Aquaporin-4 (AQP4) exists as two major isoforms that differ in the length of the N-terminus, the shorter AQP4-M23 and the longer AQP4-M1. Both isoforms form tetramers, which can further aggregate in the plasma membrane to form typical Orthogonal Arrays of Particles (OAPs) whose dimension depends on the ratio of the M1 and M23. In this study, we tested the hypothesis that the M23 isoform can be produced directly by the M1 mRNA. In cells transiently transfected with AQP4-M1 coding sequence we observed beside AQP4-M1 the additional presence of AQP4-M23 isoform associated with the formation of typical OAPs observable by 2D BN/SDS-PAGE and TIRF microscopy. The mutation of the second in-frame methionine M23 in AQP4-M1 (AQP4-M1^{M23I}) prevented the expression of the M23 isoform and the formation of OAPs. We propose “leaky scanning” as translational mechanism for the expression of AQP4-M23 protein isoform and that the formation of OAPs may occur even in the absence of M23-AQP4 mRNA. This mechanism can have important pathophysiological implications for the cell regulation of the M1/M23 ratio and thus OAP size. In this study we also provide evidence that AQP4-M1 is mobile in the plasma membrane, that it is inserted and not excluded into immobile OAPs and it is an important determinant of OAP structure and size.

INTRODUCTION

Aquaporin-4 (AQP4) is a water channel protein abundantly expressed in the central nervous system (1,2). It is mainly localized in astrocyte end-feet surrounding blood vessels, in the astrocyte processes that form the glia limitans, and in ependymal cells lining the ventricles. This water channel has been demonstrated to have a key role in fluid movement in mammalian brain, in cell migration and glial scar formation, and in signal transduction (3,4,5). The AQP4 gene encodes for two different mRNAs with different translation initiating methionines, M1 or M23 (6,7,8). Thus, AQP4 is expressed as two major isoforms of 32 kDa (AQP4-M1) and 30 kDa (AQP4-M23), which differ by 22 amino acids in the N-terminus. These two major AQP4 isoforms are organized in the plasma membrane as heterotetramers (9,10,6), appearing as regularly spaced intramembrane particles (IMPs) in freeze-fracture electron microscopy images (FFEM) (11,12,13,14). Multiple IMPS aggregate to form structures known as orthogonal arrays of particles (OAPs) or square arrays (12,13,15). Correlations have been reported between OAP alteration and various neuromuscular disease including brain ischemia, edema, stroke, brain tumor, focal cortical freeze injury, acute bacterial meningitis and brain abscess (16,17,18,19,20,21), and muscular dystrophy (22,23,24,25,26,27). Recently, AQP4 was correlated to the neuromyelitis optica (28), an inflammatory demyelinating autoimmune disease associated with AQP4 water channel antibodies (NMO-IgG) detectable in the serum of patients (29,30,31,32,33). Little is known about the molecular target of AQP4 autoantibodies. We recently reported (34) the ability of NMO-IgG to specifically recognize AQP4 only when present as OAPs indicating that NMO-IgG epitope is not present in the AQP4 protein “per se”, but it is intrinsic in AQP4 assembled into OAPs.
The physiological role of AQP4 organization into OAPs and of their size is as yet largely unknown. It has been speculated that this assembly might enhance water permeability (35,36,37), although the water permeabilities of the individual isoforms are similar (4). Alternately, formation of OAPs may confer higher-level plasma membrane stability necessary for AQP4 polarization in particular zones such as astrocyte foot processes (38). Such functional differences in plasma membrane lateral diffusion have been documented. AQP4-M23 isoform was shown to have lower mobility in the plasma membrane compared to the AQP4-M1 isoform (39).

FFEM analysis of AQP4-M1 and AQP4-M23 isoforms, selectively expressed in cultured cells, has shown that AQP4-M23 alone forms large-size OAPs, whereas AQP4-M1 alone is unable to form OAPs. When coexpressed, they form OAPs of intermediate size, similar to those seen in native tissues, suggesting that the ratio of the two isoforms is central to the determination of OAP size (14,37). Biochemical analysis using BN/SDS-PAGE has recently shown the expression of several AQP4 multi-subunit complexes (pools) ranging from several MDa to approximately 400 kDa in the brain, indicative of OAPs of various sizes (40,41). These pools contain different ratios of the 30/32 kDa isoforms. AQP4-M23 is mainly expressed in the higher molecular weight pools in contrast with AQP4-M1 mainly expressed in the lower molecular weight pools.

