologically and embryologically unrelated to the other cell types of the nervous system. These cells normally exist in the CNS in small numbers, but they multiply and become activated during infection, injury, and seizures. Activated microglia have processes that are stouter and more branched than those of inactivated cells, and they express a range of antigens, suggesting that they may serve as the major antigen-presenting cell in the CNS (Kandel E.R. et al., 2000).

2. Megalencephalic leukoencephalopathy with subcortical cysts (MLC).

Megalencephalic leukoencephalopathy with subcortical cysts (MLC, OMIM 604004) is a rare congenital and incurable leukodystrophy characterized by early–onset macrocephaly, subcortical cysts and swollen appearance of the white matter which was first described by Van der Knaap et al. (1995).

2.1 Clinical phenotype

MLC patients manifest:

- Macrocephaly. Patients develop macrocephaly during the first year of life, but some children are born with it.
- Slow deterioration of motor functions occurs with cerebellar ataxia and mild spasticity, which eventually leads to the inability to walk.
- Cognitive deterioration is usually late and mild.
- Epiletic Seizures. Almost all patients have epileptic seizures from early on, but are usually easily controlled with medication.

The disease is also characterized by a slowly progressive clinical course that can be worsened by minor stress, particularly minor head trauma and common viral infections. (van der Knaap et al., 1995; Goutieres et al., 1996; Singhal et al., 1996; Topçu et al., 1998; Ben-Zeev et al., 2002).

2.2 MRI and histopathological analysis

Magnetic resonance imaging analysis of the brain indicates diffuse white matter swelling and invariant bilateral subcortical cysts, in the anterior-temporal and often in the fronto-parietal regions (Figure 2.1 B), two main features characterizing MLC disease (Van der Knaap et al., 1995, 1996). The cerebral white matter is diffusely abnormal in signal and slightly swollen; the occipital white matter often looks a bit less abnormal and less swollen. Central white matter structures are relatively spared, including the corpus callosum, internal capsule, and brain stem, although they are not entirely normal. Usually, the cerebellar white matter has a mildly abnormal signal and is not swollen (Figure 2.1, A and B) (van der Knaap et al., 1995; Goutieres et al., 1996). As the disease progresses, the white matter swelling decreases and cerebral atrophy ensues. In general, the subcortical cysts increase in size and number. In some patients,

the cysts become very large and occupy a large part of the frontoparietal white matter (van der Knaap et al., 1995, Leegwater et al., 2002).

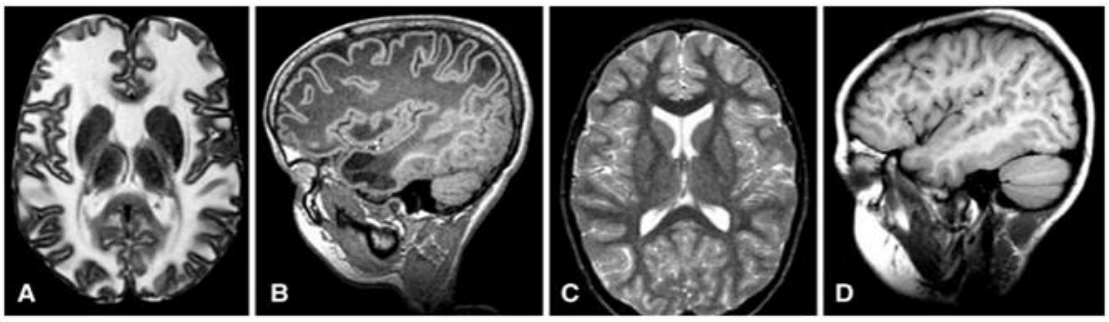

Fig.2.1 MRI of the brain of a MLC patient (A, B) versus an unaffected child (C, D). The transverse T2-weighted image show diffusely abnormal, slightly swollen white matter in the patient (A compared with C). The sagittal T1-weight image shows subcortical cysts in the anterior-temporal and parietal subcortical region (B compared with D) (adapted from Leegwater et al., 2002).

Analysis of brain biopsies showed that spongiform white matter changes are related to the presence of vacuoles between the outer lamellae of the myelin sheaths, probably generated by splitting of these lamellae along the intraperiod line or their incomplete compaction (Figure 2.2, A and B) (van der Knaap et al., 1996). According to these histopathological findings, the disease can be classified as one of the spongiform leukoencephalopathies

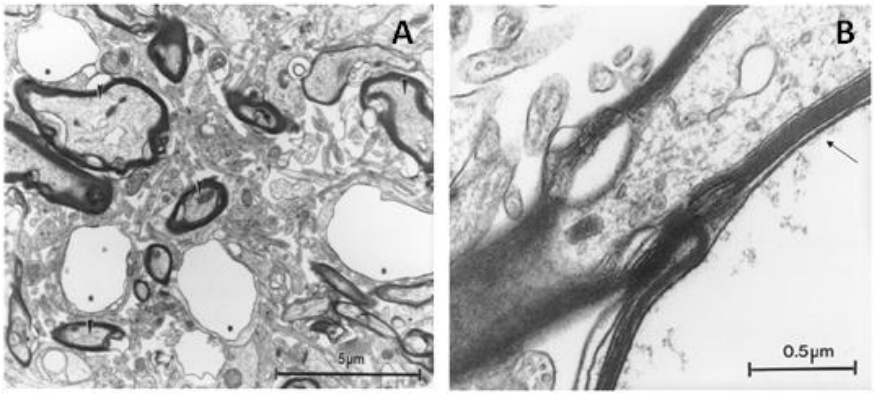

Fig.2.2 Electron microscopy examination of the brain of a MLC biopsy. (A) Electron microscopy image demonstrates numerous membrane covered vacuoles (asterisks) amidst several myelinated fibers with well-preserved axons (arrowheads). (B) Detail of a multi-lamellar covering of a vacuole (arrow) (adapted from van der Knaap et al., 1996).

Astrogliosis, enlargement of extracellular spaces and alterations in the structure of some blood vessels were also reported (Van der Knaap et al., 1996; Pascual-Castroviejo et al.,

2005). Recently the presence of vacuoles in the end-feet of astrocyte contacting blood-vessels has been reported (Duarri et al., 2012).

2.3 *MLC1* gene and the pathogenetic mutations.

Based on the pedigree analysis of affected families, the mode of inheritance for the MLC disease has been determined to be autosomal recessive. In 2000, by a genome wide linkage analysis in 13 affected Turkish families, the locus of the disease gene was mapped at the very end of the long arm of chromosome 22 and named *MLC1* (Topku et al., 2000) and formerly known as KIAA0027. MLC1 spans at least 24 kb genomic DNA and contains 11 coding exons and a first non-coding exon, with a start codon in exon 2 and a 3'untranslated region (UTR) end of 2.2 kb (Figure 2.3). The gene exhibits two alternative splice variants (accession numbers NM_015166.2 and NM_139202.1), which differ only in their 5'UTRs (Boor et al., 2006). The transcription start site and the promoter region have not yet been fully characterized.

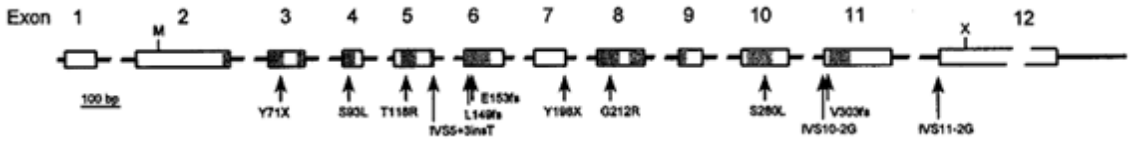

Fig.2.3 Organization of MLC1 gene. The exons are shown as boxes; introns, as solid lines. The gray parts indicate the eight regions that encode transmembrane domains of the protein. The positions of the first identified mutations that were found in DNA of patients with MLC are shown (indicated) by arrows. Interrupted lines indicate deviations from scale. M = start codon, X = stop codon, and fs = frameshift (adapted from Leegwater et al., 2001).

The MLC1 cDNA was cloned from the immature myeloid cell-line KG-1, and the expression in these cells proved to be high in comparison with the expression in a variety of human tissues (Nomura et al., 1994). The expression of MLC1 is relatively high in the all brain regions examined including cortex, hippocampus, caudate nucleus, amygdala, thalamus, and cerebellum (Steinke et al., 2003). It is also expressed in peripheral white blood cells and spleen, and the expression in ovary, prostate, placenta, thymus, and lung is relatively low (Nomura et al., 1994). In situ hybridization studies performed in the mouse and human brain report MLC1 mRNA in glial cells such as astrocytes, Bergmann glia and ependymal cells (but not in oligodendrocytes and microglia (Schmitt et al., 2003; Teijido et al., 2004; Boor et al., 2005).

Almost 75% of MLC patients carry different types of mutations in the *MLC1* gene, which include formation of stop codons, frameshifts, splice-site mutations, and amino acid substitutions of conserved residues in predicted transmembrane domains or outside them (Leegwater et al., 2001; Boor et al., 2005; Teijido et al., 2004; Jeworutzky et al., 2012), suggesting that alterations of the function of the MLC1 gene product are the leading cause of this disease. However, to date no correlation between genotype and phenotype has been found. (Patrono et al., 2003; Boor et al., 2006). Interestingly, although some patients share the same mutation, they present a wide variability in clinical symptoms and disease severity. Some patients have a more severe clinical course and never learn to walk, or lose their ability to walk within a few years. In contrast, some patients have a milder clinical course and have, even as teenagers, only macrocephaly as a symptom. The differences in phenotype between patients carrying the same mutations suggest that modifier genes and/or environmental factors have an effect in determining the final phenotype. Recently, mutations in the Hepacam/Glialcam gene encoding an adhesion-like molecule of unknown function have been found in about 60% of MLC affected patients without mutations in MLC1, indicating the genetic heterogeneity of the disease (Jeworutzki et al., 2012; Lòpez-Hernàndez et al., 2011; Blattner et al., 2003). MLC patients exhist in which no mutations either in *MLC1* and in *Hepacam/Glialcam* gene have been found (Lòpez-Hernàndez et al., 2011). It is possible that in these patients mutations are distribuited in regions of the MLC1/Hepacam genes that have not been analysed yet, like the promoter region, the non coding exon 1, introns, and the 3' UTR, all of which may influence the expression of the MLC1 gene (Leegwater et al., 2001).

Even if MLC is a very rare pathology, the disease occurs with relatively high frequency in an Asian-Indian tribe and Turkish populations (Singhal et al., 1996; Topçu et al., 1998). In 1996, Singhal et al described MLC disorder in thirty-one patients from a specific Indian community, namely the Agarwals from India: all the patients showed the same homozygous mutation (135insC) in exon 2 of the MLC1 gene, suggesting a possible founder effect. The same mutation was reported also in another large series among Turkish patients (Topçu et al., 1998). There have been reports of similar cases from Croatia, Brazil, France and Japan. Overall these data indicate that MLC disease is present in several regions of the world, affecting patients of different ethnicities (Singhal et al., 2003) and particularly frequent in areas of high levels of consanguinity as usually observed for disease with recessive mode of inheritance.

2.4 MLC1 protein

The *MLC1* gene encodes for a 377-amino acid protein of still unknown function, containing eight putative transmembrane domains and short amino- and carboxylic-cytoplasmic terminals named MLC1 (Leegwater et al., 2001; Teijido et al., 2004; Boor et al., 2005) wich is highly expressed in brain.

Immunohistochemical studies performed in the mouse and human brain showed that MLC1 protein is preferentially expressed in in distal astrocytic processes in perivascular and subpial regions, in ependymal cells lining the ventricles and in Bergmann glia, in the cerebellum, but not in oligodendrocytes and microglia (Teijido et al., 2004; Boor et al., 2005).

MLC1 shows no similarities with proteins of known function, except for a very low homology with a subunit of the voltage-gated potassium (K) channel (Kv1.1) and an ABC type of transporter (Meyer et al., 2001; Leegwater et al., 2001). Moreover, MLC1 contains an internal amino acid repeat that is found in several ion channel proteins (Teijido et al., 2004). A search in the Swissprot database indicated that most of the human proteins containing eight transmembrane domains have a transporter or channel function (Boor et al., 2005). Taken together with the pathological alterations observed in MLC patients and the predominant localization of MLC1 in astrocytic processes in perivascular, subpial and subependymal regions, these observations suggest that MLC1 may be involved in astrocyte-mediated processes regulating fluid homeostasis and transport of ions or other substances between the central nervous system (CNS) tissue and the blood or cerebrospinal fluid. Astrocytes are the most abundant glial cell population of the CNS and play an essential role in the maintenance of neural tissue homeostasis through the regulation of ions, neurotransmitters and metabolites in the extracellular space (Kimelberg, 2010). Recent findings from our and other groups indicate that MLC1 interacts with components of the dystrophin–glycoprotein-associated complex (DGC) (Ambrosini et al., 2008; Boor et al., 2007; Lanciotti et al., 2010). This multiprotein complex is expressed in brain astrocytes (Waite et al., 2009), where it is involved in electrolyte balance and water movement by targeting and stabilizing the inwardly rectifying K+ (Kir) channel Kir4.1 and the water channel aquaporin-4 (AQP4) at the astrocytic end-foot domains (Neely et al., 2001; Connors et al., 2004,). Although it is conceivable that via association with DGC proteins MLC1 might take part in water and ion homeostasis in the brain, no evidence supporting this hypothesis was available since the start of the present research project.

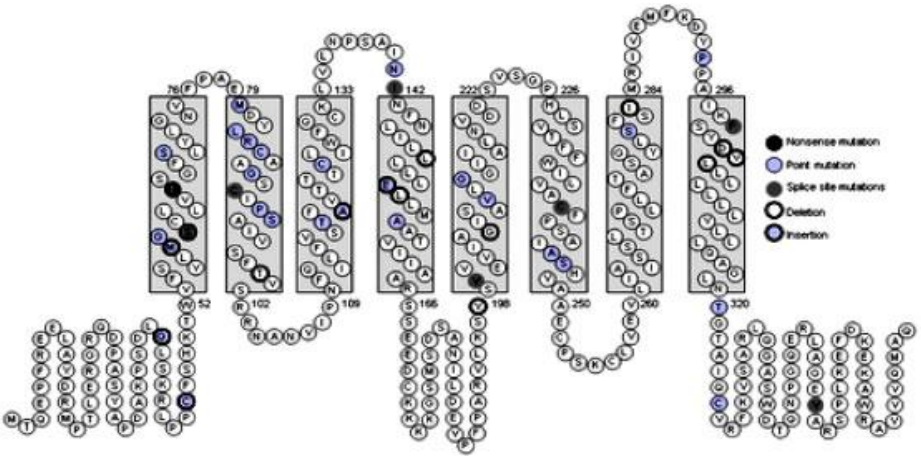

Fig.2.4 Predicted MLC1 protein structure. Amino acid position of the different mutations are indicated (adapted from Boot et al., 2006).

Scope and outline of this thesis

Megalencephalic leukoencephalopathy with subcortical cysts (MLC, OMIM 604004) is a rare congenital and incurable leukodystrophy characterized by early–onset macrocephaly, subcortical cysts and swollen appearance of the white matter (van der Knaap M.S. et al., 1995). The disease is characterized by ataxia, seizures, degeneration of motor functions and late-onset cognitive impairment. MLC patients have a slowly progressive clinical course that can be worsened by minor stress, particularly minor head trauma and common infections (van der Knaap M.S. et al., 1995; Singhal B.S. et al., 2003; Topku M. et al., 1998). Histopathologically, the brain of MLC patients shows intramyelinic vacuole formation, structural alterations of the blood–brain barrier and astroglial activation (van der Knaap M.S. et al., 1995; 1996). Enlarged intracellular vacuoles localized in the end-feet of vessels contacting astrocytes have also been recently described (Duarri A. et al., 2011).

In the majority of patients (about 75-80%) MLC disease is caused by mutations in a single gene (MLC1) encoding a protein highly expressed in CNS astrocytes. However, to date no correlation between genotype and phenotype has been found (Leegwater P.A. et al., 2001; 2002; Patrono C. et al., 2003; Boor I.P.K. et al., 2006). Although MLC1 function is still unknown, the neuropathological features observed in MLC patients, in particular, swollen white matter, fluid cysts and myelin and astrocyte vacuolation and the localization of MLC1 in astrocyte end-feet-contacting brain barriers led to hypothesize a role for MLC1 in fluid and ion homeostasis in the central nervous system. Thereby, brain damage in MLC patients might be generated by abnormalities in the regulation of specific cellular process related to ion and water influx and as a consequence in cell volume regulation that in the brain are exerted by astrocytes. Based on this evidence the main working hypotheses of this thesis are: i) MLC1 expressed in astrocytes plays a key role in ion and fluid homeostasis in the CNS; ii) pathological mutations in the MLC1 gene affect MLC1 protein function by altering its intracellular distribution and trafficking and modifying molecular interactions that are critical to perform the above functions.

Elucidation of MLC1 protein function represents an essential step to understand MLC pathogenesis and provide the rationale basis to develop a therapy for MLC patients.

To identify MLC1 function we performed study aimed at investigating the functional pathways in which MLC1 is involved in astrocytes.

To this aim we approached the identification of MLC1 molecular interactors as one of the most promising strategies to investigate MLC1 functional pathways.

By applying a panel of biochemical, immunofluorescence and proteomic techniques on primary rat astrocyte cultures and on a new human pathological model generated in our laboratory by stably overexpressing MLC1 WT or carrying pathological mutations in astrocytoma cells, we have first identified molecular interactors and specific functional pathways associated to MLC WT and then we have described mutation specific defects.

RESULTS

To shed light on MLC1 function in the brain and in particular in astrocytes where MLC1 protein is abundantly expressed, we searched for MLC1 intracellular interactors . The identifcation of MLC1 partners is an important step to understand the functional patways in wich MLC1 is involved in astrocytes.

- By using the two-hybrid system assay, we identified the β1 subunit of the Na,K-ATPase pump, the ubiquitous enzyme responsible for the maintenance of the Na+ and K+ gradients across the plasma membrane and cell volume regulation, as an MLC1-interacting clone. Using biochemical assays as pull-downs, co-fractionation assays and immunostainings, we further characterized and confirmed the direct interaction between MLC1 and Na,K-ATPase β1 subunit in human and rat astrocytes in vitro and in brain tissue. We found that, by binding to Na,K-ATPase, MLC1 takes part to a multiprotein complex that includes the inward rectifier potassium channel 4.1 (Kir4.1), the water channel aquaporin-4 (AQP-4), the membrane raft associated protein caveolin-1 (cav-1) and the dystrophin associated proteins syntrophin (Synt) and dystrobrevin (DB) functionally involved in the regulation of cell volume changes occurring after osmotic imbalance (Brignone M.S. et al., 2011). These findings show for the first time the direct association of MLC1 with a known protein involved in intracellular osmotic control and volume regulation, thus providing new insights into potential mechanisms underlying MLC pathogenesis.

- To elucidate the role of MLC1 in astrocyte physiology and the possible functional consequences of MLC1 mutations, we have generated and characterized human astrocytoma cell lines overexpressing wild-type (WT) MLC1 and three missense mutations described in MLC patients (Lanciotti A. et al., 2012). This work shows that distinct pathological mutations differently alter MLC1 intracellular localization and interaction with the Na,K-ATPase-associated multiprotein complex in astrocytoma cells and affect the response to osmotic changes.

- Moreover, it demonstrates that MLC1 functionally interacts with the transient receptor potential vanilloid-4 cation channel (TRPV4) to increase intracellular calcium influx in cell subjected to osmotic stress. This calcium influx is essential to regulate the rescue of the correct cell volume after hyposmosis induced cell swelling. Conversely, mutations hampering MLC1 expression in the plasma membrane fail to activate calcium influx leading to abnormal response to osmotic changes (Lanciotti A. et al., 2012).

These findings represent the first evidence that MLC1-induced defects in the intracellular calcium response in astrocytes may contribute to the pathogenesis of MLC disease.These main results has been object of two publications reported above.

Human Molecular Genetics, 2011, Vol. 20, No. 1 **90–103**
doi:10.1093/hmg/ddq435
Advance Access published on October 6, 2010

The β1 subunit of the Na,K-ATPase pump interacts with megalencephalic leucoencephalopathy with subcortical cysts protein 1 (MLC1) in brain astrocytes: new insights into MLC pathogenesis

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Received August 30, 2010; Revised and Accepted October 3, 2010

Megalencephalic leucoencephalopathy with subcortical cysts (MLC) is a rare congenital leucodystrophy caused by mutations in MLC1, a membrane protein of unknown function. MLC1 expression in astrocyte end-feet contacting blood vessels and meninges, along with brain swelling, fluid cysts and myelin vacuolation observed in MLC patients, suggests a possible role for MLC1 in the regulation of fluid and ion homeostasis and cellular volume changes. To identify MLC1 direct interactors and dissect the molecular pathways in which MLC1 is involved, we used NH_2-MLC1 domain as a bait to screen a human brain library in a yeast two-hybrid assay. We identified the β1 subunit of the Na,K-ATPase pump as one of the interacting clones and confirmed it by pull-downs, co-fractionation assays and immunofluorescence stainings in human and rat astrocytes *in vitro* and in brain tissue. By performing ouabain-affinity chromatography on astrocyte and brain extracts, we isolated MLC1 and the whole Na,K-ATPase enzyme in a multiprotein complex that included Kir4.1, syntrophin and dystrobrevin. Because Na,K-ATPase is involved in intracellular osmotic control and volume regulation, we investigated the effect of hypo-osmotic stress on MLC1/Na,K-ATPase relationship in astrocytes. We found that hypo-osmotic conditions increased MLC1 membrane expression and favoured MLC1/Na,K-ATPase-β1 association. Moreover, hypo-osmosis induced astrocyte swelling and the reversible formation of endosome-derived vacuoles, where the two proteins co-localized. These data suggest that through its interaction with Na,K-ATPase, MLC1 is involved in the control of intracellular osmotic conditions and volume regulation in astrocytes, opening new perspectives for understanding the pathological mechanisms of MLC disease.

INTRODUCTION

Megalencephalic leucoencephalopathy with subcortical cysts (MLC, OMIM 604004) is a rare inherited, autosomal recessive form of spongiform leucodystrophy affecting children (1). MLC patients manifest macrocephaly, motor function deterioration, ataxia, spasticity, epileptic seizures and variable levels of mental impairment. The disease is also characterized by a slowly progressive clinical course that can be worsened by minor stress, particularly minor head trauma and common viral infections (1,2). White matter swelling and the presence of subcortical cysts in the fronto-parietal and temporal regions are the two main features characterizing MLC disease by magnetic resonance imaging (2). In brain biopsies of MLC patients, the white matter was vacuolated and liquid vacuoles were localized between the outer lamellae of the myelin sheaths, probably generated by their splitting along the intraperiod line or incomplete compaction (2). Alterations in blood–brain barrier structure and astrogliosis have also been reported (2,3). To date, the pathogenetic mechanisms

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underlying MLC disease are still unknown and no therapy is available for patients. The locus of the disease gene was mapped at the very end of the long arm of chromosome 22 and named MLC1 (4). Different types of pathological mutations, mostly missense mutations, have been found in this gene without any clear correlation with the severity of the phenotype (2,5). Furthermore, the involvement of a second MLC-associated gene has been hypothesized (6–8).

The *MLC1* gene encodes a protein of still unknown function, containing eight putative transmembrane domains and short amino and carboxylic cytoplasmic domains. MLC1 proteic sequence shows the presence of an internal amino-acidic repeat that is also found in several ion channel proteins (2,9) but, with the exception of a low homology with a potassium Kv1.1 channel subunit, it does not show any similarities with known proteins. In the human brain, MLC1 protein is highly expressed in distal astrocytic processes in perivascular and subpial regions, in ependymal cells lining the ventricles and in Bergmann glia in the cerebellum (9–11). These observations led to hypothesize that brain damage in MLC patients might be generated by abnormalities in astrocyte function. In the mouse, expression of MLC1 in some neuronal populations of the central and peripheral nervous systems has been reported (9).

Altogether, the pathological alterations observed in MLC patients, the localization of MLC1 in astrocyte end-feet contacting brain barriers and its molecular structure suggest a role for MLC1 in astrocyte-mediated processes regulating fluid homeostasis and transport of ions or other substances between the central nervous system (CNS) tissue and the blood or cerebrospinal fluid. Astrocytes are the most abundant glial cell population of the CNS and play an essential role in the maintenance of neural tissue homeostasis through the regulation of ions, neurotransmitters and metabolites in the extracellular space (12). Recent findings from our and other groups indicate that MLC1 interacts with components of the dystrophin–glycoprotein-associated complex (DGC) (11,13,14). This multiprotein complex is expressed in brain astrocytes (15), where it is involved in electrolyte balance and water movement by targeting and stabilizing the inwardly rectifying K^+ (Kir) channel Kir4.1 and the water channel aquaporin-4 (AQP4) at the astrocytic end-foot domains (16,17). Although it is conceivable that via association with DGC MLC1 might take part in water and ion homeostasis in the brain, no evidence supporting this hypothesis is available yet.

To shed light on MLC1 function in the brain, we searched for MLC1 intracellular interactors. By using the two-hybrid system assay, we identified the β1 subunit of the Na,K-ATPase pump, the ubiquitous enzyme responsible for the maintenance of the Na^+ and K^+ gradients across the plasma membrane, as an MLC1-interacting clone. Using biochemical assays and immunostainings, we further characterized the interaction between MLC1 and Na,K-ATPase β1 subunit in cultured astrocytes and whole-brain tissue. We found that, by binding to Na,K-ATPase, MLC1 is part of a multiprotein complex that includes Kir4.1 and the DGC proteins syntrophin and dystrobrevin and is functionally involved in the regulation of cell volume changes occurring after osmotic imbalance. These findings show for the first time the direct association of MLC1 with a known protein involved in intracellular osmotic control and volume regulation, thus providing new insights into potential mechanisms underlying MLC pathogenesis.

RESULTS

Yeast two-hybrid screening of a human fetal cDNA library identified the Na,K-ATPase β1 subunit as MLC1-interacting partner

In order to isolate intracellular MLC1-interacting proteins in the human brain, we performed a yeast two-hybrid screening of the human fetal brain cDNA library using the MLC1-NH_2 domain (from amino acid M1 to amino acid Y55 of the protein sequence) as bait. We used a yeast-mating strategy with a high level of stringency to isolate proteins that interact strongly with this bait. The human MLC1-NH_2-domain-coding sequence obtained by RT–PCR from a human brain extract was fused in-frame to the DNA-binding domain of Gal4 (pGBKT7), transformed into yeast strain AH109 and used to screen a human fetal brain cDNA library subcloned into the Gal4 activation domain vector pGADT7-rec and pre-transformed into yeast strain Y187. Approximately 2×10^6 independent clones were screened for the expression of the reporter genes *HIS3*, *ADE2* and *Mel1/lacZ*, and a total of 350 positive clones were found. Because these clones could contain more than one AD/library plasmid, we allowed segregation and recollected them on SD/-TLHA/X-α-Gal plates. We selected 144 diploids strongly activating *Mel1/lacZ* reporter genes, isolated their plasmid DNA and sorted them using PCR followed by restriction analysis. We rescued AD/library plasmids via transformation of *Escherichia coli*, obtaining 27 different types of insert. End sequencing and BLAST searching revealed that one of them encoded for the C-terminal half (residues 156–303) of the Na,K-ATPase β1 subunit isoform A (NaK-β1) (GenBank Accession no. NM_001677). The specificity of the direct interaction between MLC1 and NaK-β1 polypeptides was confirmed by glutathione-S-transferase (GST) pull-down experiments performed with recombinant GST-MLC1 N-terminal domain (GST-MLC1-NH_2) and ^{35}S-labelled NaK-β1 C-terminal domain (NaK-β1-COOH). In this experiment, we found that NaK-β1-COOH specifically interacted with the GST-MLC1-NH_2 protein, although no interaction with GST-MLC1-COOH protein (residues 322–377) or with GST protein, used as negative control, was detected (Fig. 1A).

Interaction between MLC1 and NaK-β1 in *in vivo* systems

To verify whether MLC1-NH_2 bound NaK-β1 in *in vivo* systems, we performed a pull-down assay using GST-MLC1-NH_2 recombinant protein and protein extracts of rat primary astrocytes, which can be obtained in large numbers and with a high degree of purity from the newborn rat brain. As shown in Figure 1B, we observed a specific interaction between MLC1-NH_2 domain and the endogenous astrocyte-derived NaK-β1. Neither GST-MLC1-COOH nor GST proteins interacted with NaK-β1. Since both our in-house-developed and the commercially available anti-MLC1 pAbs failed to immunoprecipitate MLC1, to further validate the observed interaction

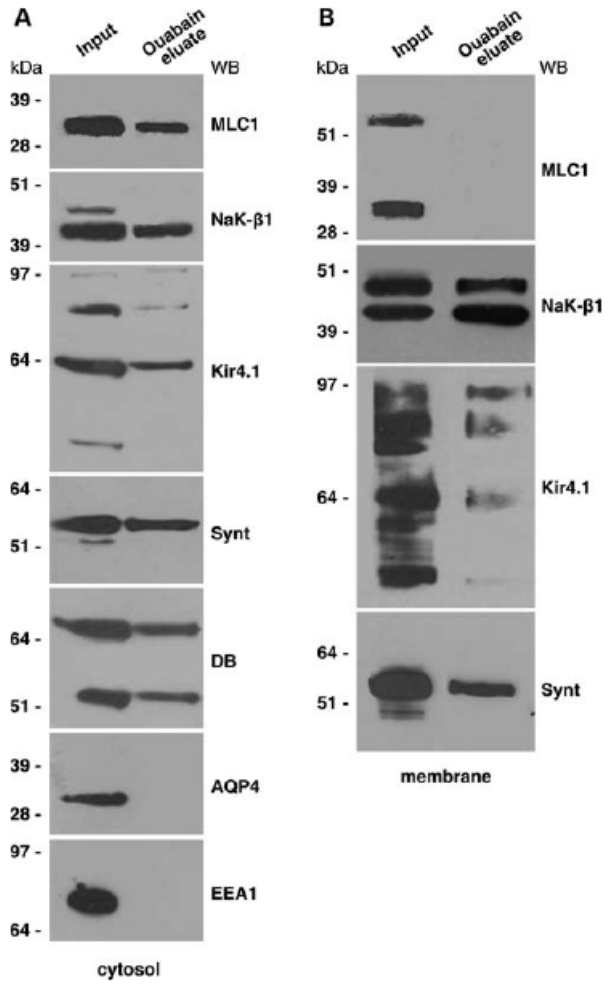

Figure 2. Co-purification of MLC1 and Na,K-ATPase by ouabain affinity chromatography. Fractions eluted from ouabain affinity chromatography of cytosol (A) and membrane (B) extracts from the rat brain. Chromatography fractions were analysed by SDS–PAGE and WB and probed with Abs against MLC1, Na,K-ATPase β1 (NaK-β1), early endosome antigen1 (EEA1), Kir4.1, syntrophin (synt), dystrobrevin (DB) and aquaporin 4 (AQP4). MLC1, NaK-β1, Kir4.1, synt and α1 and α2 dystrobrevin (DB) isoforms (87 and 55 kDa revealed by the monoclonal antibody), but not AQP-4 and EEA1, were detected in the ouabain eluate from the cytosolic extract of rat brain (A). No MLC1 was found in the membrane eluate, although NaK-β1, Kir4.1 and syntrophin were present (B).

in brain cytosolic extract and partially co-localizes with MLC1 in immunofluorescences on cultured rat astrocytes (14), was not detected in the ouabain-eluate (Fig. 2A). When we analysed the ouabain-eluted fraction obtained from the rat brain membrane extract, we failed to reveal both the 60 and 30 kDa MLC1 membrane components (14; Fig. 2B), although NaK-β1 and the associated proteins Kir4.1 and syntrophin were present. It is possible that the amount of membrane MLC1 bound to the ouabain column is too low to be detected by WB or that several proteins known to interact with Na, K-ATPase in the brain (20) might interfere with its binding to MLC1.

Co-localization of MLC1 and NaK-β1 in cultured astrocytes and their re-distribution around intracellular vacuoles during hypo-osmotic shock

To identify the subcellular compartments where MLC1–NaK-β1 interaction occurs, we next performed immunolocalization experiments in rat primary astrocytes. Immunofluorescence stainings with an anti-MLC1 pAb raised against the intracellular MLC1-NH_2 domain confirmed that MLC1 mainly localized in endoplasmic reticulum (ER) areas and endolysosomal compartments (Fig. 3A, Iso, red), as described previously (14). The distribution of NaK-β1, as revealed by an mAb recognizing the COOH domain of the protein, indicated that NaK-β1 was expressed both at the plasma membrane level and in discrete perinuclear areas and some cytoplasmic vesicles (Fig. 3A, Iso, green). The intracellular localization of the Na,K-ATPase enzyme in cytoplasmic structures has been described previously in different cell types and demonstrated to be due to its trafficking through the endolysosomal compartment (23–26). The merge of the immunostainings indicated that in most astrocytes, MLC1 and NaK-β1 co-localized primarily in cytoplasmic vesicles distributed around the nuclei, most likely in ER domains and endolysosomal organelles (Fig. 3A Iso, merge), thus suggesting a possible MLC1/Na,K-ATPase association during endocytosis processes or transport from ER to the plasma membrane (14).

In MLC patients, magnetic resonance imaging reveals diffuse signal abnormalities, swelling of the cerebral white matter and cysts, suggesting that MLC1 may have a role in the regulation of ion and water transport associated with cell volume regulation in the brain. Since Na,K-ATPase regulates cell swelling and regulatory volume decrease (RVD) occurring in astrocytes after exposure to hypo-osmotic medium (27,28), we cultured rat astrocytes in hypo-osmotic solution (29,30) for different time lengths (6 and 12 h) and analysed NaK-β1 and MLC1 intracellular distribution in comparison with untreated cells. Phase contrast microscopy analysis of control (Fig. 3B, Iso) and treated astrocytes (Fig. 3B, Hypo) indicated that the latter were slightly swelled with enlarged nuclei showing a 25–30% increase in size compared with controls, as assessed by confocal microscopy comparative analysis (data not shown), as described previously (27). We also observed the formation of intracellular vacuoles that increased in number and size over time and were more evident in cells subjected to 12 h hypo-osmotic treatment (Fig. 3B, Hypo, arrowheads).

The finding that osmotic imbalance can lead to the formation of intracellular vacuoles has been already reported in different cell types (31,32). Moreover, ER-derived watery vacuole formation induced by long-term hypo-osmotic shock was described previously in 3T3L1 cells (33). By performing immunofluorescence stainings of astrocytes subjected to 6 h hypo-osmotic treatment with anti-MLC1 and anti-NaK-β1 Abs, we observed that MLC1 was distributed around the newly formed vacuoles, most likely in the membranes bordering the vacuolar structures, where it partially co-localized with NaK-β1 (Fig. 1A, Supplementary Material). After the 12 h hypo-osmotic treatment, we observed an enlargement of the vacuoles and an almost complete co-localization between MLC1 and NaK-β1 in the vacuolar rims and around nuclear

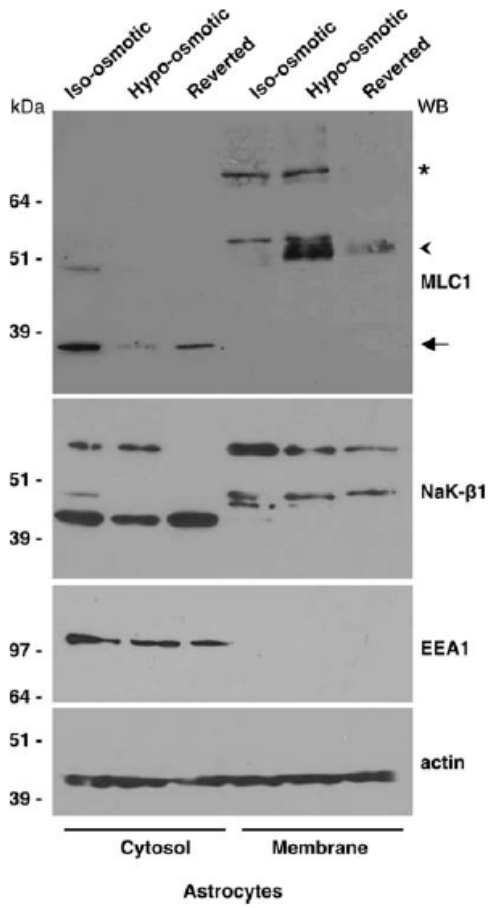

Figure 4. Hypo-osmotic treatment of rat astrocytes increases the membrane components of MLC1. Rat astrocytes were incubated overnight with iso-osmotic or hypo-osmotic buffer or were put back in iso-osmotic cell culture medium for 12 h after hypo-osmotic treatment. Then, the cytosolic and membrane fractions were analysed by SDS–PAGE and WB. Hypo-osmotic treatment causes an increase in the membrane MLC1 components, the 60 kDa (arrowhead) and the higher molecular weight oligomers (asterisk), and a decrease in the cytosolic 36 kDa MLC1 component (arrow). NaK-β1 protein levels do not change, with the only exception of the higher molecular weight component, probably a multimeric proteic aggregate that is not present in the cytosol after the reversion treatment. No differences were detected in EEA1 and actin protein levels. Actin was also used as internal loading control.

in the vacuolar membranes (Fig. 2B, Supplementary Material), suggesting the participation of Kir4.1 channel to the hypo-osmosis-induced changes observed in astrocytes.

Hypo-osmotic treatment increases the membrane component of MLC and its association with the Na,K-ATPase enzymatic complex in astrocytes

To investigate whether hypo-osmosis could also induce changes in the expression levels of the membrane and cytosolic MLC1 protein components, we performed WB analysis using extracts of control astrocytes, astrocytes exposed to hypo-osmotic solution for 12 h and astrocytes reverted to iso-osmotic condition after hypo-osmotic treatment. Figure 4 shows that the 60 kDa MLC1 membrane protein markedly increased during hypo-osmotic treatment. Conversely, the 36 kDa cytosolic MLC1 protein component was decreased. Both MLC1 components returned to control levels after the removal of the hypo-osmotic medium (Fig. 4). RT–PCR performed on control and hypo-osmotic astrocytes revealed no differences in MLC1 mRNA levels (data not shown), indicating that the higher levels of MLC1 membrane protein observed in WB experiments are due to an increase in protein stability and/or trafficking to the plasma membrane, and not to neosynthesis. No significant differences were observed in NaK-β1 protein levels, with the only exception of the highest molecular weight component, probably a multimeric protein aggregate that was found to decrease in the cytosol after the removal of hypo-osmotic medium. Similarly, no differences were detected in the protein levels of EEA1 and actin, used as internal loading control (Fig. 4), as well as of Kir4.1, dystrobrevin and syntrophin (data not shown). Since it has been proposed that the 60 kDa MLC1 component could represent the dimeric functional complex (9,14), these data suggest that MLC1 may be functionally involved in the processes regulating cell volume and vacuole formation under hypo-osmotic condition.