Little is known about the molecular mechanism that controls the tissue-specific expression ratios AQP4-M1/M23 isoforms, and the mechanism by which AQP4-M23 isoform is expressed from the AQP4 gene. The AQP4-M23 isoform was described as a transcript or spliced variant of the AQP4 gene (42,6), and more recently, AQP4-M23 has been assumed as translational variant of the AQP4-M1 isoform mRNA (8). According to the scanning model for translational initiation (43,44,45), the 40S ribosomal subunits enter at the 5′ m7G cap end of the mRNA and scan in a 5′ to 3′ direction until they find a suitable start codon, at which point they initiate translation. However, if this first start codon is not in a “good nucleotide context” as defined by the Kozak consensus sequence (43,44), the scanning can be “leaky” which means that some ribosomes may fail to initiate at this AUG and continue scanning to the next AUG. Comparison of the sequences surrounding the two start codons in AQP4 indicates that the second downstream AUG (M23) is in an optimal nucleotide context (ATCAUGG) whereas the upstream AUG (M1) is in a sub-optimal context (GGCAUGA). Therefore, we hypothesized that the shorter M23 isoform of AQP4 can be translated from the AQP4-M1 mRNA through a leaky scanning mechanism (LSM). In order to test this hypothesis we analyzed whether cells transiently transfected with AQP4-M1 cDNA also synthesize the AQP4-M23 isoform, and consequently, form OAPs. As control, a mutated form of AQP4-M1 was in parallel studied in which the methionine 23 was substituted with isoleucine (AQP4-M1M23).

**EXPERIMENTAL PROCEDURES**

**SDS-PAGE and Western Blot Analysis** - Cerebrum, cerebellum, spinal cord, kidney, stomach and skeletal muscle were removed and cut into small pieces in seven volumes of Lysis Buffer [10 mM Trizma base; 150 mM NaCl, 0.2 Na-orthovanadate; 1 % Triton X-100, 0.5% IGEPAL; 1 mM EDTA, 1 mM EGTA; protease inhibitors cocktail (Roche, Diagnostic, Indianapolis, IN)]. The lysis was performed on ice for 2 h and the samples were then centrifuged at 22,000xg for 1 h. The protein content of the supernatant was measured with BCA Protein Assay Kit (BioRad, Rockford, IL, USA). Membrane proteins were dissolved in SDS loading buffer and 2.5 % β-mercaptoethanol, heated to 37°C for 10 minutes, and resolved on a 13% polyacrylamide gel. Immunoblotting was performed as previously described (46,47). Reactive proteins were revealed with an enhanced chemiluminescent detection system (ECL-Plus; Amersham Biosciences) and visualized on a Versadoc imaging system (Bio-Rad laboratories).

**Plasmids and Mutagenesis** - The wild type human AQP4-M1 and AQP4-M23 cDNAs were cloned into pTarget Mammalian Expression Vector System (Promega). The mutant construct named pTarget human AQP4-M1M23I was constructed in parallel in which the methionine 23 was substituted with isoleucine (AQP4-M1M23I). AQP4-M1 cDNA also synthesize the AQP4-M23 isoform, and consequently, form OAPs. As control, a mutated form of AQP4-M1 was in parallel studied in which the methionine 23 was substituted with isoleucine (AQP4-M1M23I). The amplified product was digested with Dpn I and sub-cloned in E. coli XL1-Blue supercompetent cells (Stratagene).
The same method was used to convert the methionine 23 translational context (ATCATGG) into an inefficient translational context (CTCATGC). This mutation allows us to maintain the apolar methionine 23 context converting the isoleucine 22 and the valine 24 into two leucines.

The wild type human AQP4-M1 cDNA was also cloned into pcDNA3.1/NT-GFP-TOPO vector, containing the GFP. Protein samples for Blue Native/PAGE were prepared as described earlier were mixed with 5% deoxycholate, 1%; protease inhibitors cocktail (Roche, Diagnostic, Indianapolis, IN). After 30min. of incubation on ice, the samples were centrifuged at 22,000g for 30 min and the protein content of the supernatant was measured with CBB G-250 (Coomassie Blue G-250) dye. The electrophoresis was stopped when the tracking line of CCB G-250 dye had left the edge of the gel. Lanes from the first dimension were cut into individual strips and equilibrated in denaturation buffer (1% SDS, 1% mercaptoethanol) 1-2 h at room temperature. A single strip was then placed into a second dimension gel of the same thickness and subject to SDS-PAGE performed according to standard protocols. At the end of the run, the gel was blotted onto a PVDF membrane for Western blot analysis.