To analyse the effects of hypo-osmotic treatment on MLC1 interaction with the whole Na,K-ATPase enzyme and the associated multiprotein complex observed after ouabain affinity chromatography on rat brain tissue (Fig. 2), we used protein extracts derived from the cytosol and membrane fractions of control and hypo-osmosis-treated astrocytes. WB analysis of ouabain-eluted proteins showed the presence of small amounts of the cytosolic 36 kDa MLC1 along with Na,K-ATPase complex components, Kir4.1, dystrobrevin and EEA1 without quantitative differences between normal and hypo-osmosis-treated astrocytes (Fig. 5A). Nonetheless, syntrophin appeared decreased in the ouabain eluate derived from hypo-osmosis-treated cells. In these experiments, we used a more sensitive in-house-generated antibody to detect dystrobrevin isoforms (45) (Fig. 5A and B). Similar to what observed in rat brain, AQP4 was not found in the ouabain eluate, although two bands of molecular weight ~32 and 60 kDa, which represent the monomeric and dimeric forms of AQP4, respectively (46), were detected by the pAb used in the starting material (Fig. 5A and B, Input). When we analysed the fractions obtained from astrocyte membrane extracts, we found that in astrocytes, in contrast to what observed with rat brain membrane extracts, the 60 kDa and the higher molecular weight MLC1 oligomers (9,11,14) were eluted from the ouabain column along with Na,K-ATPase, Kir4.1, dystrobrevin and syntrophin (Fig. 5B). In the same eluted sample, we also found caveolin-1 (cav-1), the specific structural component of caveolae that has been reported to directly bind Na,K-ATPase (47,48) and MLC1 (14). Most importantly, after hypo-osmotic shock, the amount of the 60 kDa MLC1 and of the higher molecular weight oligomers eluted from the column increased along with NaK-β1, although no major differences were seen in the other proteins analysed (Fig. 5B). These data confirm that the interaction between MLC1 membrane components and NaK-β1is favoured by hypo-osmotic treatment, indicating that it may be functionally important in astrocytes during changes in homeostatic conditions. The presence of MLC1 in

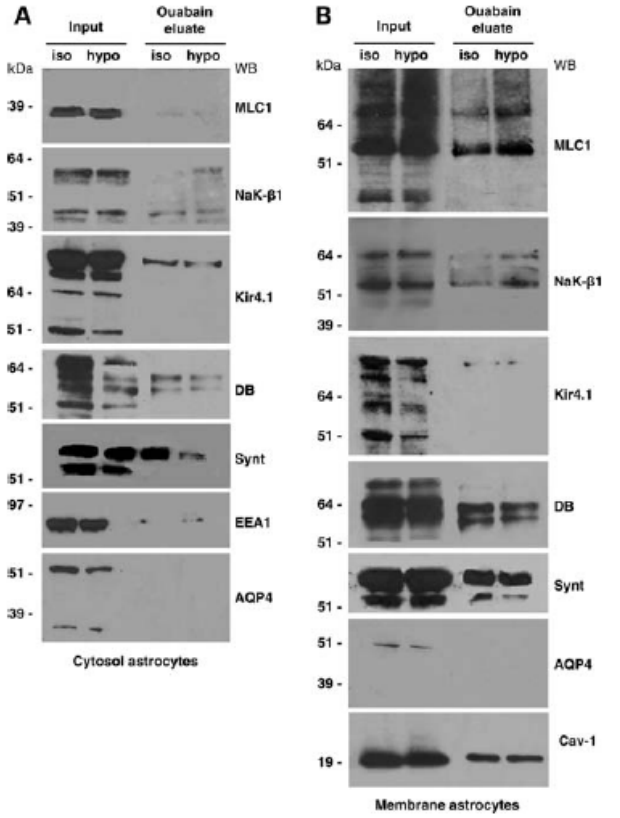

Figure 5. MLC1 is a component of a multiprotein complex associated with Na,K-ATPase in astrocytes, and hypo-osmotic treatment increases its association with Na,K-ATPase. Analysis of the eluted fractions from ouabain affinity chromatography of cytosol (A) and membrane (B) extracts of cultured astrocytes grown for 12 h in iso-osmotic and hypo-osmotic mediums. Fractions were analysed by SDS–PAGE and WB and probed with Abs against MLC1, Na,K-ATPase β1 (NaK-β1), Kir4.1, dystrobrevin (DB), syntrophin (Synt), early endosome antigen1(EEA1), aquaporin-4 (AQP4) and caveolin-1 (Cav-1). Following hypo-osmotic shock, syntrophin decreases in the ouabain eluate of cytosol extract (A), whereas MLC1 and NaK-β1 increase in the ouabain eluate of membrane extract (B). AQP4 was not detected in the ouabain eluate of both cytosol and membrane fractions.

the Na,K-ATPase-associated protein complex in the eluates derived from astrocyte membranes but not in those derived from brain membrane extracts could be due to enrichment of the MLC1 60 kDa protein in purified astrocytes relatively to whole-brain-derived samples. Alternatively, because rat astrocytes prevalently express the α2 isoform of the Na,K-ATPase that shows a lower affinity for ouabain relative to the neuronal specific α1 isoform (49), we cannot exclude that, in the chromatography experiments performed with brain membrane extract, the competition between the two isoforms limits the binding of the α2 astrocytic-specific isoform to the ouabain column. Experiments are in progress to evaluate this possibility.

Association of MLC1 and Na,K-ATPase in hypertrophic astrocytes in the inflamed human brain

Altogether, the above results suggested that MLC1 may cooperate with Na,K-ATPase in processes regulating cell volume and osmotic balance in astrocytes. To investigate this issue further, we analysed MLC1/Na,K-ATPase relationship in an inflammatory condition of the CNS. Using immunohistochemistry, we analysed the localization of NaK-β1 and MLC1 in post-mortem brain samples from a patient with multiple sclerosis (MS), a disease characterized by chronic inflammation accompanied by reactive astrogliosis and astrocyte hyperthophy (50). As already reported (9–11), we found that, in the normal brain, MLC1 was expressed in astrocytes contacting blood vessels and that its expression increased in the MS brain in the perivascular areas of immunologically active, demyelinated lesions in the white matter (Fig. 3, Supplementary Material). We also observed that numerous small blood vessels, compared with the normal brain sample, were MLC1-immunopositive, probably reflecting the increased levels of expression of the MLC1 protein in MS brain (Fig. 3, Supplementary Material). Double-immunofluorescence stainings with anti-MLC1 and anti-NaK-β1 Abs on a brain section containing an active lesion with numerous perivascular inflammatory cell infiltrates revealed a partial co-localization of MLC1 with the NaK-β1 around blood vessels (Fig. 6A and B). Interestingly, in some hypertrophic astrocytes, the two molecules co-localized in cytoplasmic vesicles (Fig. 6C, arrowheads). Virtually, no co-localization between the two proteins was seen in normal brain tissue (not shown). These findings suggest that in MS lesions, MLC1–NaK-β1 interaction in activated astrocytes might be induced by osmotic changes caused by inflammation.

DISCUSSION

In this study, we demonstrate a direct association between MLC1, the protein product of the MLC gene whose mutations cause MLC disease, and the β1 subunit of Na,K-ATPase, the ubiquitous enzyme that is responsible for the maintenance of Na^+ and K^+ gradients across the plasma membrane. This molecular association was detected and characterized *in vitro*, in cultured astrocytes and in brain tissue. We also found that MLC1 along with Na,K-ATPase is a component of a macromolecular complex that encompasses the K^+ channel Kir4.1 and the DGC proteins syntrophin and dystrobrevin and undergoes reversible dynamic association in early endosome-derived vacuoles that form in cultured astrocytes during hypo-osmotic shock. Because brain pathology in patients carrying MLC1 mutations is thought to be caused by alterations in the mechanisms regulating osmotic balance and cell volume changes, the demonstration that MLC1 associates with the Na,K-ATPase enzymatic complex represents the first experimental evidence of the hypothesized involvement of MLC1 in astrocyte functions controlling these processes.

We first identified the β1 subunit of Na,K-ATPase among the MLC1-interacting clones screened by the yeast two-hybrid assay from a human fetal brain cDNA library. The Na,K-ATPase enzyme is a heterodimer made of the assembly of α catalytic and β subunits (51), with some cell types, including astrocytes, expressing an additional regulatory γ subunit (52–54). By pumping three Na^+ ions from the cytoplasm in exchange for two extracellular K^+ ions coupled to the hydrolysis of one molecule of ATP,

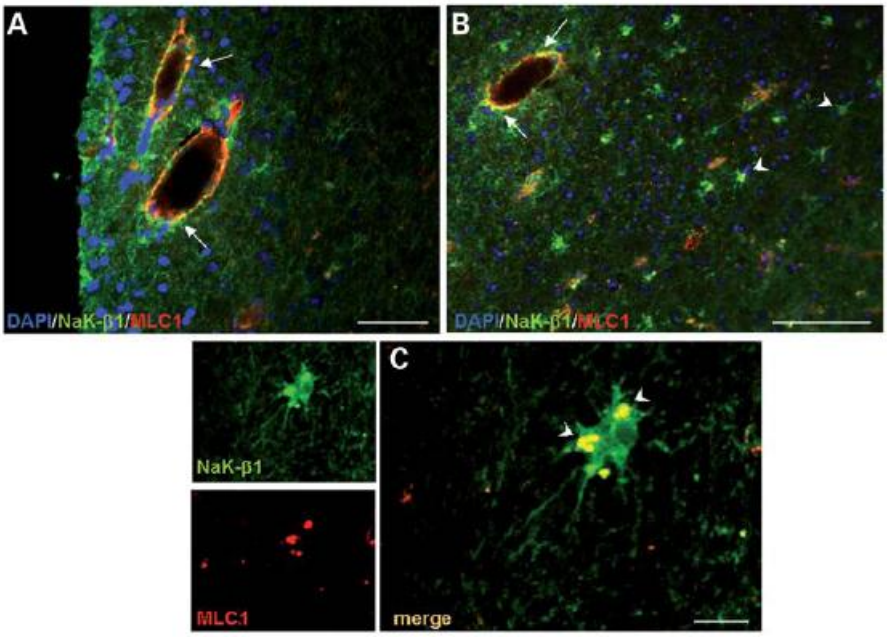

Figure 6. Co-immunolocalization of NaK-β1 and MLC1 in multiple sclerosis (MS) brain lesions. (A and B) Immunofluorescence staining of MS brain tissue with anti-MLC1 pAb (red) and anti-Na,K-ATPase β1 (NaK-β1) mAb (green) shows that MLC1 and NaK-β1 immunoreactivities partially overlap around inflamed blood vessels (A and B, arrows) and in hypertrophic astrocytes in the lesioned periventricular white matter (B, arrowheads). (C) High-power magnification of a confocal image shows the overlapping vesicular localization of MLC1 and NaK-β1 in reactive astrocytes (arrowheads). Scale bars: A: 50 μm; B: 100 μm; C: 10 μm.

Na,K-ATPase produces ion gradients across the plasma membranes, thus maintaining the electrochemical gradient of the membrane (51,55). Cells use these ion gradients in many essential processes, such as osmoregulation, generation of plasma membrane potential and maintenance of intracellular pH and Ca^{2+} concentration, vectorial transport of many solutes and excitability in muscle fibres and neurons.

The β1 subunit is a highly glycosylated membrane protein of 303 amino acids that is responsible for the functional expression of the Na,K-ATPase pump. It contains a short NH_2 cytoplasmic domain and a large extracellular portion that includes the C-terminal and, by binding the nascent α subunit, leads to the correct conformation of the whole enzymatic complex allowing its release from ER and targeting to the plasma membrane (56). By performing biochemical analyses on cultured rat and human astrocytes and brain tissue, we confirmed the specific direct interaction between the MLC1-NH_2 terminal (1–55 amino acids) and the extracellular portion of the NaK-β1 that encompasses the COOH domain (156–303 amino acids). Although at the plasma membrane level the two interacting peptides show an opposite orientation (intracellular for the MLC1-NH_2 terminal and extracellular for the COOH domain of NaK-β1), it can be hypothesized that the interaction described here can occur in other intracellular compartments where the proteins are correctly oriented in order to interact. This hypothesis is supported by the observation that in cultured astrocytes, MLC1 and NaK-β1 immunoreactivities co-localize in intracellular structures, like cytoplasmic vesicles and organelles. Interestingly, a similar structural interaction between the extracellular portion of the NaK-β1 subunit (229–298 amino acids) and the intracellular COOH domain of the large conductance Ca^{2+}-activated K channel (BK_{Ca} channels or Slo1 protein) has been reported recently (57). Although also in this case the interacting regions of the two proteins are localized one inside and the other outside the cell membrane, the functional outcome of this interaction is supported by the finding that NaK-β1 plays a role in regulating the steady-state expression of BK_{Ca} channels on the cell surface (57). It has been suggested that the NaK-β1 subunit contributes to the targeting and stabilization of membrane proteins, particularly ion channels, to specific cellular domains to form a larger protein complex involved in ion homeostasis and signalling pathways (57), as indicated already by previous studies (58,59). Of note, in the mouse brain, the COOH domain of the NaK-β1 subunit was found to interact with MONAKA (60) and NKAIN family proteins (NKAIN 1, 2, 3, 4; 61), all recently cloned membrane proteins of unknown function that have been proposed to cooperate in the regulation of NaK-β1 function.

In line with the hypothesis that the extracellular domain of the β1 subunit of Na,K-ATPase could play a role in determining multiproteic interactions that are important for the different functions of the whole Na,K-ATPase enzymatic complex (see above), we show that MLC1, through its binding to NaK-β1, interacts with the whole Na,K-ATPase pump and is part of a multiproteic complex that includes the K^+ channel Kir4.1, the DGC adaptor proteins syntrophin, dystrobrevin and caveolin-1 and could be assembled in specific cellular domains in response to functional demands. The presence of caveolin-1 among the proteins isolated by ouabain affinity

chromatography supports the possible caveolar localization of the whole complex. Although in cultured astrocytes we observed very low levels of MLC1/NaK-β1 co-localization at the plasma membranes where caveolae are structured, we cannot exclude that in certain physiological conditions, by binding to the C-terminal of the NaK-β1, MLC1 can be recruited and stabilized to the caveolae to fulfil specific functions. After having exerted their functions, both proteins could be endocytosed in caveosomes, as reported previously for MLC1 (14), to be sorted to specific endolysosomal organelles for storing, recycling or degradation. This scenario could explain, at least in part, the intracellular vesicular localization of MLC1-NH_2 and NaK-β1-COOH domains in astrocytes *in vitro*, indicating that their interaction may occur during intracellular sorting and trafficking route. It has been demonstrated that the presence of the Na,K-ATPase pump in early endosomes is functionally related to its activity of internal pH regulation (35,36,62). These organelles maintain a slightly acidic pH, which is directly responsible for their ability to ensure proper sorting of incoming receptors and ligands during endocytosis. Na,K-ATPase, which appears to be a functional component of the endosomal membrane, contributes to the early endosome pH control by inhibiting excessive acidification (23,37,62). This activity was found only in early endosomes, consistent with their limited acidification capacity relative to late endosomes and lysosomes (62). Our finding that MLC1 preferentially co-localizes with NaK-β1 in vacuolar structures of early endosome origin (EEA1- and Rab5-positive endosomes) that are generated in rat astrocytes during hypo-osmotic treatment supports the idea that the intracellular interaction between MLC1 and NaK-β1 occurs in early endosomes and may be functionally relevant during osmotic stress and cell swelling. The observations that in astrocytes MLC1/NaK-β1 co-immunostaining is maintained around the limiting membrane of vacuoles induced during stress condition and that the two molecules co-localize around blood vessels and in hypertrophic astrocytes in the inflamed MS brain but not in the non-pathological brain are consistent with the idea that this interaction is not constitutive but dynamic and might reflect specific functional requirements during osmotic stress or cell volume alterations. It is worth noting that alterations of Na,K-ATPase functionality in the brain have been observed in a fatal spongiform encephalopathy (63). Moreover, in mice, disruption of the β2 isofom of Na,K-ATPase, initially identified as the adhesion molecule on glia that mediates neuron–glia interaction (64), results in swelling and subsequent degeneration of astrocyte end-feet in the brainstem.

It has been suggested that brain pathology in patients carrying MLC1 mutations may be caused by alterations in the processes regulating osmotic balance and cell volume changes. MLC pathological features like fluid cysts, myelin vacuolation, enlargement of extracellular space and swelling of the white matter (1–3) are compatible with alterations in astrocyte-mediated processes controlling brain volume and ion and fluid exchanges. It is known that astrocytes, by contacting neurons and cells lining fluid-filled compartments, play a crucial role in regulating ion and water homeostasis through the selective transmembrane movements of organic and inorganic molecules and the balance of osmotic gradients which involve alterations of cell volume (reviewed in 12). Cell survival is ensured by avoiding excessive alterations of cell volume, a process that requires the strictly regulated and cooperative action of different ion and water channels (65), including the Na,K-ATPase pump (66,67). In astrocytes, Na,K-ATPase is involved in cell volume regulation and swelling induced by glutamate and hypo-osmotic treatment (27,67–71), water exchange (22) and the generation of calcium waves (72,73). Moreover, it plays a major role in K^+ and glutamate uptake following neuronal excitation and takes part in astrocyte–neuron metabolic coupling (22,49,74,75). Our experiments suggest that the long-term exposure of astrocytes to hypo-osmotic solution may deregulate the pH control activity of the Na,K-ATPase, leading to organelle osmotic imbalance and swelling (23,37). The presence of MLC1 along with NaK-β1 in the membranes lining the swelled organelles indicates their possible cooperation in the molecular mechanism generating these structures. In support of this hypothesis, we also found that hypo-osmotic shock induced an increase in the amount of the MLC1 functional component (60 kDa) and favoured the association of MLC1 and NaK-β1 to the ouabain-eluted macromolecular complex. Collectively, these results suggest that, along with Na,K-ATPase, MLC1 is functionally involved in osmotic imbalance-induced volume alterations. Although we do not know yet the specific function of MLC1, we hypothesize that this protein works as an ion channel itself, or as a chaperone subunit of some other channel(s) cooperating with the Na,K-ATPase enzyme in astrocyte swelling and/or RVD occurring during hypo-osmotic shock.

Exposure of cells to hypo-osmotic extracellular fluid leads to water influx, along the osmotic gradient across the cell membrane and consequent cell swelling which involves the collaborative activity of different transporters, exchangers, water and ion channels, including AQPs, K^+ channels and/or anion channels, KCl^- co-transporter, K^+/H^+ and Cl^-/HCO_3^- exchanger. Cell volume response of astrocytes in hypo-osmotic medium involves the net movement of osmoles by a mechanism that is dependent on cellular energy and tightly coupled to the Na,K-ATPase ion pump (27). It is noteworthy that Kir4.1, the K^+ channel previously found to bind MLC1 (5,14), was also present in the macromolecular complex of the Na,K-ATPase, thus indicating that the two proteins may participate in the same process. In addition, the presence of syntrophin and dystrobrevin suggests that these scaffolding proteins may mediate the link between different components of the Na,K-ATPase transmembrane complexes. In glial cells, Kir channels, and particularly Kir4.1, control extracellular K^+ homeostasis by uptake of K^+ ions from the extracellular space and their release into the microvasculature. In addition, in glial cells, Kir4.1 has been implicated in K^+-associated water influx and cell swelling in hypo-osmotic conditions, *in vitro* and *in vivo* systems (76,77). It has been shown that the activity of Kir4.1 on cell volume regulation is mediated by its association with the water channel AQP4, although this finding has not been reproduced in all studies (76). In our experiments, we could not detect AQP4 in the ouabain-eluted fraction, despite its recently reported interaction with the α subunit of Na,K-ATPase (22). It is possible that AQP4 is present in the Na,K-ATPase-associated macromolecular complex in lower amounts relative to other proteins and its detection may depend on the antibody used.

To understand whether the loss of MLC1 observed in MLC brain patients (5) may affect the functionality of the Na,K-ATPase/MLC1 macromolecular complex, we performed preliminary RNA-interfering experiments using lentiviral infection. Although MLC1 mRNA was downregulated to some extent (~30% reduction), we did not observe a reduction of MLC1 protein levels, as assessed by WB and immunostainings (data not shown). We are currently exploring the possibility of using multiple combinations of different interfering RNA sequences to induce efficient MLC1 protein downregulation.

MLC1 patients show variability in both disease progression and severity of clinical symptoms (2,5,78). So far, about 50 different mutations distributed along the whole MLC1 gene have been identified with no evident correlation between genotype and phenotype (5). In fact, clinical phenotypes may be different in patients with the same mutation, whereas patients with genetically proved mutations may share similar clinical features with patients without mutations in the MLC1 gene (7,9). In exon 2, which encodes the N-terminal region and part of the first transmembrane domain of MLC1, different types of mutations have been identified: either deletion/insertion mutation types that cause frameshifts and the appearance of premature truncation codons (probably leading to protein loss of functions) or missense mutations that do not disrupt synthesis of full-length MLC1 protein (5,79). However, the observed clinical phenotypes are variable and do not correlate with the severity of the mutations, as observed for mutations affecting other regions of the protein (5). Interestingly, we have recently found that in the cytoplasmic N-terminal domain of MLC1, S27 can be phosphorylated *in vitro* by both protein kinase A and protein kinase C (14), suggesting a potential regulatory function of this domain in MLC1-binding properties. Based on these findings, we shall investigate further the association of MLC1 with Na,K-ATPase in human astrocytoma cell lines stably transfected with wild-type and mutated MLC1 in both normal and hypo-osmotic conditions, with particular attention to the phenotype of cells expressing mutations localized in the NH_2 region of the protein that interacts with the β1 subunit of the Na,K-ATPase.

In conclusion, by showing a dynamic interaction between MLC1 and Na,K-ATPase β1, this study provides the first experimental evidence for an involvement of MLC1 in processes controlling cellular volume and ion homeostasis in astrocytes. These findings will pave the way for a better understanding of the pathogenetic mechanisms underlying MLC and thus for the development of the most appropriate strategies to cure this disease.

MATERIALS AND METHODS

Plasmid constructs

The coding region of the MLC1 N-terminal domain (MLC1-NH_2; amino acids 1–55) obtained by RT–PCR from human brain tissue RNA samples, as described previously (11), was cloned into pGBKT7 vector (Clontech) to create pGBKT7/MLC1-NH_2. PCR reactions were performed using the following primers: forward 5′-CAT GCC ATG GCA ACC CAG GAG CCA TTC AGA-3′ and reverse 5′-CGG AAT TCC ACA GAG AAG ACC CAC GT-3′. cDNA was subjected to PCR amplification for 35 cycles in the following conditions: 30 s/94°C, 30 s/50°C and 30 s/72°C for the first 5 cycles and 60 s/94°C, 30 s/65°C and 30 s/72°C for the remaining 30 cycles. After *Nco*I and *Eco*RI digestion, the MLC1-NH_2 fragment was inserted into the *Nco*I–*Eco*RI site of pGBKT7 vector. The correct orientation of the cDNA insert was verified by sequencing service (M-Medical).

Yeast two-hybrid screen

Two-hybrid screening was carried out by yeast mating, using the Matchmaker Gal4 Two-Hybrid System 3 (Clontech) as reported previously (45). Briefly, pGBKT7/MLC1-NH_2 was tested negative for auto-activation of reporter gene activity in the yeast two-hybrid reporter strains, *Saccharomyces cerevisiae* AH109 (*MAT***a**, *trp1–901, leu2–3, 112, ura3–52, his3–200, gal4D, gal80D, LYS2::GAL1*$_{UAS}$*–GAL1*$_{TATA}$*–HIS3, GAL2*$_{UAS}$*–GAL2*$_{TATA}$*–ADE2, URA3::MEL1*$_{UAS}$*–MEL1*$_{TATA}$*–lacZ MEL1*) and *S. cerevisiae* Y187 (*MAT***α**, *ura3–52, his3–200, ade2–101, trp1–901, leu2–3, 112, gal4*△, *gal80*△,*met*$^-$, *URA3::GAL1*$_{UAS}$*–GAL1*$_{TATA}$*–lacZ MEL1*). The Gal4 DNA-binding domain construct pGBKT7/MLC1-NH_2 was used to transform the *MAT***a** yeast strain AH109. AH109[pGBKT7/MLC1-NH_2] was then employed as a bait strain to screen a human fetal brain cDNA library (Clontech), which was cloned into the activation domain vector pGADT7-Rec, and pre-transformed in the *MAT***α** yeast strain Y187. The yeast mating screening was performed according to the manufacturer's instructions. Diploids were selected by culture on minimal synthetic dropout medium lacking Trp, Leu, His and Ade (–TLHA), and including 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal) for 7–14 days. Single colonies of yeast obtained from the library screen growing on SD/-TLHA/X-α-Gal were re-streaked at least twice onto SD/-TL/X-α-Gal to allow segregation, and then transferred to SD/-TLHA/X-α-Gal to verify that they maintained the correct phenotype. Duplicates containing the same AD/library plasmids were eliminated by yeast colony PCR followed by restriction digestion with frequently cutting enzymes. AD/library plasmids from sorted colonies were isolated and rescued using *E. coli* strain DH5α on ampicillin-resistant plates. Unique inserts were sequenced and DNA and protein sequence analyses performed with the BLAST algorithm at the National Center for Biotechnology Information (NCBI). pGADT7-Rec plasmids encoding the library clones were tested for auto-activation of the reporter gene in yeast. Isolates growing in SD/-TLHA/X-α-Gal and developing blue staining without the presence of the MLC1-NH_2 bait were excluded from further investigation. Activation of the reporter genes in the positive colonies was confirmed in the same experiments.

In vitro transcription and translation

In vitro transcription and translation of pGADT7/Na,K-ATPase β1 was carried out using the TNT T7 Quick Coupled Transcription/Translation System (Promega) in the presence of EasyTag L-[^{35}S] Methionine (PerkinElmer)

according to the manufacturer's protocol. Newly synthesized proteins were separated by SDS–PAGE and analysed with an Instant-Imager (Packard).

Recombinant protein preparation and pull-down assays

GST, GST-MLC1-N terminal (GST-MLC1-N) and GST-MLC1-C terminal (GST-MLC1-C) pre-bound to glutathione-Sepharose beads were used in *in vitro* protein-binding assays, as described previously (14). Briefly, primary rat astrocyte lysate obtained by lysis with 1% Triton X-100, 0.5% sodium deoxycholate, 150 mM NaCl, 150 mM Hepes (pH 7.4) and centrifugated at 16 000*g* at 4°C for 20 min, was pre-cleared by incubation with the GST-bound to glutathione-agarose and then incubated with agarose-bound GST-MLC1-N terminal or -C terminal peptides. Following exhaustive washes, protein-bound beads were eluted with 0.1 M glycine, pH 3. Aliquots (0.5 ml) of eluted proteins were precipitated with acetone (1:4 v/v) overnight at 4°C and analysed by SDS–PAGE and WB.

Biochemical enrichment of His-tagged proteins

Lysates obtained from an astrocytoma cell line stably overexpressing His-tagged MLC1 (U251-HisMLC1) were incubated overnight at 4°C with 100 µl (50% v/v suspension) of Ni-NTA Agarose (Qiagen, Hilden, Germany); after extensive washings, protein elution was carried out using imidazole, at a concentration of 50 and 200 mM (14). The eluted proteins were analysed by SDS–PAGE and WB.

Cell cultures and treatments

Astrocyte-enriched cultures (95% purity) were generated from 1–2-day-old newborn Wistar rats, as described previously (80). Cells were maintained in culture in DMEM medium (Euroclone, UK) supplemented with 10% FCS (Gibco, BRL, Gaithersburg, MD, USA) and antibiotics (penicillin/streptomycin, Euroclone) in 5% CO_2 atmosphere. For cell treatments, primary cultured astrocytes were plated in polylysinated 60 mm diameter dishes, washed three times in PBS and treated for different time lengths with iso-osmotic medium (122 mM NaCl, 3.3 mM KCl, 0.4 mM $MgSO_4$, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 10 mM D-glucose, 25 mM HEPES, pH 7.4) or in the same buffer in which NaCl concentration was half-reduced to 50 mM NaCl (hypo-osmotic buffer) as described previously (30,81). After stimulation, cells were washed three times in PBS, collected by scraping, and centrifuged at 2700*g* at 4°C for 20 min. Cell pellets were solubilized as described below. For immunofluorescence stainings, cells were treated with the same buffers. For the reversion experiments after 12 h of hypo-osmotic treatment, cells were put back in cell culture medium and left for additional 12 h before immunofluorescence or WB analysis.

Ouabain affinity chromatography

The ouabain affinity matrix was prepared according to a previously described procedure (20) with some modifications. For the cytosolic and membrane brain extracts, 0.5 g of epoxy-activated agarose (Sigma) with a 12 atom spacer was added to 7.5 ml of a solution containing 12.5 mM ouabain and 100 mM sodium carbonate, pH 8.5, at RT. The mixture was then incubated at 37°C for 20 h with gentle shaking after which the resin was washed with 10 ml of 100 mM sodium carbonate buffer, pH 8.5, followed by 10 ml of water. The resin was incubated with 1 M ethanolamine, pH 8.5, for 4 h at 37°C to block unreacted coupling sites. The column was washed with 200 mM Tris–HCl, pH 8.5, and stored in 50 mM imidazole containing 0.1% NaN_3, pH 7.4. Before purification, the ouabain affinity column was washed with 50 bed volumes of 50 mM imidazole, pH 7.4, and equilibrated with two bed volumes of K^+ loading buffer (60 mM KCl, 25 mM imidazole, 1 mM EDTA, 1 mM $CaCl_2$ and 0.1% Triton X-100, pH 7.4). A mock elution was performed with 5–10 ml of elution buffer (12.5 mM ouabain, 25 mM imidazole, 150 mM NaCl and 1x protease inhibitor cocktail, pH 7.4), followed by 4x bed-volume wash with K^+ loading buffer. The sample was incubated with the ouabain affinity matrix overnight at 4°C and the flow-through collected (ouabain unretained fraction). The column was then washed with 3x bed volumes of K^+ loading buffer. One ml of elution buffer was then added to the column and after 50 min incubation at 4°C collected (ouabain-eluted fraction). Fractions were analysed by SDS–PAGE and WB. For hypo-osmotic and iso-osmotic astrocyte extracts, 1 g of epoxy-activated agarose was used for 15 ml solution of the cytosol and membrane fractions.

Immunofluorescence and confocal microscopy analysis

Astrocytes grown on polylysine-coated coverslips were incubated in control or hypo-osmotic solution for 6 or 12 h, then fixed for 10 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 RT with the following primary antibodies (Abs) diluted in PBS, 0.025% Triton X100: affinity-purified anti-MLC1 pAb (1:50, Atlas AB, AlbaNova University Center, Stockholm, Sweden), anti-Na,K-ATPase β1 mAb (1:50, Millipore, Temecula, CA, USA), anti-EEA1 mAb (1:50, BD Transduction Laboratories, Lexington, KY, USA). A biotinilated secondary antibody (4.3 µg/ml, Biotin-SP-AffiniPure goat anti-rabbit IgG H+L; Jackson Immunoresearch Laboratories, West Grove, PA, USA) followed by incubation with 2 µg/ml streptavidin-TRITC (Jackson, UK), or a fluorescein-conjugated donkey anti-mouse (1:100, Jackson) was used. Coverslips were washed, sealed in Vectashield medium (Vector Lab, Burlingame, CA, USA) and analysed with a laser scanning confocal microscope (LSM 5 Pascal, Carl Zeiss, Jena, Germany).

Immunostaining of human brain tissue

Indirect immunofluorescence technique was used to detect MLC1 and NaK-β1 in autopsy brain tissues obtained from the UK MS Tissue Bank at Imperial College London. Post-mortem MS tissues were obtained via a UK prospective donor scheme with full ethical approval (08/MRE09/31). Brain tissue was fixed in 4% paraformaldehyde in PBS, cryoprotected in 30% sucrose for 1 week, frozen in dry ice-cooled

isopentane and stored at −75°C. Air-dried, acetone-fixed (4°C), 10 μm thick cryosections were rehydrated with PBS and post-fixed sections were subjected to the antigen retrieval procedure with microwave in citrate buffer 10 mM (pH 6.0), as described previously (11). For double-immunofluorescence stainings, sections were incubated for 1 h with 20% of normal goat serum (NGS) (Jackson) and then overnight at 4°C with a mixture of rabbit anti-MLC1 pAb (1:250, ATLAS) and anti-Na,K-ATPase β1 mAb (1:200 Millipore) in PBS containing 2% BSA and 0.05% Triton X-100. After extensive washing, sections were incubated for 1 h at RT with a mixture of fluorescein-conjugated goat anti-mouse and rhodamine-conjugated goat anti-rabbit Abs diluted in PBS containing 10% NGS. Images were analysed with a fluorescence microscope (Leica DM-4000B Microsystems, Bannockburn, IL, USA) and with a laser scanning confocal microscope, as above.

Brain and astrocyte subcellular fractionation procedures and WB

Cytosolic and membrane fractions from rat brains and cultured astrocytes were extracted as described previously (14). In some experiments, cytosolic supernatant was ultracentrifuged at 100 000*g* for 2 h at 4°C. Protein samples were subjected to SDS–PAGE using gradient (4–12%)-pre-casted gels (Invitrogen) (14), transferred to a nitrocellulose membrane and immunoblotted overnight at 4°C with the following Abs: anti-MLC1 pAb (1:500, in-house generated), anti-Na,K-ATPase β1 mAb (1 μg/ml, Millipore), anti-Kir4.1 pAb (1:400, Alomone, Israel), anti-AQP4 (4/18) mAb (1:500, Santa Cruz Biotecnology, Inc., Santa Cruz, CA, USA), anti-syntrophin mAb (1:2000, MA-1-745, Affinity BioReagents, Co., USA), anti-β-DG mAb (1:25, NCL-43 DAG, Novocastra Laboratories Ltd, Newcastle-upon-Tyne, UK), anti-EEA1 mAb (1:5000, BD Transduction Laboratories), anti-dystrobrevin mAb (1:750, BD Transduction Laboratories), anti-dystrobrevin pAb (1:500, in-house generated), anti-caveolin-1 pAb (1:1000, Santa Cruz Biotechnology) in PBS, 3% BSA followed by extensive washings and then incubated for 1 h with horseradish peroxidase-conjugated anti-mouse or anti-rabbit Abs (1:10 000; Thermo Scientific, MO, USA), for 1 h at RT. Immunoreactive bands were visualized using an enhanced chemiluminescence reagent (Pierce), according to the manufacturer's instructions and exposed on X-ray films.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGEMENTS

We thank Dr Antonella Bernardo for providing astrocyte cell cultures. Brain tissue samples were kindly provided by the UK Multiple Sclerosis Tissue Bank (www.ukmstissuebank.imperial.ac.uk), funded by the MS Society of Great Britain and Northern Ireland (registered charity 207495).

Conflict of Interest statement. None declared.

FUNDING

This work was supported by ELA Foundation (grants ELA 2006-001/4 and ELA 2009-002C5A to E.A.) and ISS-NIH Collaborative Programme on Rare diseases (grant 7/DR1 to P.M.).

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SUPPLEMENTARY MATERIAL AND METHODS

Immunofluorescence

Astrocytes were incubated over night at 4°C with anti-MLC1 pAb (1:50, Atlas AB) and anti-Rab5 (D-11) mAb (1:25, Santa Cruz Biotecnology) and then for 1 h at RT with a rhodamine-conjugated donkey anti-goat (1:100, Jackson, UK) and a fluorescein-conjugated donkey anti-mouse (1:100, Jackson, UK) antibody. For double staining with anti-EEA1 mAb (1:50, BD Transduction Laboratories) and anti-Kir4.1 pAb (1:50, Alomone), cells were incubated for 1 h at RT. A biotinilated secondary antibody (4.3 µg/ml ,Biotin-SP-AffiniPure goat anti-rabbit IgG H+L, Jackson Immunoresearch Laboratories) followed by incubation with 2 µg/ml streptavidin-TRITC (Jackson, UK) and a fluorescein-conjugated donkey anti-mouse (1:100, Jackson, UK) were used. Coverslips were washed, sealed in Vectashield medium (Vector Lab, Burlingame, CA) and analyzed with a laser scanning confocal microscope as previously described in Materials and Methods.

Immunohistochemistry of human brain tissue

Indirect immunofluorescence technique was used to detect MLC1 in post-mortem brain tissues from a control patient without neurological disease and from a patient with multiple sclerosis (MS) obtained from the UK MS Tissue Bank at Imperial College London. As described in Materials and Methods, sections were incubated overnight at 4°C with a mixture of rabbit anti-MLC1 pAb (1:250, ATLAS) and anti-Na,K-ATPase ß1 mAb (1:200, Millipore). After extensive washing, sections were incubated for 1 h at RT with a mixture of fluorescein-conjugated goat anti-mouse and rhodamine-conjugated goat anti-rabbit Abs and images were analysed with a fluorescent microscope (Leica DM-4000B Microsystems, Bannockburn, IL).

Supplementary figures

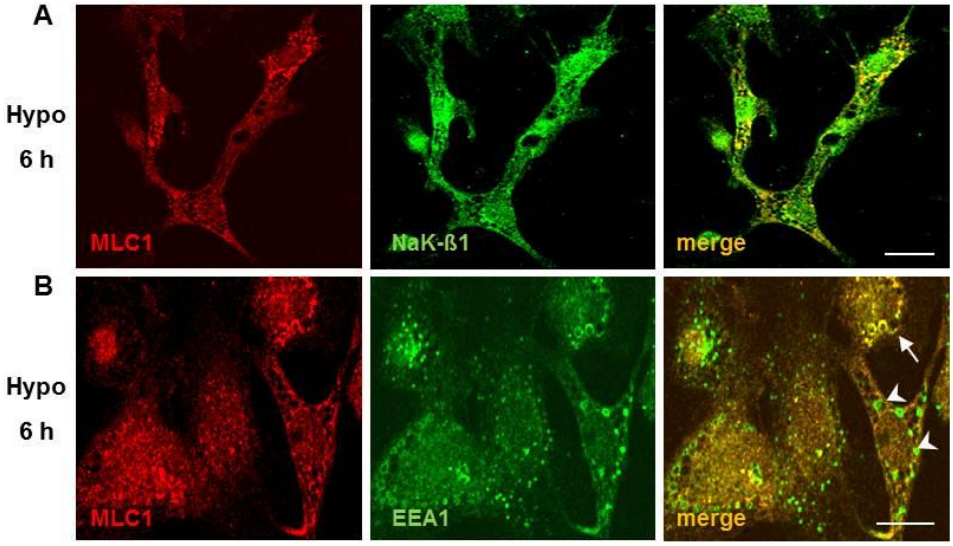

Figure S1. Characterization of vacuoles induced by 6h hypo-osmotic treatment of rat astrocytes. (A) Double immunofluorescence stainings with anti-MLC1 pAb (red) and anti-Na,K- β1 mAb (green) show partial colocalization of MLC1 and NaK-β1 in the membranes bordering the newly formed vacuoles. (B) Double immunostainings with anti-MLC1 pAb (red) and anti-EEA1 mAb (green), a marker of early endosomes, reveal a partial colocalization of MLC1 and EEA1 around some vacuoles (Hypo 6h, arrow) while others are immunopositive only for EEA1 (Hypo 6h, arrowhead). Scale bars: 20 μm

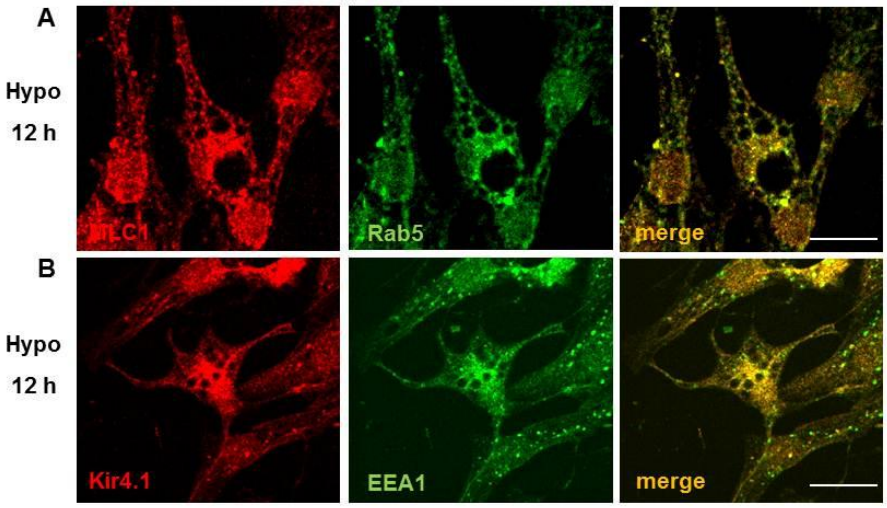

Figure S2. Characterization of vacuoles induced by twelve hours hypo-osmotic treatment of rat astrocytes. (A) In astrocytes treated for 12 h with hypo-osmotic medium there is a high level of colocalization between MLC1 (red) and Rab5 (green), a marker of the early endosomes, along the vacuole limiting membranes and in the perinuclear area. (B) Staining with anti-Kir4.1 pAb (red) and anti-EEA1 mAb (green) shows Kir4.1 and EEA1 colocalization along vacuole membranes. Scale bars: 20 μm

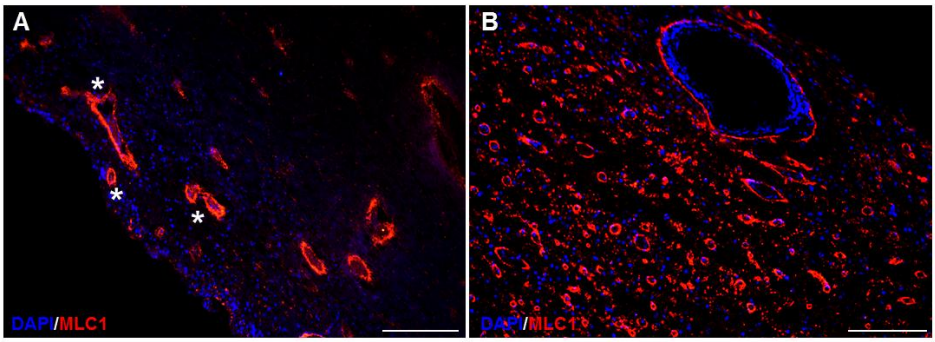

Figure S3. Immunohistochemical detection of MLC1 in non-pathological human brain (A) and in multiple sclerosis (MS)-affected brain (B). (A) Immunofluorescence staining of a control human brain section with anti-MLC1 pAb shows strong immunoreactivity around blood vessels (asterisks) in the periventricular white matter. (B) In the MS brain MLC1 is more abundantly expressed in perivascular areas of an active white matter lesion. Numerous small blood vessels appeared positive for MLC1. Scale bars: 200 μm

Human Molecular Genetics, 2012, Vol. 21, No. 10 **2166–2180**
doi:10.1093/hmg/dds032
Advance Access published on February 9, 2012

Megalencephalic leukoencephalopathy with subcortical cysts protein 1 functionally cooperates with the TRPV4 cation channel to activate the response of astrocytes to osmotic stress: dysregulation by pathological mutations

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Received December 15, 2011; Revised and Accepted February 4, 2012

Megalencephalic leukoencephalopathy with subcortical cysts (MLC), a rare leukodystrophy characterized by macrocephaly, subcortical fluid cysts and myelin vacuolation, has been linked to mutations in the *MLC1* gene. This gene encodes a membrane protein that is highly expressed in astrocytes. Based on MLC pathological features, it was proposed that astrocyte-mediated defects in ion and fluid homeostasis could account for the alterations observed in MLC-affected brains. However, the role of MLC1 and the effects of pathological mutations on astrocyte osmoregulatory functions have still to be demonstrated. Using human astrocytoma cells stably overexpressing wild-type MLC1 or three known MLC-associated pathological mutations, we investigated MLC1 involvement in astrocyte reaction to osmotic changes using biochemical, dynamic video imaging and immunofluorescence techniques. We have found that MLC1 overexpressed in astrocytoma cells is mainly localized in the plasma membrane, is part of the Na,K-ATPase-associated molecular complex that includes the potassium channel Kir4.1, syntrophin and aquaporin-4 and functionally interacts with the calcium permeable channel TRPV4 (transient receptor potential vanilloid-4 cation channel) which mediates swelling-induced cytosolic calcium increase and volume recovery in response to hyposmosis. Pathological MLC mutations cause changes in MLC1 expression and intracellular localization as well as in the astrocyte response to osmotic changes by altering MLC1 molecular interactions with the Na,K-ATPase molecular complex and abolishing the increase in calcium influx induced by hyposmosis and treatment with the TRPV4 agonist 4αPDD. These data demonstrate, for the first time, that MLC1 plays a role in astrocyte osmo-homeostasis and that defects in intracellular calcium dynamics may contribute to MLC pathogenesis.