Antibodies - Goat anti-AQP4 polyclonal antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) were used for Western Blot and immunofluorescence analysis. For AQP4 and NMO double immunofluorescence experiments a rabbit anti-AQP4 antibody was used (1). Peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnologies. The secondary antibodies used for IF were from Molecular Probes (Eugene). A pool of 4 different NMO-patients sera at the dilution 1:1000 in buffers containing 4% bovine serum albumin (BSA) were used for IF. Goat anti-AQP4 polyclonal antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) were used for Western Blot and immunofluorescence analysis. Only for AQP4 and NMO double immunofluorescence experiments a rabbit anti-AQP4 antibody was used (1).

Chemicals - ε-aminocaproic acid, Imidazole, Ferritin were from Sigma (St Louis, MO). Acrylamide/bisacrylamide was from Serva (Heidelberg, Germany). All other chemicals were obtained from Sigma (St Louis, MO).

Total Internal Reflection Fluorescence Microscopy (TIRFM) analysis for the measurement of AQP4 dots - A Nikon Laser TIRF setup was used, consisting of a 488 nm Argon laser mounted on a Nikon Laser TE2000U Microscope. The objective of this microscope is to achieve a higher numerical aperture. The TIRF microscope also allows phase-contrast and epifluorescence techniques to be combined with TIRF technology. An incidence angle greater than the critical angle was achieved by the use of a 100X CFI Plan Apo of 1.45 numerical aperture. Fluorescence excited by TIR evanescent field was collected with the same objective and images were collected by a cooled charge-coupled device camera (Hamamatsu Orca). Transfected HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. Six hours before transfection the cells were plated at sub-confluency using antibiotic-free medium. Cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and analyzed after 24–48 h.

Immunofluorescence - HeLa cells plated on coverslips were fixed in 4% paraformaldehyde, washed in phosphate buffered saline (PBS), and permeabilized with 0.3% Triton X-100 in PBS. After blocking with 0.1% gelatin in PBS, cells were incubated with primary antibodies for 1 h at RT. After washings in PBS, cells were incubated for 30 min with Alexa conjugated secondary antibodies. The staining with NMO sera was performed on unfixed living cells. Coverslips were mounted on slides with mounting medium and examined by using a Nikon photomicroscope equipped for epifluorescence (DMRXA; Leica, Heidelberg GmbH, Mannheim, Germany). Digital images were obtained with a DMX 1200 camera (Nikon, Tokyo, Japan).

Protein Samples for Blue Native/PAGE - Transfected HeLa cells were washed once in ice-cold PBS and dissolved in BN lysis buffer (48) (ε-aminocaproic acid, 500 mM; Imidazole, 50 mM; EDTA, 2 mM; NaCl, 12 mM; glycerol, 10%; Triton X-100, 1%; protease inhibitors cocktail (Roche, Diagnostik, Indianapolis, IN). After 30 min. of incubation on ice, the samples were centrifuged at 22,000g for 30 min and the protein content of the supernatant was measured with BCA Protein assay kit (BioRad, Rockford, IL).

Blue Native/SDS-PAGE - Polyacrylamide native gradient gels (3-9%) were prepared as described (40,41). Twenty micrograms of protein sample prepared as described earlier were mixed with 5% of CBB G-250 (Coomassie Blue G-250) and loaded in each lane. Twenty micrograms of ferritin were used as molecular weight standard (440 and 880 kDa). The running buffers were the anode buffer (25 mM Imidazole, pH 7) and blue cathode buffer (50 mM Tricine; 7.5 mM Imidazole; 0.02% Coomassie Blue G-250; pH 7). After the blue running front has moved about one-third of the desired total running distance, the blue cathode buffer was removed and the run was continued using slightly blue cathode buffer B/10 (50 mM Tricine; 7.5 mM Imidazole; 0.002% Coomassie Blue G-250; pH 7) for better detection of faint protein bands, and improve native blotting. The electrophoresis was stopped when the tracking line of CCB G-250 dye had left the edge of the gel. Lanes from the first dimension were cut into individual strips and equilibrated in denaturation buffer (1% SDS, 1% β-mercaptoethanol) 1-2 h at room temperature. A single strip was then placed into a second dimension gel of the same thickness and subject to SDS-PAGE performed according to standard protocols. At the end of the run, the gel was blotted onto a PVDF membrane for Western blot analysis.

Antibodies - Goat anti-AQP4 polyclonal antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) were used for Western Blot and immunofluorescence analysis. Only for AQP4 and NMO double immunofluorescence experiments a rabbit anti-AQP4 antibody was used (1). Peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnologies. The secondary antibodies used for IF were from Molecular Probes (Eugene). A pool of 4 different NMO-patients sera at the dilution 1:1000 in immunofluorescence experiments was used.