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INTRODUCTION

Megalencephalic leukoencephalopathy with subcortical cysts (MLC, OMIM 604004) is a rare autosomal recessive white matter disease in children characterized by macrocephaly at birth, subcortical cysts and swollen appearance of the white matter evidenced by magnetic resonance imaging (1). Motor functions degenerate along with cerebellar ataxia and spasticity, while cognitive functions appear less affected and patients show a variable degree of mild cognitive impairment (1,2). The disease is also characterized by a slowly progressive clinical course that can be worsened by minor stress, particularly minor head trauma and common infections (3–5). Epileptic seizures are frequently observed in MLC. Histopathological analysis of MLC brain biopsies revealed myelin splitting and intramyelinic vacuolation, structural alterations of the blood–brain barrier, along with astroglia activation and presence of enlarged vacuoles in the end-feet of vessel-contacting astrocytes (1,6,7).

A broad spectrum of pathogenic mutations has been identified in the *MLC1* gene without any clear correlation with MLC disease severity (8,9). Some mutations are quite frequent in certain populations indicating a founder effect (10,11). Recently, mutations in a second gene have been found in a percentage of MLC-affected patients that do not carry mutations in *MLC1*, indicating the possible polygenic origin of this disease (12). The human *MLC1* gene product is a 377-amino acid protein (MLC1) (8,13,14) that is highly expressed in brain astrocytes, Bergmann glia and ependymal cells lining the ventricles (13,15,16). In the human brain, MLC1 is predominantly localized in the plasma membrane of perivascular and subpial astrocytic processes and in astrocyte–astrocyte junctions (7,13,15). MLC1 shows no similarities with proteins of known function, except for a very low homology with a subunit of the voltage-gated potassium (K) channel (Kv1.1) and an ABC type of transporter (2,17). Altogether, the pathological alterations observed in MLC patients (in particular, swollen white matter, fluid cysts and myelin and astrocyte vacuolation) and the localization of MLC1 in astrocyte end-feet-contacting brain barriers led to hypothesize a role for MLC1 in astrocyte-mediated processes regulating fluid and ion exchanges in the central nervous system. In support of this hypothesis, we recently demonstrated that MLC1 directly binds the beta1 subunit of the Na,K-ATPase enzyme and is part of a multiprotein complex involved in the regulation of ion transport and cellular volume in brain astrocytes during hyposmotic stress (18).

Astrocytes are multifunctional cells that play an essential role in myelin formation, neuronal activity and maintenance of neural tissue homeostasis through the regulation of ions, neurotransmitters and metabolites in the extracellular space (19,20). By expressing water and ion channels at their end-foot processes at the interface between the brain and liquid spaces, astrocytes are the first cells to be exposed and react to environmental changes (21,22). Astrocyte alterations are associated with an increasing number of disease conditions (23–27), including leukodystrophies (21,28–30).

To elucidate the role of MLC1 in astrocyte physiology and the possible functional consequences of MLC1 mutations, we have generated and characterized human astrocytoma cell lines overexpressing wild-type (WT) MLC1 and three missense mutations described in MLC patients. Here, we show that distinct pathological mutations differently alter MLC1 intracellular localization and interaction with the Na,K-ATPase-associated multiprotein complex in astrocytoma cells and affect the response to osmotic changes. Moreover, we demonstrate that MLC1 functionally interacts with the transient receptor potential vanilloid-4 cation channel (TRPV4) to increase intracellular calcium influx in response to hyposmosis and to the TRPV4 agonist 4-alpha-phorbol 12,13-didecanoate (4αPDD). Conversely, mutations hampering MLC1 expression in the plasma membrane fail to activate calcium influx both after hyposmotic shock and 4αPDD treatment. These data show for the first time that MLC1-induced defects in the intracellular calcium response in astrocytes may contribute to the pathogenesis of MLC disease.

RESULTS

Generation and characterization of astrocytoma cell lines overexpressing WT and mutated MLC1

To study the functional changes induced by MLC1 mutations in astrocytes, we have generated human astrocytoma (U251MG) cell lines that overexpress three single-missense mutations (S246R, S280L, C125R) associated with MLC (9 and references therein) (Fig. 1A). These mutations were previously characterized for their effect on MLC1 protein localization and stability after expression in heterologous cell systems and rat astrocytes. S246R was characterized as a mild mutant, while S280L and C125R were characterized as more severe mutants (14). Because U251 cells express MLC1 mRNA but very low levels of the MLC1 protein (16) and originate from astrocytes, we reasoned that they could represent a better experimental model to study the functional consequences of WT and mutated MLC1 expression in the appropriate cellular context.

MLC1 mutations were generated by site-directed mutagenesis (31) and the cDNAs were subcloned with a His-tag at the NH_2 moiety in a retroviral vector system to originate stably infected cell lines, as described in Materials and Methods. Expression of recombinant proteins was evaluated with western blot (WB). By loading 40 μg of protein extracts derived from the infected cell lines, we observed a high level of expression of recombinant MLC1 proteins, while endogenous MLC1 was undetectable in the parental cell line infected with the mock vector (Fig. 1B). The expression level of MLC1 S246R was higher than that of MLC1 S280L and MLC1 C125R (Fig. 1B) most probably due to different degradation kinetics of MLC1-mutated proteins (14).

Possible changes in MLC1 intracellular localization due to pathological mutations were investigated with double immunofluorescence techniques. Using an anti-MLC1 Ab and FITC-conjugated phallacidin which stains actin filaments, we observed that in both WT and S246R cells, MLC1 immunoreactivity localized in cytoplasmic organelles and membranes, particularly in the plasma membrane, astrocyte extensions and at the level of astrocyte–astrocyte contacts, showing a distribution similar to that of actin filaments (Fig. 1C). Conversely, S280L and C125R mutant proteins

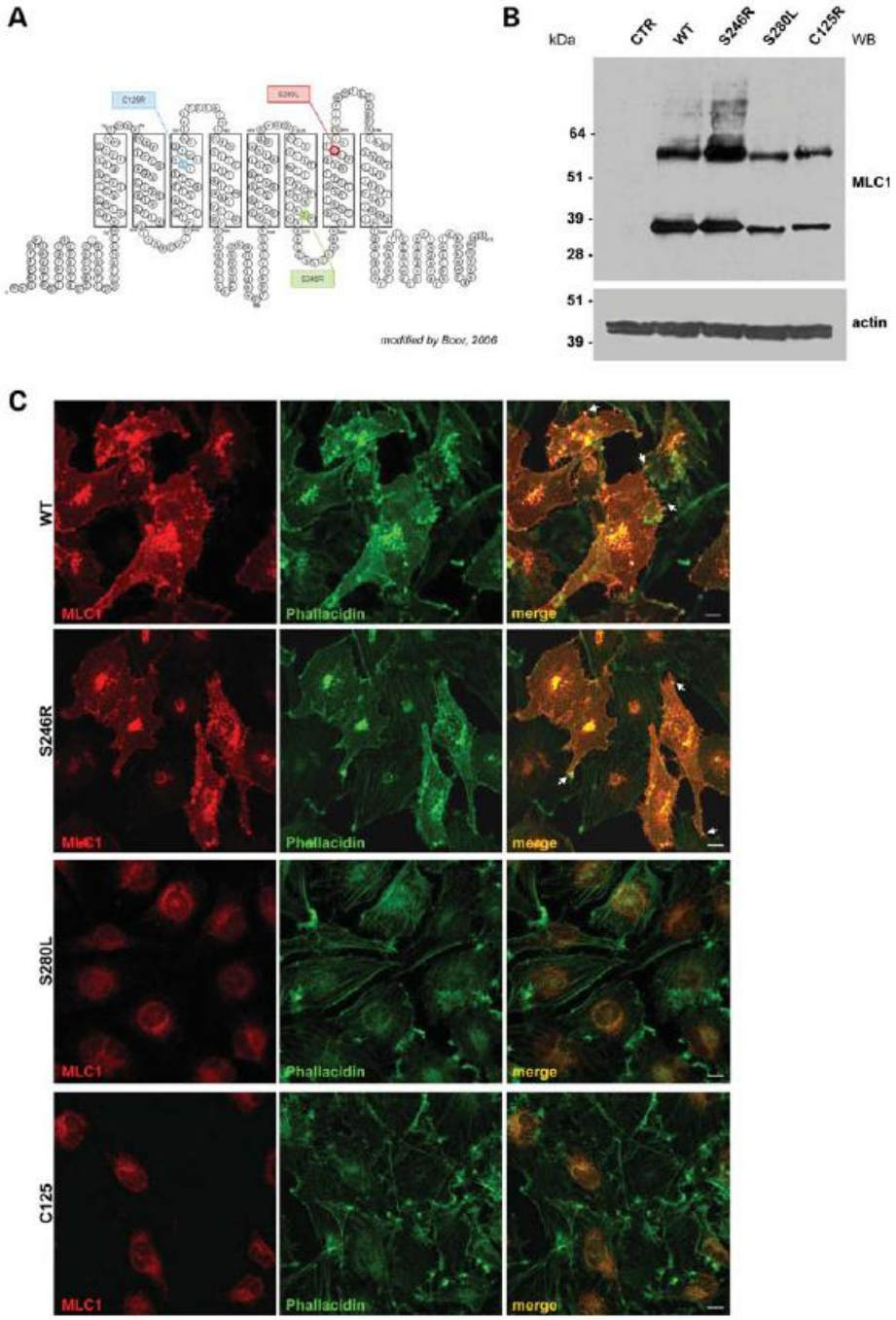

Figure 1. Characterization of U251 astrocytoma cell lines overexpressing WT and MLC1 pathological mutations. (A) Schematic representation of the MLC1 protein supposed molecular structures [modified by Boor (9)]. MLC1 mutations overexpressed in astrocytoma cell lines are indicated in colours. (B) WB analysis of protein extracts (40 μg) of astrocytoma cells infected with empty vector (CTR), or with vectors expressing WT and MLC1 mutations S246R, S280L and C125R. Expression levels of MLC1 WT and MLC1 S246R are higher than those of MLC1 S280L and MLC1 C125R. MLC1 endogenous protein is undetectable in control cells. Actin is used as internal loading control. Molecular weight markers are indicated on the left (kDa). (C) Immunofluorescence stainings of U251 astrocytoma cell lines overexpressing WT and mutated MLC1 with anti-MLC1 pAb (red) and FITC-conjugated phallacidin (green) to stain actin filaments show a similar distribution of MLC1 in the perinuclear region and along astrocytic plasma membranes in MLC1 WT and S246R mutant-expressing cells. MLC1 and actin colocalize in astrocytic extensions and astrocyte intercellular contacts (arrows). A different distribution of MLC1 (red) is observed in S280L and C125R mutant-expressing cells where MLC1-mutated proteins fail to reach the plasma membrane and are retained in the cytoplasmic perinuclear area. Scale bars = 10 μm.

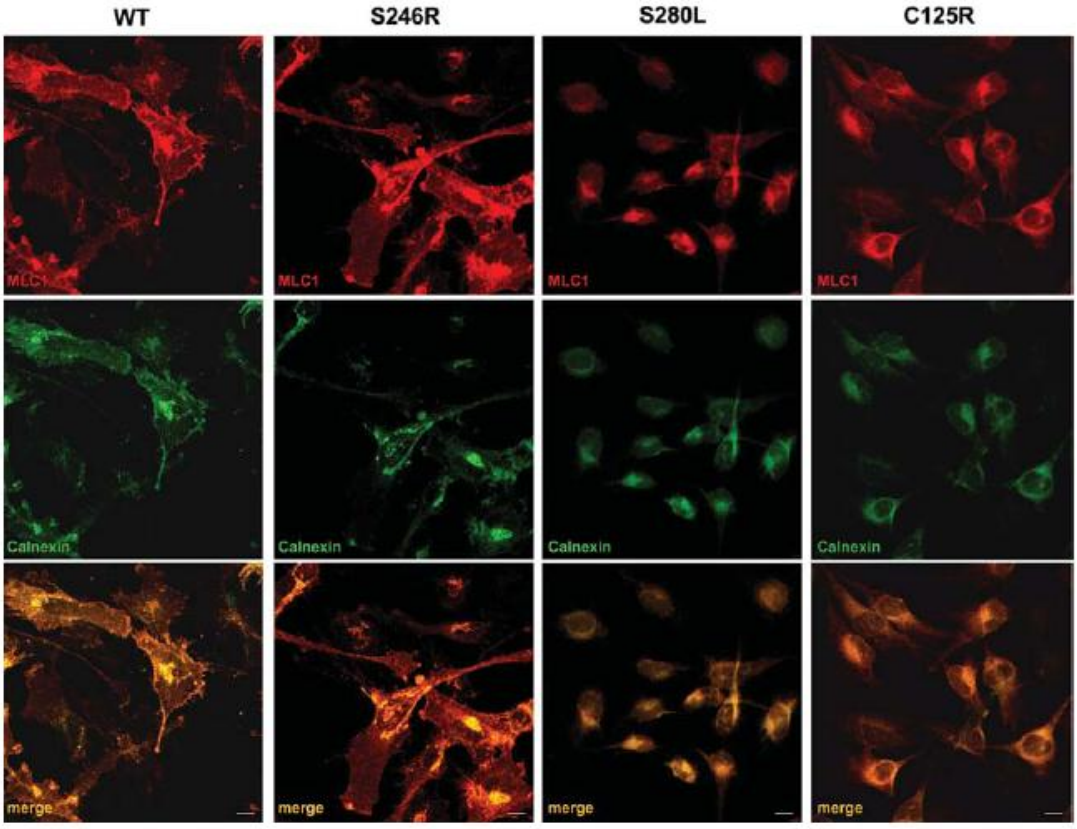

Figure 2. MLC1 intracellular distribution in U251 astrocytoma cell lines overexpressing WT and pathological MLC1 mutations. Double immunofluorescence staining with anti-MLC1 pAb (red) and a mAb specific for the ER marker calnexin (green) shows that WT MLC1 and S246R mutant are partially expressed in the ER, whereas S280L and C125R MLC1 proteins are almost exclusively localized in this compartment. Scale bars = 10 μm.

failed to reach the plasma membrane and were mostly retained in the cytoplasmic perinuclear areas (Fig. 1C). By combining anti-MLC1 Ab with an Ab specific for the endoplasmic reticulum (ER) marker calnexin, we observed that similarly to WT MLC1, the S246R mutant was only in part localized in the ER while both S280L and C125R MLC1 proteins were almost exclusively confined to this compartment (Fig. 2).

Effect of pathological mutations on MLC1 interaction with the Na,K-ATPase pump

We recently showed that in rat, primary astrocyte MLC1 interacts with the Na,K-ATPase pump and, through this, binding is part of a multiprotein complex that includes the inwardly rectifying potassium channel 4.1 (Kir4.1), syntrophin and caveolin-1, all proteins involved in the response of astrocytes to hyposmotic stress (18). We therefore investigated the interaction of MLC1 with the Na,K-ATPase protein complex and the pathophysiological relevance of this interaction in astrocytoma cells overexpressing WT and mutated MLC1. Double immunofluorescence stainings with Abs specific for MLC1 and the Na,K-ATPase β1 subunit (NaK-β1) showed that in WT and S246R, astrocytoma cells MLC1 colocalized with NaK-β1 in the plasma membrane, where NaK-β1 is normally abundantly expressed, perinuclear region and intracellular organelles (Fig. 3), as observed in rat astrocytes (18). Conversely, in S280L- and C125R-expressing astrocytoma cells, colocalization of MLC1 and NaK-β1 was restricted to the perinuclear area and absent at the level of the plasma membrane (Fig. 3). To validate this result with a biochemical approach, we set up the ouabain-affinity chromatography assay which takes advantage of the strong affinity of the alpha-subunit of Na,K-ATPase for the cardiac glycoside ouabain and allows to isolate the Na,K-ATPase whole protein complex and its interactors (18,32,33). Protein extracts derived from astrocytoma cell lines overexpressing WT and mutant MLC1 were purified on ouabain-affinity chromatography and, after elution, proteins associated with Na,K-ATPase were revealed by WB. In WT cell lines, MLC1 was specifically eluted along with NaK-β1 by K^+ and a high concentration of ouabain from the ouabain-affinity column, indicating specific association (Fig. 4). Relatively to WT MLC1, a lower amount of S246R MLC1 was found associated with the Na,K-ATPase complex, while almost no MLC1 was eluted from the ouabain column when cellular extracts derived from S280L and C125R-expressing astrocytoma cells were analysed (Fig. 4). Importantly, in the presence of all MLC1 mutations, including S246R, other components of the Na,K-ATPase macromolecular complex, such as the Kir4.1 potassium channel and the adaptor protein syntrophin, were not

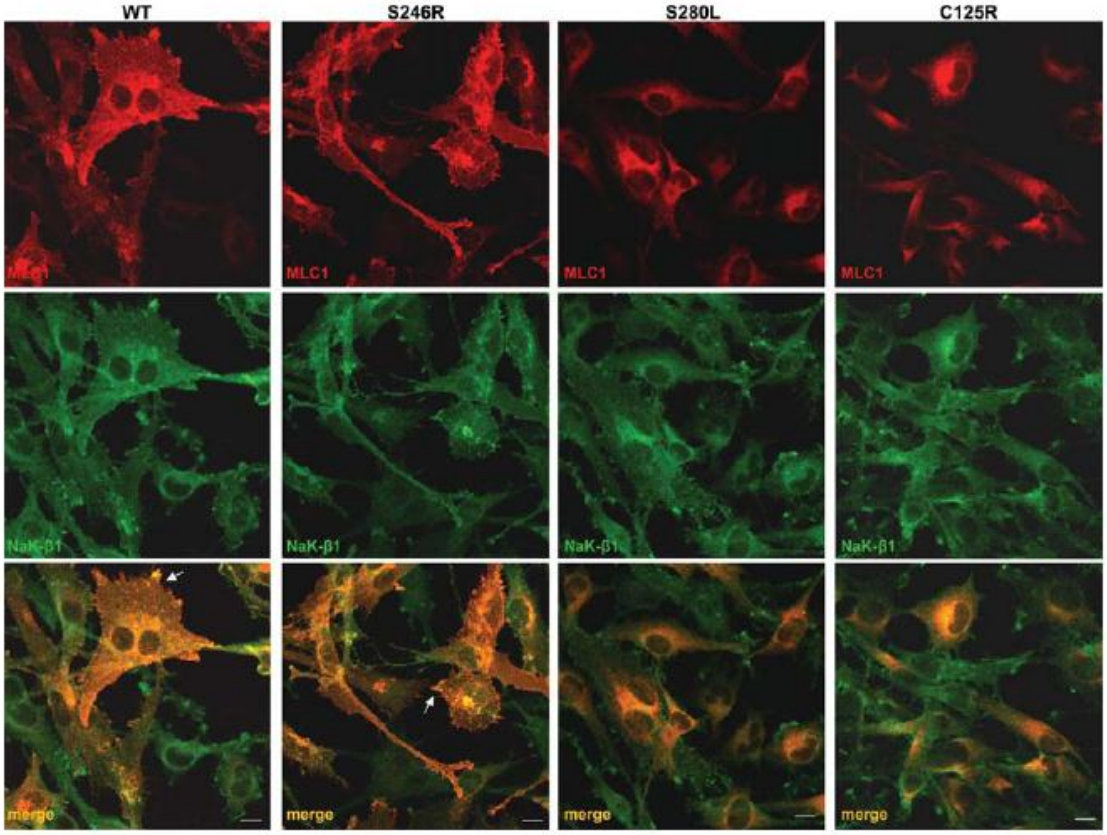

Figure 3. Immunolocalization of MLC1 and NaK-β1 in U251 astrocytoma cell lines overexpressing WT and pathological MLC1 mutations. Double immunofluorescence staining with anti-MLC1 pAb (red) and anti-Na,K-ATPase β1 subunit (NaK-β1) mAb shows that in WT and S246R-expressing astrocytoma cells MLC1 and NaK-β1 colocalize in the plasma membrane (arrows) and in the perinuclear region. A lower level of colocalization is observed in intracellular structures of S280L- and C125R-expressing cells, while in the same cells, no colocalization is found at the plasma membrane level. Scale bars = 10 μm.

detectable or their binding was strongly reduced (Fig. 4). Similar results were obtained with a His-protein co-purification assay that allows to isolate MLC1 interactors by exploiting the His-tag expressed at the NH_2 terminal of the MLC1 recombinant proteins (Supplementary Material, Fig. S1).

Since we demonstrated previously that hyposmotic treatment of primary rat astrocytes increased the association of MLC1 with the Na,K-ATPase complex (18), we examined MLC1/Na,K-ATPase interactions using ouabain-affinity chromatography in WT and mutated MLC1-overexpressing astrocytoma cells undergoing hyposmotic shock. These experiments showed that the association between MLC1 and the Na,K-ATPase complex was favoured by hyposmotic shock in WT-expressing cells. Conversely, no increase in the binding of MLC1 to the Na,K-ATPase complex was detected in any of the cell lines expressing mutant MLC1 (Supplementary Material, Fig. S2).

MLC1 mutations affect intracellular calcium influx in hyposmotic conditions

Although the above findings indicate that alterations in MLC1 association to the Na,K-ATPase complex could play a role in MLC pathogenesis through modification of the astrocyte response to osmotic imbalance, the precise function of MLC1 in this context remains to be elucidated. It has been shown that the decrease in extracellular osmolarity induces a transient and rapid swelling in astrocytes, followed by corrective processes of volume recovery named regulatory volume decrease (RVD) (34,35). The whole process occurs through sequential activation of different types of water and ion channels, exchangers and pumps (36,37), including Na,K-ATPase (38,39) and Kir4.1 (40). A recent study demonstrated that astrocytes rapidly respond to hyposmosis by increasing intracellular calcium (Ca^{2+}) influx and that this influx is essential to activate RVD processes (41). To understand the possible role of MLC1 in this regulatory loop, we investigated the swelling-induced changes in intracellular Ca^{2+} concentration in WT and mutant MLC1-overexpressing astrocytoma cell lines using the FURA-2 video-imaging technique. In preliminary experiments, mock-infected astrocytoma cells loaded with the Ca^{2+} indicator FURA-2 AM were challenged with a hyposmotic solution (60 mM NaCl) and Ca^{2+} changes were analysed during a 10 min period. A fraction of astrocytoma cells reacted to hyposmotic shock with heterogeneous Ca^{2+} transients that differed for lag time of onset, ranging from immediate to late-occurring profile of the transient (i.e. peak shaped, sustained or a combination of the two), and maximal amplitude of the Ca^{2+} changes (Fig. 5A).

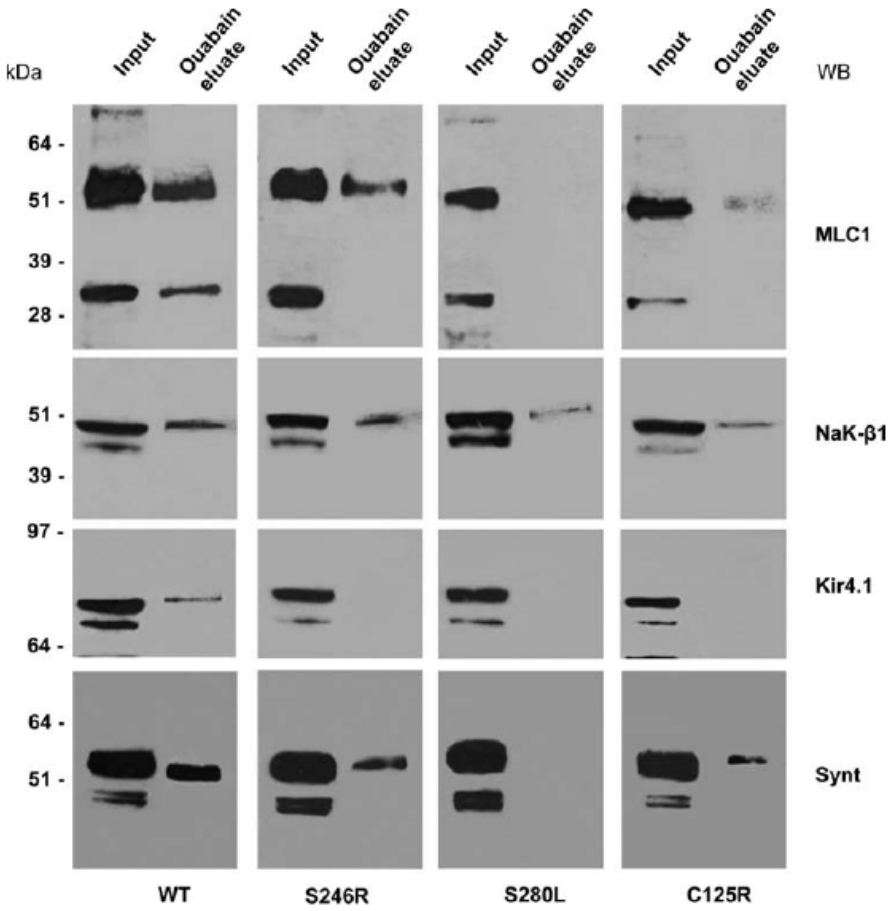

Figure 4. Copurification of MLC1 and Na,K-ATPase by ouabain-affinity chromatography. Analysis of the eluted fractions from ouabain-affinity chromatography of U251 astrocytoma cell lines overexpressing WT and mutated MLC1. Fractions were analysed by SDS–PAGE and WB and probed with Abs against MLC1, Na,K-ATPase β1 (NaK-β1), Inward Rectifier K Channel 4.1 (Kir4.1) and syntrophin (Synt) (input). MLC1, Kir4.1, NaK-β1 and Synt were detected in the ouabain eluate (eluate) from the protein extract derived from WT cells. A lower amount of S246R MLC1 was found associated to the Na,K-ATPase complex relatively to WT protein, whereas almost no S280L and C125R MLC1-mutated proteins were found associated to the Na,K-ATPase complex. NaK-β1 was present in all the eluates. Kir4.1 was absent in the eluate derived from all MLC1 mutations. Syntrophin was found decreased in S246 and C125R and absent in S280L-derived eluates. Molecular weight markers are indicated on the left (kDa).

Hyposmotic shock never induced a Ca^{2+} raise in the absence of extracellular Ca^{2+} (Ca^{2+} replaced by Mg^{2+} and 0.5 mM EGTA added to the medium), confirming Ca^{2+} influx as the main event causing the observed raise of intracellular Ca^{2+} in these cells (Fig. 5B).

When comparing the lag time of onset and the profile of the Ca^{2+} transients, no differences were observed between control astrocytoma cells and cells infected with WT or mutated MLC1. However, we found that overexpression of WT MLC1 influenced the probability of occurrence (i.e. the fraction of responding cells) of the hyposmosis-induced Ca^{2+} transient, the latter being 58% higher than in control cells, while overexpression of mutated MLC1 did not alter this parameter (Fig. 5C). Moreover, Ca^{2+} transients registered in cells overexpressing WT and S246R MLC1 were higher in amplitude compared with control cells, while the average amplitude of Ca^{2+} transients in S280L and C125R cells was smaller than that in control cells (Fig. 5D). Taken together, these data demonstrate that the MLC1 protein when expressed in the plasma membrane (MLC1 WT and S246R) regulates the response of astrocytes to hyposmosis by promoting Ca^{2+} influx and that this process is inhibited by MLC1 mutations (S280L and C125R) reducing membrane-associated MLC1.

MLC1 mutations affect TRPV4-mediated calcium influx

Recent studies have shown that TRPV4 is the channel responsible for the increase of intracellular Ca^{2+} and the consequent activation of RVD in astrocytes under hyposmotic stress (41,42). Preliminary WB experiments indicated that U251 cells express detectable levels of TRPV4 channel without quantitative differences between WT and mutated MLC1-expressing cells (Supplementary Material, Fig. S3). To understand whether the variations in intracellular Ca^{2+} induced by hyposmotic shock in MLC overexpressing astrocytoma cells could be due to an effect on TRPV4 functionality,

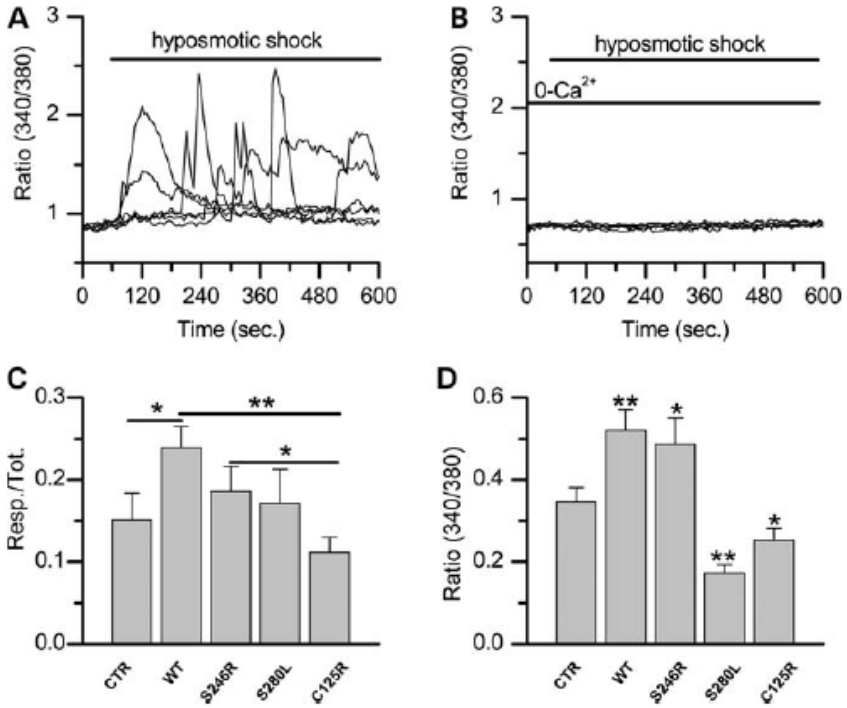

Figure 5. In astrocytoma cells, hyposmotic shock induces different Ca^{2+} signals in control cells and cells overexpressing WT and pathological MLC1 mutations. (**A** and **B**) Fura-2-loaded astrocytoma cells were challenged for 10 min with a hyposmotic solution (60 mM NaCl), while the cytoplasmic Ca^{2+} level was monitored as Fura-2 fluorescence emission in single cells. (A) Representative Ca^{2+} traces recorded in the presence of Ca^{2+} show the different profile of the signals in the single cells. (B) The same challenge did not induce any Ca^{2+} signal when Ca^{2+} was omitted and 0.5 mM EGTA was added to the bath solution, depicting Ca^{2+} influx as the event-causing Ca^{2+} signals. (**C**) The probability of occurrence of Ca^{2+} transients, expressed as the fraction of responding cells, was calculated in control and MLC1-expressing lines. The fraction of hyposmosis-responding cells was higher in WT MLC1-expressing cells than in control and C125R cells ($^{*}P < 0.05$ versus CTR; $^{**}P < 0.01$ versus WT). (**D**) Average amplitudes of the Ca^{2+} signals were calculated from responding cells in the different astrocytoma cell lines (control and MLC1-expressing lines). Average Ca^{2+} signals in WT and S246R lines were higher than in control cells and lower in S280L cells (total cells examined: 5888, in four to nine experiments.$^{*}P < 0.05$; $^{**}P < 0.01$).

astrocytoma cell lines were acutely treated with 4αPDD, a selective activator of the TRPV4 channel (43). Application of a low concentration of 4αPDD (500 nM) induced a Ca^{2+} rise in a fraction of cells, 13 ± 2 and 3 ± 1% in WT MLC1- and S280L-expressing cells, respectively. At this low concentration of the agonist, the amplitude of Ca^{2+} transients did not differ between cells, being 1.0 ± 0.2 and 0.7 ± 0.15 R in control (Ctr) and WT cells, respectively, but the amount of responding cells was too low and the variability of the transients too large to allow a reliable comparison of the responses recorded in the different cell lines (data not shown). The application of a higher dose of 4αPDD (2 μM) induced slowly raising Ca^{2+} transients which, similarly to those induced by hyposmotic shock, did not differ according to the lag time and profile (Fig. 6A). Moreover, also in this case in the absence of Ca^{2+} in the external solution, the Ca^{2+} responses were not observed (Fig. 6B), while on the readmission of Ca during 4αPDD application an abrupt increase in Ca^{2+} could be recorded. The readmission of Ca^{2+} did not trigger Ca^{2+} transients in the absence of 4αPDD, depicting that Ca^{2+} influx was due to 4αPDD-induced opening of TRPV4 channels and not to Ca^{2+} depletion-induced capacitative Ca^{2+} influx (data not shown). Compared with mock-infected control cells, the probability of occurrence of the Ca^{2+} transients increased by 54 and 63% in WT and S246R cells, respectively, but was unchanged or significantly reduced in S280L and C125R cells, respectively (Fig. 6C). Lastly, also with a higher concentration of 4αPDD (2 μM), the average amplitude of the transients did not differ significantly among cell lines, being 1.7 ± 0.1 and 1.4 ± 0.2 R in control and S280L cells, respectively (Fig. 6D). Thus, similarly to the hyposmosis-induced Ca^{2+} influx, the TRPV4 agonist-induced Ca^{2+} influx was also greatly potentiated when MLC1 was expressed in the plasma membrane. Taken together, these data depict a functional role of MLC1 in promoting a more efficient Ca^{2+} influx through TRPV4 channels. In keeping with the selective involvement of these channels, both hyposmosis- and 4αPDD-induced Ca^{2+} transients were completely abolished by ruthenium red (RR), a general blocker of TRPV ion channels (44) (Fig. 7).

MLC1 interacts with TRPV4: effect of MLC1 mutations

To investigate the possibility that MLC1 directly interacts with TRPV4 ion channels in astrocytes in preliminary experiments, we performed a pull-down assay using protein extracts derived from the cytosol and membrane fraction of rat astrocytes and the recombinant His-MLC1 protein bound to an His-affinity column. Figure 8 shows that TRPV4 was eluted from the MLC1-containing column along with the NaK-β1

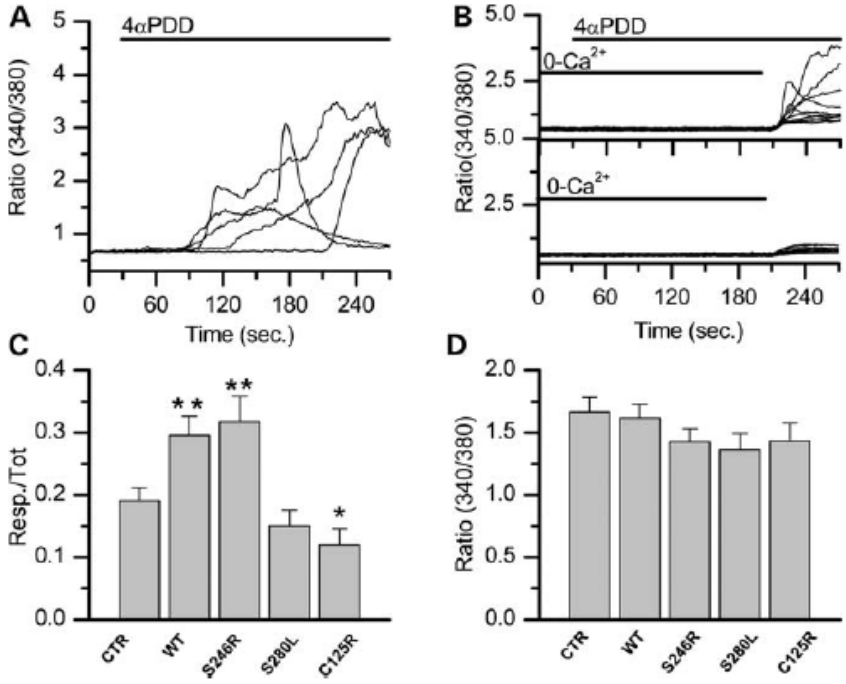

Figure 6. The application of 4αPDD induces different Ca^{2+} signals in control astrocytoma cells and cells overexpressing WT and pathological MLC1 mutations. (**A** and **B**) Fura-2-loaded astrocytoma cells were challenged for 4 min with 2 μM 4αPDD, while the cytoplasmic Ca^{2+} level was monitored as Fura-2 fluorescence emission in single cells. The TRPV4 agonist caused the slow raising of intracellular Ca^{2+} (A) which was abrogated by the removal of Ca^{2+} from the bath (B). The rapid increase in Ca^{2+} on the readmission of the ion was observed only when 4αPDD was present, indicating that it was not due to Ca^{2+} influx through capacitative channels but only through TRPV4 channels. (**C**) The probability of occurrence of Ca^{2+} transients, expressed as fraction of responding cells, was significantly higher in WT and S246R cells and lower in C125R compared with control. (**D**) Conversely, the average amplitude of the 4αPDD-induced Ca^{2+} transients did not differ in control and MLC1-expressing lines (total cells examined: 5935 in three to five experiments.*$P < 0.05$; **$P < 0.01$).

used as a positive control in the cytosolic and membrane astrocyte fractions but not from a control column bound with bacterial non-specific proteins. We next verified the association of TRPV4 with the MLC1/Na,K-ATPase multiprotein complex in human astrocytoma cells and the possible effects of MLC1 mutations in isosmotic and hyposmotic conditions. To this purpose, we performed ouabain-affinity chromatography on WT and mutant MLC1-expressing human cells with or without hyposmotic treatment. We found that in WT-expressing cells, TRPV4 was associated to the MLC1/Na,K-ATPase complex in control conditions and after hyposmotic shock (Fig. 9). TRPV4 was also found in the Na,K-ATPase complex isolated from untreated S246R and C125R cells, albeit at lower levels compared with WT cells, but was almost undetectable after hyposmotic shock (Fig. 9). In S280L-expressing cells, no TRPV4 was co-eluted with the ouabain complex, both in control conditions and after hyposmotic shock (Fig. 9).

Since in rat and mouse astrocytes, TRPV4 activates the cell response to hyposmosis in cooperation with the water channel aquaporin-4 (AQP-4) (41), we analysed whether AQP-4 is also part of the Na,K-ATPase/MLC1 complex. In WT-expressing cells, AQP-4 was detected in the Na,K-ATPase/MLC1 macromolecular complex only after hyposmosis treatment. In contrast, although some AQP-4 was found associated with the complex in untreated S246R and C125R cells, no AQP-4 protein was detected in any of the MLC1 mutant-expressing cells after hyposmotic shock (Fig. 9).

Recently, a second gene linked to MLC disease has been identified that encodes for Hepacam/GlialCAM, a protein expressed in liver and brain whose function is still unknown (12). It has been shown that this protein binds directly MLC1 (12,45). By WB analysis of ouabain eluates, we found that Hepacam/GlialCAM was present in the Na,K-ATPase/MLC1 macromolecular complex and that all MLC1 mutations analysed markedly reduced the association of Hepacam/GlialCAM to the protein complex both in control conditions and after hyposmotic shock (Fig. 9).

DISCUSSION

With the aim of unravelling MLC1 protein function and the role of *MLC1* gene mutations in MLC pathogenesis, we have generated human astrocytoma cell lines overexpressing WT and mutated MLC1. Using this *in vitro* system, we provide evidence that MLC1 functionally interacts with a macromolecular protein complex that includes Na,K-ATPase, Kir.4.1, AQP-4, TRPV4 and syntrophin. Most importantly, we show that through the functional interaction with the TRPV4 cation channel, MLC1 has a direct role in swelling-induced intracellular calcium influx in astrocytes and that pathological MLC1 mutations dysregulate this mechanism which is essential to activate the response of astrocytes to changes in the ionic environment.

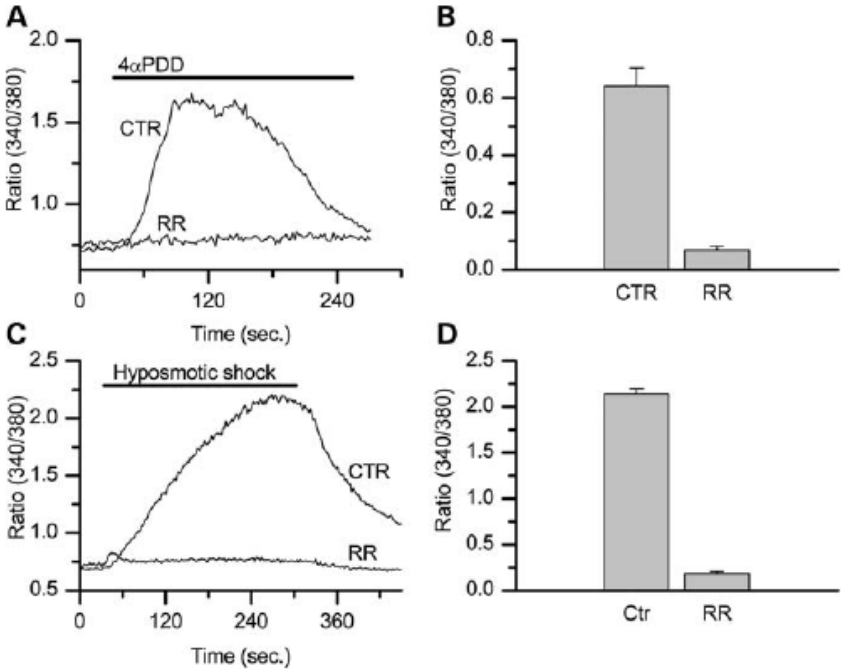

Figure 7. RR inhibits the Ca^{2+} transient induced by 4αPDD and hyposmotic shock. The TRPV channel inhibitor RR was used to characterize the nature of the channels involved in Ca^{2+} influx triggered by hyposmotic shock. In these experiments, the potassium ionophore gramicidin (10 μg/ml), known for its ability to strengthen the hyposmosis-induced Ca^{2+} influx, was used. When used in Fura-2 experiments, the totality of cells reacted to hyposmosis with a sudden and synchronous Ca^{2+} raise, allowing an easier evaluation of the pharmacological sensitivity of hyposmosis-induced Ca^{2+} raise. In this condition, RR (10 μM) completely abrogated both the 4αPDD- (A and B) and hyposmosis-induced (C and D) Ca^{2+} signals, confirming the involvement of TRPV4 channels in the hyposmosis-induced Ca^{2+} influx in astrocytoma cells.

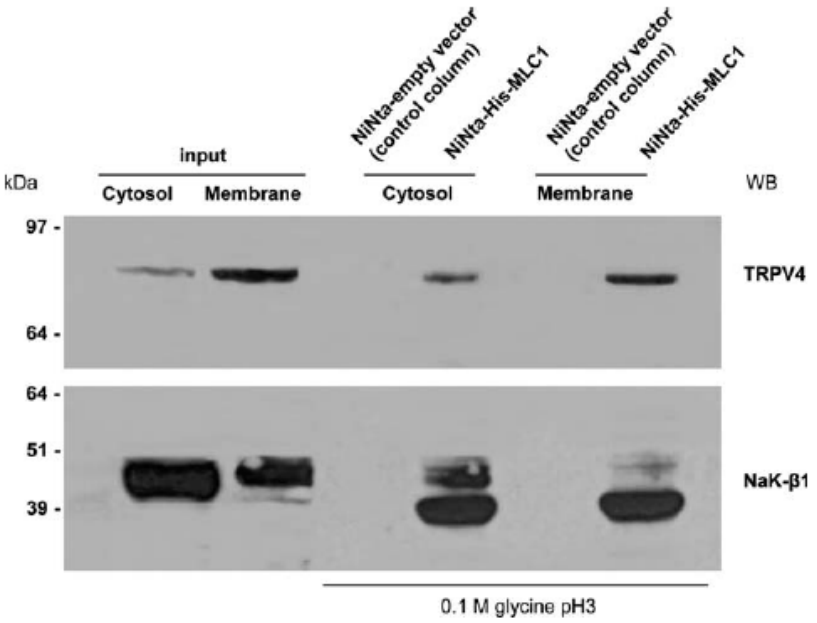

Figure 8. Pull-down assay shows the interaction between MLC1 and TRPV4 in rat astrocytes. Cytosolic and membrane fractions from primary cultures of rat astrocytes (input) pulled-down by His-MLC1 and His-empty vector used as control bound to Ni-NTA agarose were eluted with 0.1 M glycine, pH 3. WB analysis using Abs against TRPV4 and Na,K-ATPase β1 subunit (NaK-β1) shows that TRPV4 interacts with His-MLC1, along with the NaK-β1 used as a positive control, in both cytoplasmic and membrane fractions. No bands are observed in the eluates derived from control column bound with bacterial unspecific proteins. Molecular weight markers are indicated on the left (kDa).

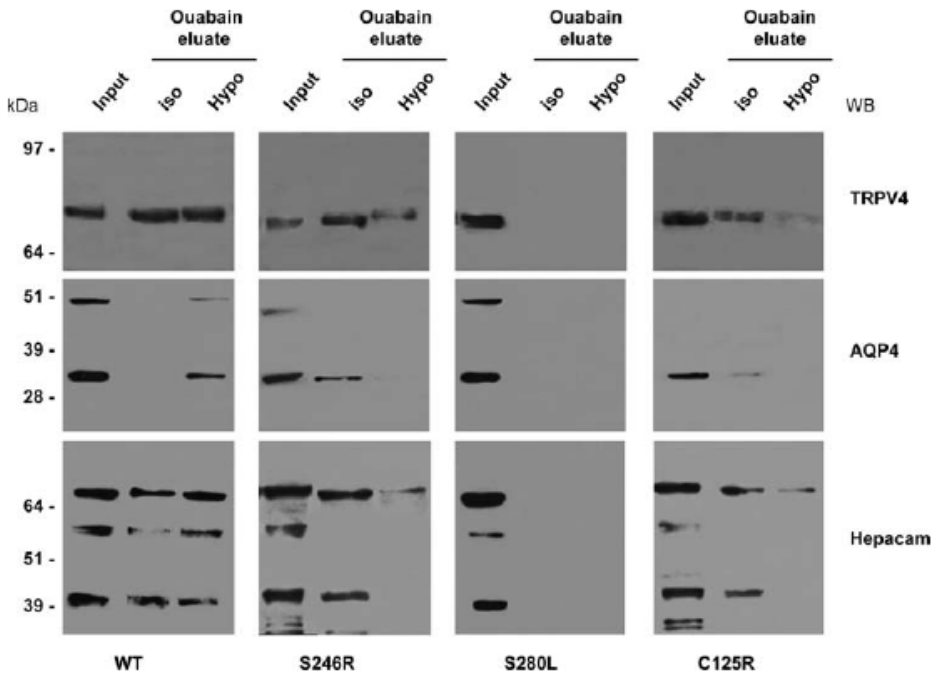

Figure 9. Analysis of the eluted fractions from ouabain-affinity chromatography of U251 astrocytoma cell lines overexpressing WT and MLC1 mutation grown for 6 h in iso- and hypo-osmotic medium. Fractions were analysed by SDS–PAGE and WB and probed with Abs against TRPV4, aquaporin-4 (AQP-4) and Hepacam. In isosmotic conditions, TRPV4 and Hepacam were eluted from WT-expressing cells and, at lower levels, in S246 and C125R-expressing cells. Low levels of AQP-4 were observed in S246 and C125R-derived eluates. Hyposmotic shock increased TRPV4, AQP-4 and Hepacam in the ouabain eluate of MLC1-WT. A decrease in TRPV4, AQP-4 and Hepacam was observed in S246R and particularly in C125R-expressing cells under hyposmosis. No TRPV4, AQP-4 and Hepacam were found in the ouabain eluates of S280L-expressing cells both in isosmotic and hyposmotic conditions.

Characterization of the newly generated pathological model: effects of MLC1 mutations on MLC1 localization and molecular interactions

The generation of human astrocytoma cell lines overexpressing WT or mutated MLC1 has allowed, for the first time, to model MLC disease in a human background taking into account that MLC1 is mainly expressed in astrocytes and the current view that mutated MLC1 may cause alterations in astrocyte functions leading to brain damage. Altogether, the low level of endogenous MLC1 expression, the relatively easy and fast generation procedures, the possibility to obtain a considerable amount of material to performs biochemical assays and the correspondence with data obtained in rat primary astrocytes (see below) demonstrates that human astrocytoma cells carrying MLC1 mutations represent a useful model to investigate MLC1 functions and MLC pathogenesis.

The detailed characterization of astrocytoma cells expressing WT MLC1 and three MLC1 mutations confirm previous observations in human HeLa cells that distinct pathological mutations differently influence MLC1 membrane expression (14). Similarly to WT MLC1, the S246R mutated protein localizes in the plasma membrane, while S280L and C125R mutants are mainly confined to the ER. Similarly to what observed in rat astrocytes (18), we show that in human astrocytoma cells expressing the WT protein MLC1 interacts with Na,K-ATPase and the associated multiprotein complex which includes Kir4.1 and syntrophin and is involved in the astrocyte response to osmotic imbalance (18 and references therein). Our extended analysis of the MLC1/Na,K-ATPase-associated proteins reveals that in astrocytoma cells, TRPV4 and AQP-4 proteins also participate to the complex, and that hyposmotic treatment influences their association. The pathological relevance of these associations is highlighted by the finding that all the pathological missense mutations analysed influence the interaction of MLC1 with the Na,K-ATPase multiprotein complex. In particular, S280L and C125R mutations strongly affect MLC1 interaction with all the Na,K-ATPase-associated components analysed, whereas in cells overexpressing the S246R mutation MLC1 still interacts with Na,K-ATPase, TRPV4 and AQP-4, albeit at a lower level relatively to WT MLC1, but not with Kir4.1, in isosmotic conditions. However, S246R protein binding to Na,K-ATPase, TRPV4 and AQP-4 is markedly reduced after hyposmotic treatment. It is conceivable that the pathological effects of this mutation might be related to dynamic alterations in the molecular pathways in which these interactors are functionally involved in astrocytes. In light of these findings, the study of the pathological consequences of each single MLC1 mutation expressed in an astrocytic background emerges as a key step in the identification of specific, patient-tailored therapeutic strategies.

Among the Na,K-ATPase-associated proteins, we also found Hepacam/GlialCAM, the hepatic-glial adhesion molecule that was recently reported to interact with MLC1 (45) and whose gene mutations are associated with a fraction of MLC patients that do not carry mutations in the *MLC1* gene (12). Interestingly, the association of Hepacam/GlialCAM to

the Na,K-ATPase complex was affected by MLC1 mutations similarly to TRPV4 and AQP-4 and further reduced by hyposmotic treatment of mutation-expressing astrocytoma cells, suggesting a possible involvement of Hepacam in astrocyte osmoregulatory processes. Because no difference in Hepacam localization was found in the brain of a patient carrying the MLC1 missense mutation S69L (45), we speculate that MLC1 mutations may affect the molecular relationships of Hepacam within the Na,K-ATPase-associated complex without altering its cellular localization. Alternatively, different mutations may affect MLC1–Hepacam interaction in a different manner.

Recently, the junctional Zonula Occludens -1 (ZO-1) protein was found to coimmunoprecipitate with MLC1 (46). We have not investigated yet whether ZO-1 interacts with MLC1 or the Na,K-ATPase complex in astrocytoma cells. However, since the beta-1 subunit of the Na,K-ATPase, which binds MLC1 (18), is involved in the formation and maintenance of adherens and tight junctional complexes (47 and references therein), it cannot be excluded that ZO-1 interacts with this multimolecular complex. Interestingly, also the cation channel TRPV4 has been reported to be present at cell–cell junctions (47). Although the functional outcome of the molecular interactions described in this study is not yet fully understood, the finding that MLC1 mutations affect multiple components of the Na,K-ATPase complex protein underlines the pathological importance of these dynamic multiprotein associations.

MLC1 is a key player in the response of astrocytes to hyposmotic shock

Since pathological brain damages (1,2,7) and recent biochemical studies in cultured rat astrocytes (18) suggest possible defects in the control of water and ion homeostasis in MLC pathogenesis, in this study we have explored further the hypothesis that MLC1 is involved in the regulation of ion and fluid exchange in astrocytes. We demonstrate that in astrocytoma cells, MLC1 physically and functionally associates to a specific calcium-permeable cation channel, TRPV4. This channel belongs to the TRPV subfamily of the transient receptor potential (TRP) proteins, which includes six members (TRPV1–6) homologous to the vanilloid receptor. Originally identified as a hyposmotically activated channel (48,49), TRPV4 is now recognized as a multimodal transducer that is activated by different stimuli, such as phorbol derivatives, moderate heat and mechanical stress (43,50,51). TRPV4 is the cation channel responsible for swelling-induced cytosolic calcium increase and for the consequent RVD in response to hyposmosis in different type of cells (52). Recent data obtained in rat astrocytes indicate that TRPV4 in cooperation with AQP-4 constitutes the main astroglial osmosensor (41,42) regulating RVD also in hyposmotic astrocytes. Using FURA-2 analysis, we show that overexpression of MLC1 at the plasma membrane significantly enhances Ca^{2+} influx induced by hyposmotic shock. Accordingly, in the presence of the specific TRPV4 agonist 4αPDD, we observed an increased probability of occurrence of intracellular calcium response in WT and S246R cells where MLC1 is present in the plasma membrane but not in S280L- and C125R-expressing cells, indicating that MLC1 interacts and functionally cooperates with the TRPV4 cation channel only when it is localized in the cell membrane. These findings suggest that defects in intracellular calcium transients in response to osmotic challenges may be involved in the pathogenetic mechanism underlying MLC disease. Unexpectedly, the average amplitude of 4αPDD-induced calcium signals did not differ in control and MLC1-expressing cells. This could not be explained by a saturating effect due to the high dose of agonist, since it was observed also at a lower concentration, but can be explained by considering this calcium signal in this cell type as an 'all or none' phenomenon. In this respect, it has to be recalled that TRPV4 is a multimodal transducer of a variety of stimuli and belongs to a multiprotein complex (see above), in which reciprocal regulations and cooperation among different players could ultimately determine a sort of 'threshold effect', compatible with our effect on the probability of occurrence rather than on the average amplitude of the event.

Although at present, we do not know whether MLC1 binds TRPV4 directly or by means of other intermediate proteins, the data obtained by pull-down and ouabain-chromatography clearly demonstrate that both in primary rat astrocytes and human astrocytoma cells, MLC1 and TRPV4 interact and participate to the same functional multiprotein complex. Interestingly, in the brain, the TRPV4 channel is strongly expressed in astrocyte end-feet surrounding blood vessels, ependyma and meninges (42), a localization that mimics that of MLC1 and suggests the possibility of their interaction *in vivo*. At the subcellular level, TRPV4 has been found in caveolae (52) where MLC1 and Na,K-ATPase are also expressed (31,53,54). Therefore, it is conceivable that in astrocytes, MLC1-associated proteins can functionally cooperate in these compartments where the activity of mechanosensitive channels is strictly regulated during cell swelling (55).

The increase in intracellular calcium in response to cell swelling is a key molecular event in the biochemical pathway responsible for initiation of volume recovery which enables cells to regain their former volume. RVD occurs in different cell types, including astrocytes (36,41,56–58), and leads to a rapid efflux of organic and inorganic osmolytes followed by obligatory water extrusion (59,60). The presence of AQP-4 in the Na,K-ATPase/TRPV4 protein complex found associated to WT MLC1 confirms that in astrocytoma cells these proteins are functionally linked to MLC1 and that MLC1 can contribute to RVD. Our preliminary experiments aimed at evaluating RVD in astrocytoma cells suggest that WT MLC1-expressing cells activate RVD more rapidly and efficiently than mutation-expressing cells (unpublished data), further supporting a key role for MLC1 in the osmoregulatory function and volume control processes in astrocytes.

During the preparation of this manuscript, a study by van der Knaap and co-authors (61) has been published showing that defects in chloride currents and hyposmosis-induced RVD are observed in patient-derived lymphoblasts and in rat astrocytes in which MLC1 expression is downregulated by siRNA. Because calcium influx in response to hyposmotic stimulus has been demonstrated to be the earliest event leading to activation of RVD and the associated chloride

conductance, it is possible that the effects reported in that study (61) are linked to a defect in calcium channel activation (41,62,63). Although this possibility deserves further investigations, our data corroborate this model by showing that astrocytoma cells overexpressing MLC1 in the plasma membrane activate calcium influx in response to hyposmosis and 4αPDD more efficiently than cells expressing mutations that hamper MLC1 localization in the plasma membrane and empty control cells. These findings indicate that defects in processes regulating calcium influx during hyposmosis are part of the pathogenetic mechanisms leading to MLC disease.

Conclusions

We have demonstrated that MLC1 mutations impair the functional interaction with the macromolecular protein complex that includes Na,K-ATPase, Kir4.1, AQP-4, TRPV4 and syntrophin. Although the molecular events underlying the cooperative actions of these proteins, such as factors regulating the relationship among individual proteins of the macromolecular complex, remain to be elucidated, the effects observed in astrocytoma cells expressing mutated MLC1 relatively to WT MLC1 support the idea that alterations in the astrocyte response to osmotic changes contribute to the brain abnormalities observed in MLC disease. The ability of cells to respond to anisotonic environment is a fundamental physiological function. Changes in the ionic composition of the extracellular space dynamically occur in physiological conditions, such as during neuronal electrical activity, brain development and aging (64), and in pathological conditions such as trauma, ischemic insult, diabetes, inflammatory and infectious conditions (65–70). In the absence of astrocytic ionic homeostasis control, perturbations in the composition of the extracellular space can adversely affect normal brain function. It is worthwhile recalling that clinical conditions of MLC patients worsen after minor head trauma and common infections that often lead to epileptic seizures and coma (3–5). It is conceivable that MLC1 mutations induce astrocytic dysfunction in regulating extracellular ion homeostasis and cause the abnormal reactions to pathological insults observed in MLC patients.

In conclusion, results presented here open new perspectives for the elucidation of the molecular pathogenesis of MLC that is essential to develop a specific therapeutic approach to be offered to patients.

MATERIALS AND METHODS

Cloning of MLC1 mutations and generation of U251 astrocytoma cells overexpressing mutations

C125R mutation was generated by site-direct mutagenesis as previously described (31). To insert the His-tag at its NH_2 terminal, MLC1 carrying C125R mutations have been cloned in pcDNAV4-HIS as previously reported for WT MLC1 and S280L and S246R mutations (31). All the cDNA-encoding MLC proteins along with the His-tag were subcloned in a retroviral bicistronic vector (pQCXIN, Clontech) by using *Pac*I and *Eco*RI digestion and transfected in a packaging cell line (GP2 Hek293) to generate replication incompetent retroviral particles. Viral suspensions were used to infect U251 astrocytoma cell lines as previously described (71). Cell lines infected with virus carrying the empty vector, used as control cells (CTR), or overexpressing recombinant MLC1 proteins (WT, S246R, S280L and C125R) were obtained by growing infected cells in G418 (gentamicin, Euroclone, Ltd, UK) containing selective medium.

Cell cultures and treatments

The glioblastoma U251-MG cell line was obtained from the American Type Culture Collection (Rockville, MD, USA) and grown in Dulbecco's modified Eagle's medium high glucose (DMEM, Euroclone, Ltd) supplemented with 10% FBS (Gibco BRL, Paisley, UK), 1% penicillin/streptomycin (Sigma Ltd, Irvine, UK) and 600 μg/ml G418 (Euroclone, Ltd) for selection at 37°C in a 5% CO_2/95% air atmosphere. Hyposmotic cell treatment was performed as previously described (18) for different time lengths. After stimulation, cells were washed in PBS, collected by scraping and centrifuged at 2.700*g* at 4°C for 20 min. Cell pellets were solubilized as described below. Primary rat astrocytes were generated as previously described (72).

Immunofluorescence and confocal microscopy analysis

Cells were grown subconfluent on polylysine-coated cover slips, fixed for 10 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 RT with the following primary antibodies (Abs) diluted in PBS, 0.025% Triton X-100: affinity-purified anti-MLC1 pAb (1:50, Atlas AB, AlbaNova University Center, Stockholm, Sweden), anti-Na,K-ATPase β1 mAb (1:50, Millipore, Temecula, CA, USA) and anti-Calnexin mAb (1:70, BD Transduction Laboratories). A biotinilated secondary antibody (4.3 μg/ml, Biotin-SP-AffiniPure goat anti-rabbit IgG H+L; Jackson Immunoresearch Laboratories, West Grove, PA, USA) followed by incubation with 2 μg/ml streptavidin-TRITC (Jackson, UK) or Alexa Fluor 488 goat anti-mouse IgG (1:300, Invitrogen, Milan, Italy) were used. To stain actin filaments, a NBD phallacidin high-affinity F-actin probe (1:30, Invitrogen) was used in combination with the primary anti-MLC1 pAb. Cover slips were washed, sealed in the Vectashield medium (Vector Lab, Burlingame, CA, USA) and analysed with a laser scanning confocal microscope (LSM 5 Pascal, Carl Zeiss, Jena, Germany).

Protein extract preparation and western blotting

Astrocytoma cell lines were lysed in buffer containing 1% Triton X-100, 0.5% sodium deoxycholate, 150 mM NaCl, 10 mM Hepes (pH 7.4) and protease inhibitor cocktail. Lysates were passed through a 26-gauge needle, incubated in ice for 20 min and centrifuged at 15.000*g* for 20 min at 4°C. Protein samples were subjected to SDS–PAGE using gradient (4–12%) precasted gels (Invitrogen) (31), transferred to a nitrocellulose membrane and immunoblotted overnight at 4°C with the following Abs: anti-MLC1 pAb (1:500, in-house generated), anti-Na,K-ATPase β1 mAb (1 μg/ml, Millipore),

anti-Kir4.1 pAb (1:400, Alomone, Israel), anti-actin mAb (1:5000, Calbiochem-Oncogene Research Products, Cambridge, MA, USA), anti-syntrophin mAb (1:2000, MA-1-745, Affinity BioReagents, CO, USA), anti-AQP4 mAb (1:200, Santa Cruz Biotecnology, Inc., Santa Cruz, CA, USA), anti-TRPV4 pAb (1:200, Alomone) and anti-Hepacam pAb (1:1500, ProteinTech, Chicago, IL, USA) in PBS+3% BSA followed by extensive washings and then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit Ab (1:10000; Thermo Scientific, MO, USA), for 1 h at RT. Immunoreactive bands were visualized using an enhanced chemiluminescence reagent (Pierce), according to the manufacturer's instructions and exposed on X-ray films.

Ouabain-affinity chromatography

The ouabain-affinity matrix was prepared according to a previously described procedure (18). Cells derived from six confluent 175 cm^2 flask for each cell line untreated and treated with hyposmotic solution were incubated with ouabain column overnight. Eluition was carried out with 2 ml of elution buffer (10 mM ouabain, 25 mM imidazole, 150 mM NaCl and 1× protease inhibitor cocktail, pH 7.4) as previously described (18). Samples obtained were analysed by SDS–PAGE and WB.

Pull-down assay with His-tagged MLC1 recombinant protein

A His-tagged recombinant human MLC1 protein was produced as previously described (16). Briefly, the entire cDNA sequence of human MLC1 (accession number BC028425) was cloned into a six histidine-tagged prokaryotic inducible expression vector (pQE-30 UA, Qiagen, GmbH, Hilden, Germany). The recombinant protein was expressed in *Escherichia coli* cells and bound to Ni-NTA agarose resin (His-MLC1 resin) following the manufacturer's instructions (Qiagen) with minor modifications (16). Protein extract derived from *E. coli* strain carrying the empty vector pQE-30 UA was used as control on Ni-Nta agarose resin (control resin). Cytosolic and membrane fractions from cultured rat astrocytes were extracted as previously described (31). Supernatants obtained, diluted to a final concentration of 10 mM imidazole, were pre-cleared by incubation with the Ni-NTA agarose resin and then incubated with His-MLC1 resin or with control resin with gentle rocking for 2 h at 4°C. Following exhaustive washes with 10–25 mM imidazole, 0.2% Triton X-100, 150 mM NaCl and 20 mM Tris–HCl, pH 7.4, protein-bound beads were eluted with 0.1 M glycine, pH 3. Aliquots (0.4 ml) of eluted proteins were precipitated with acetone (1:4 v/v) and analysed by SDS–PAGE and WB.

Intracellular Ca^{2+} measurement by FURA-2

Glass cover slips with strocytoma cells were placed in a recording chamber positioned on the stage of an inverted microscope (Axiovert 35, Zeiss, Germany), and connected to a perfusion system allowing the change of the bulk solution of the bath. In parallel, a local perfusion system (Rapid Solution Changer 100; Biologic) allowing the rapid switch between different solutions was also used. The control solution had the following composition: 122 mM NaCl, 3.3 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 0.4 mM $MgSO_4$, 10 mM D-glucose and 25 mM HEPES/NaOH (RT, pH 7.4). Ca^{2+}-free solutions were obtained by replacing the divalent ion by an equal amount of Mg^{2+} and adding 0.5 mM EGTA. Hypotonic solution was obtained by removing 60 mM NaCl from normal solution. The TRPV4 agonist 4αPDD (Sigma-Aldrich Italia, Italy) was dissolved in DMSO to obtain a 10 mM stock solution, which was kept at −20°C and utilized to make freshly made final solutions every day. Intracellular Ca^{2+} was measured by means of the video-imaging technique with the Ca^{2+} sensitive probe Fura-2 (Invitrogen). Fura-2-AM loading was achieved by exposing cell cultures to a solution containing 2.5 μM Fura-2-AM for 50 min at RT, then the culture was washed and after 10 min it was used for recording. Fura-2-loaded cells were exposed to the excitation wavelengths 340 and 380 nm by means of a monochromator (Polychrome II, Photonics, Germany), and the emission light at 510 nm was collected by a digital camera (PCO, Sensicam, Germany) and recorded on the hard disk of a PC computer (Dell, USA). Recording and analysis of the data were made possible by the use of the Imaging Workbench software package (INDEC Systems, CA, USA). For further data processing and presentation, the Origin 7.5 software package (Microcal Software, NJ, USA) was utilized. Ca^{2+} concentrations were expressed as ratio between the emission at 340 and 380 nm (R in the text). The amplitude of the Ca^{2+} signals was calculated as maximal amplitude during the challenge minus baseline amplitude recorded before the challenge.

When testing the action of RR in Fura-2 Ca^{2+} experiments, the potassium ionophore gramicidin (10 μg/ml, applied 5 min before recording), known for its capability to strengthen the hyposmosis-induced Ca^{2+} influx, was used. In this condition, the totality of cells reacted to hyposmosis with a sudden and synchronous Ca^{2+} raise, so allowing an easier evaluation of the pharmacological sensitivity of hyposmosis-induced Ca^{2+} raise. Stock solutions were made by dissolving gramicidin in ethanol (20 mg/ml) and RR in water (10 mM), kept at 4°C and used daily to make fresh solutions.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGEMENTS

We thank Dr Antonella Bernardo for providing astrocyte cell cultures and Estella Sansonetti for excellent graphical work.

Conflict of Interest statement. None declared.

FUNDING

This project was funded by ELA Foundation France (grant ELA 2009-002C5A to E.A.).

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SUPPLEMENTARY MATERIALS AND METHODS

Co-purification of His-tagged proteins

Lysates obtained from two 175 cm^2 flasks of confluent astrocytoma cell lines stably overexpressing histidine-tagged wild-type (WT) and mutated MLC1 (S246R, S280L, C125R) and control cells (U251) were incubated overnight at 4 °C with 200 µl (50% v/v suspension) of Ni-NTA Agarose (Qiagen, Hilden, Germany). After extensive washings (10 bed volumes of 10 and 25 mM Imidazole, 0.2% Triton X-100, 0.15 M NaCl, 20mM Tris-HCl pH 7.4), protein elution was carried out using imidazole, at a concentration of 50 and 200 mM (Lanciotti A. et al., 2010). The eluted proteins were precipitated with acetone (1:4 v/v) and analyzed by SDS-PAGE and WB.

Ouabain-affinity chromatography

Cells derived from six confluent 175 cm^2 flasks for each cell line (WT MLC1 and mutated S246R, S280L, C125R) untreated and treated for 6 h with hyposmotic solution were incubated with ouabain column overnight. Elution was carried out with 2 ml of elution buffer as previously described (Brignone M.S. et al., 2011). Obtained samples were precipitated with acetone (1:4 v/v) and analysed by SDS-PAGE and WB.

Protein extract preparation and Western blotting

Astrocytoma cell lines were lysed as described in Materials and Methods. Protein samples were subjected to SDS-PAGE using gradient (4–12%) precasted gels (Invitrogen) (Lanciotti A. et al., 2010), transferred to a nitrocellulose membrane, and immunoblotted overnight at 4 °C with the following Abs: anti-MLC1 pAb (1:500, in-house generated), anti-Na,K-ATPase β1 mAb (1 µg/ml, Millipore), anti-TRPV4 pAb (1:200, Alomone, Israel), in PBS, 3% BSA. After extensive washings, the membrane was incubated for 1 h at RT with horseradish peroxidase-conjugated anti-mouse or anti-rabbit Abs (1:10000; Thermo Scientific, Missouri, USA). Immunoreactive bands were visualized using an enhanced chemiluminescence reagent (Pierce), according to the manufacturer's instructions and exposed on X-ray films.

Supplementary figure

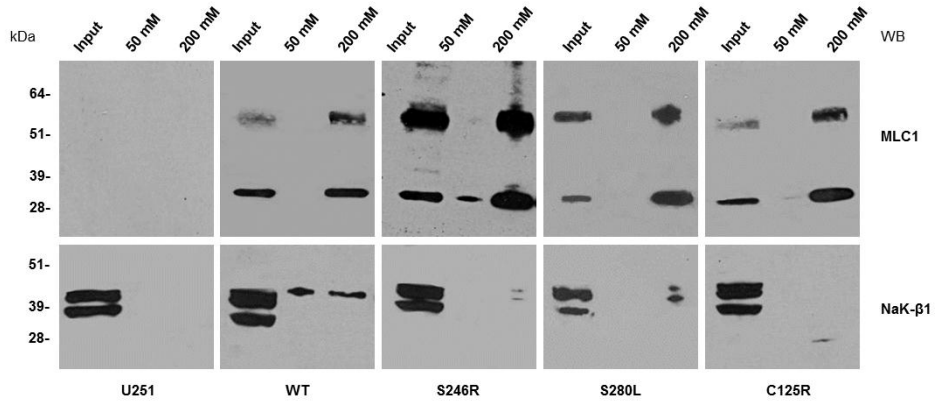


Figure S1. His co-purification assay to verify Na,K-ATPase β1 association to WT or mutated MLC1 in astrocytoma cells. Na,K-ATPase β1 (NaK-β1) was co-eluted with 50 mM and 200 mM imidazole from Ni-NTA agarose bound with WT MLC1 expressing astrocytoma proteins (WT); a decrease in the binding was observed in MLC1 S246R and S280L Ni-NTA agarose while no NaK-β1 interacts with C125R mutant. Molecular weight markers are indicated on the left (kDa).

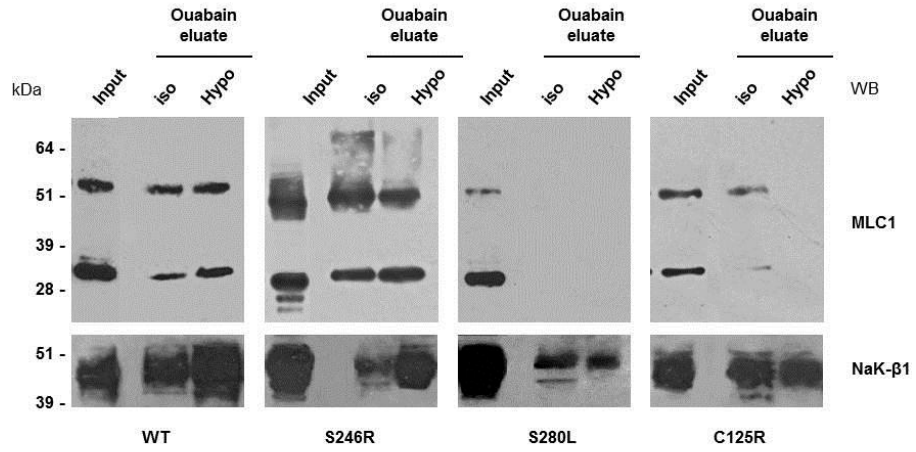
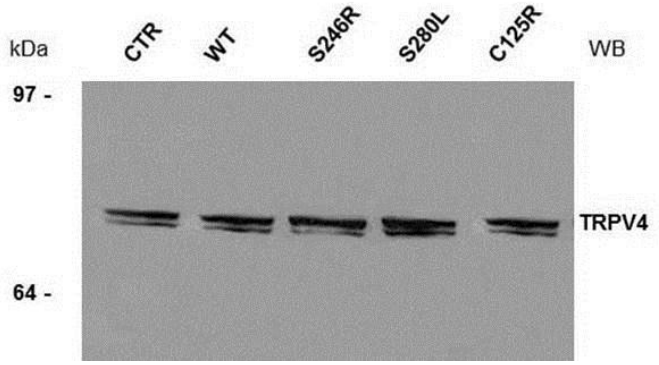


Figure S2. Hyposmotic treatment increases MLC1 association with the Na,K-ATPase.
Ouabain-affinity chromatography on astrocytoma cells subjected to hyposmotic shock revealed that in astrocytoma cells hyposmotic treatment increased the association between MLC1 and the Na,K-ATPase complex in WT and S246R expressing cells. No binding between S280L or C125R mutated MLC1 and Na,K-ATPase was observed.

Figure



S3.

Expression of TRPV4 protein in astrocytoma cell lines overexpressing WT and pathological MLC1 mutations. WB analysis of protein extracts of astrocytoma cells revealed no difference in the expression level of TRPV4 protein in control cell line (CTR), wild-type (WT) and mutated MLC1 expressing cells. Molecular weight markers are indicated on the left (kDa).

Conclusions

The results obtained in the above papers indicate that MLC1 directly binds the β1 subunit of Na,K-ATPase and through this interactions it takes part to a multiprotein complex that is involved in astrocyte response to osmotic changes. Moreover pathological mutations can affect this pathway leading to dysfunctions in astrocyte osmoregulatory activity.

These results are consistent with the starting hypothesis that brain pathology in patients carrying MLC1 mutations may be caused by alterations in the processes regulating osmotic balance and cell volume changes. MLC pathological features like fluid cysts, myelin vacuolation, enlargement of extracellular space and swelling of the white matter (van der Knaap M.S. et al., 1995; Leegwater P.A. et al., 2002; Pascual-Castroviejo I. et al., 2005, Duarri A. et al., 2011) are compatible with alterations in astrocyte-mediated processes controlling brain volume and ion and fluid exchanges. It is known that astrocytes, by contacting neurons and cells lining fluid-filled compartments, play a crucial role in regulating ion and water homeostasis through the selective transmembrane movements of organic and inorganic molecules and the balance of osmotic gradients which involve alterations of cell volume (Sofroniew M.V. and Vinters H.V. 2010; Kimelberg H.K. 2010). In astrocytes, Na,K-ATPase is involved in cell volume regulation and swelling induced by glutamate and hypo-osmotic treatment (Olson J.E. et al., 1986; Lang F. 2007; Schneider G.H. et al., 1992; Hansson E. 1994; Bender A.S. et al., 1998), water exchange (Illarionova N.B. et al., 2010) and the generation of calcium waves (Golovina V. et al., 2003; Hartford A.K. et al., 2004).

Although we do not know yet the specific function of MLC1, we hypothesize that this protein works as an ion channel itself, or as a chaperone subunit of some other channel(s) cooperating with the Na,K-ATPase enzyme in astrocyte swelling and/or regulatory volume decrease occurring during hypo-osmotic shock. Exposure of cells to hypo-osmotic extracellular fluid leads to water influx, along the osmotic gradient across the cell membrane and consequent cell swelling which involves the collaborative activity of different transporters, exchangers, water and ion channels, such as AQPs, TRPV4 and Kir4.1. To further investigate MLC1 function in astrocytes and the effect of pathological mutations in this project we have generated a human derived pathological model based on the stable transfection of astrocytoma cells with MLC1 wt or carrying pathological mutations.

The lack of an animal model and the extreme difficulty in obtaining brain biopsies from patients have slowed the research in this area and have highlighted the need of new pathological models to study MLC mutation-induced brain alterations. Since the astrocytoma cell line U251 expressed very low levels of the endogenous protein (Ambrosini E. et al., 2008) it represents an appropriate cellular model to analyze the effects of the overexpression of MLC1 wild-type or carrying pathological mutations in a human astrocytic background. By using this model we found that all the pathological missense mutations analysed alter the interaction of MLC1 with the Na,K-ATPase multiprotein complex confirming the pathological significance of the MLC1 associate molecular pathway revealed in primary astrocytes. Since MLC1 protein is involved in the astrocytes response to osmotic changes and hyposmotic shock is known to cause an abrupt (osmotic driven) cell swelling due to water movement across plasmalemma, followed by regulatory volume decrease (RVD) we have also explored the relationship between MLC1 and the calcium-permeable cation channel, TRPV4 that among its functions it is known as the cation channel responsible for swelling-induced cytosolic calcium increase and for the consequent RVD in response to hyposmosis in different type of cells (Becker D. et al., 2005). Recent data obtained in rat astrocytes indicate that TRPV4 in cooperation with AQP-4 constitutes the main astroglial osmosensor (Benfenati. et al., 2007; 2011) regulating RVD also in hyposmotic astrocytes. Our data reveal a role for MLC1 in TRPV4 mediated intracellular calcium influx under hyposmosis. Although at present we do not know whether MLC1 binds TRPV4 directly or by means of other intermediate proteins, the data obtained by pull-down and ouabain-chromatography clearly demonstrate that both in primary rat astrocytes and human astrocytoma cells MLC1 and TRPV4 interact and participate to the same functional multiprotein complex. Interestingly, in brain the TRPV4 channel is strongly expressed in astrocyte end-feet surrounding blood vessels, ependyma and meninges (Benfenati et al., 2011), a localization that mimics that of MLC1 and suggests the possibility of their interaction in vivo. At the subcellular level TRPV4 has been found in caveolae (Becker D. et al., 2005) where also MLC1 and Na,K-ATPase are expressed (Lanciotti A. et al., 2010; Liu L. et al., 2006; Cai T. et al., 2008). Therefore, it is conceivable that in astrocytes MLC1 associated proteins can functionally cooperate in these compartments where the activity of mechanosensitive channels is strictly regulated during cell swelling (Kozera L. et al., 2009). These findings suggest that defects in intracellular

calcium transients in response to osmotic challenges may be involved in the pathogenetic mechanism underlying MLC disease.

Although these data represent a considerable advance in the comprehension of MLC pathogenesis, much more work has to be done to fully understand MLC1 function in health and MLC disease and exploit this knowledge to develop effective therapies for MLC patients.

For this reason, in this project, we will continue to investigate the functional involvement of MLC1 in the cellular pathways associated to already characterized and newly discovered MLC1 interactors.

Latest results and future perspectives

Understanding the specific function of MLC1 protein whose mutations are the main cause of MLC is an essential step toward identification of disease mechanisms and development of effective therapies. Our data strongly indicate MLC1 involvement in calcium-mediated mechanisms regulating astrocyte reaction to osmotic stress and suggest an ion channel function for MLC1 at plasmamembrane.

Our latest studies (manuscript under revision) show that MLC1 is also involved in regulating ion fluxes in intracellular organelle thus affecting organelle acidity and, as a direct consequence, influencing intracellular protein trafficking. Using human astrocytoma cells overexpressing wild-type (WT) or mutated MLC1 we observed that WT-MLC1 is abundantly expressed in early and recycling endosomes and that this expression is functionally relevant to modulate organelle acidity and the recycling rate of MLC1-associated proteins like the TRPV4 channel. Furthermore, although disease-causing mutations differentially affect MLC1 localization and trafficking, all the mutated proteins fail to influence endosomal pH and protein trafficking through endosomal organelles. These findings lead to hypothesize that MLC1, by influencing the ion fluxes of endosomal vesicles in which it is expressed may favour its own recycling and the recycling of its molecular partners, as observed for TRPV4, particularly in stress conditions. It is also possible that the trafficking of other known MLC1 interactors like Na, K-ATPase and associated proteins (Brignone M.S et al., 2011), V-ATPase or junction proteins (ZO-1, occludin) (Duarri A. et al., 2011) can be influenced by MLC1 and defects in the recycling processes of MLC1 and associated proteins in conditions of altered brain homeostasis would lead to brain damage observed in patients. Interestingly abnormal recycling of junctional proteins can lead to the formation of intracellular vacuoles (Dukes J.D. et al., 2011, 2012) similar to those observed in the brain of MLC patients (Duarri A. et al., 2011). Moreover endocytosis and recycling constitute essential processes in the regulation of the expression of cell surface molecules, lipid membrane components like cholesterol and receptors that are important in the neural-glial interactions occurring during brain development (Yap C.C. and Winckler B., 2012; Shilo B.Z. and Schejter E.D., 2011; Chen J. et al., 2011) . Modification of these latter processes may results in disturbances in myelin formation as observed for other leukodystrophies in which specific defects in astrocyte maturation and functions are responsible for incorrect myelin formation (Bugiani M. et al., 2011; Messing A. et al., 2012)

Overall our data demonstrate that MLC1 regulates ion fluxes trhough plasmamembrane and intracellular organelle thus influencing the recycling of protein involved in astrocyte response to stress conditions while MLC1 disease-causing mutations deregulate these pathways.

These data suggest a possible mechanism underlying myelin and extracellular fluid abnormalities in the brain of MLC patients and open new unexpected perspective for the identification of specific therapeutic strategies for MLC patients.

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