Chemicals - ε-aminocaproic acid, Imidazole, Ferritin were from Fluka (St Louis, MO). Acrylamide/bisacrylamide was from Serva (Heidelberg, Germany). All other chemicals were obtained from Sigma (St Louis, MO).
cells were stained with commercial AQP4 antibodies and with NMO sera as described in “Immunofluorescence” paragraph. To determine the size of AQP4 spots, images were taken with a 100 X TIRF objective and 1.5 optic zoom.

**Fluorescence Recovery After Photobleaching (FRAP) experiments** - For these studies a stable cell line (HeLa) expressing a GFP fused M1 isoform of AQP4 (M1-GFP) was used. The clone that we used for this study had around 60% of transfected cells. M1-GFP expressing cells were grown on a 35-mm-diameter glass bottom dish and transiently transfected with the M23-AQP4 isoform. After one or two days, cells expressing large size OAPs (M1-GFP/M23) were identified under UV light and easily recognizable from those only expressing the M1-GFP isoform. The use of NMO-IgG at the end of the FRAP experiment confirmed the previous cell identification. FRAP experiments were performed using an Argon ion laser beam (488 nm, Nikon D Eclipse C1) which was modulated by an acousto-optic modulator and directed onto the stage of an inverted epifluorescence microscope (Nikon Eclipse TE 2000-U). The full-field and laser beams were reflected by a diachronic mirror (510 nm) onto the sample by a 100 X objective lens (Nikon Fluor, numerical aperture 1.45). The laser beam was set to 200-500 mW. Fluorescence cell images were taken before bleaching and at 10 s intervals after bleaching. All measurements were taken at 23 °C in a temperature-controlled darkroom. After the experiments, the fluorescence intensity of the spot was measured for both M1 and OAP-expressing cells present on the same dish.

**RESULTS**

**Analysis of AQP4 translation initiation signals (TIS).** The ratio of AQP4-M1/AQP4-M23 isoforms is variable among the different tissues in which AQP4 is expressed. As shown in the Western blot in Fig 1A, this ratio is lower in the CNS compared to other tissues such as skeletal muscle and stomach. To understand the mechanism by which the expression ratio of M1 and M23 isoforms is controlled, we transfected HeLa cells with cDNA encoding AQP4-M1. Surprisingly, analysis of AQP4 protein expression in these HeLa cells revealed the 32 kDa band as expected but also the additional presence of a 30 kDa band, corresponding to the M23 isoform of AQP4, was absent in cells transfected with the AQP4-M1M23I isoform indicating that a leaky scanning mechanism is likely responsible for the translation of the AQP4-M23 isoform in AQP4-M1 transfected cells. By immunofluorescence (Fig 2B) we found that the replacement of the methionine with isoleucine at position 23 did not affect the targeting of the protein to the plasma membrane. This was also confirmed by the absence of intracellular staining with confocal analysis (data not shown).

**Expression of WT and mutated AQP4 isoforms.** Initial experiments were done to analyze the expression of WT AQP4-M1 and AQP4-M23 together with the mutated AQP4-M1 (AQP4-M1M23I) isoforms in transiently transfected HeLa cells (Fig 2). By Western blot (Fig 2A), the presence of a 30 kDa band together with the expected 32 kDa band was clearly detectable in WT AQP4-M1 transfected cells. The 30 kDa band, corresponding to the M23 isoform of AQP4, was absent in cells transfected with the AQP4-M1M23I isoform, indicating that a leaky scanning mechanism is likely responsible for the translation of the AQP4-M23 isoform in AQP4-M1 transfected cells. By immunofluorescence (Fig 2B) we found that the replacement of the methionine with isoleucine at position 23 did not affect the targeting of the protein to the plasma membrane. This was also confirmed by the absence of intracellular staining with confocal analysis (data not shown).

**OAP formation in AQP4-M1 transfected cells.** Because both isoforms of AQP4 were expressed in AQP4-M1 transfected cells, we determined whether OAPs were present in these cells. Analysis of the plasma membrane organization of the different AQP4 isoforms was performed by both TIRFM (Fig 3A) and by BN-SDS/PAGE (40,41,49) (Fig 3B). The TIRFM analysis of WT AQP4-M1 transfected cells stained with commercial AQP4 antibodies revealed the linear staining typical of the expression of M1 isoform. In addition, many cells exhibited punctate staining indicating the presence of OAPs. In agreement with this data, the BN-SDS/PAGE analysis showed the presence of several larger size spots corresponding to discrete OAPs in addition to the smallest ~ 440 kDa pool. In contrast, OAPs and larger sized pools were completely absent in cells transfected with AQP4-M1M23I. Staining in AQP4-M1M23I cells was found to be continuous and evenly distributed in the plasma membrane with no visible dots by TIRFM and only the 440 kDa pool by BN-SDS/PAGE. The size of the dots visualized by TIRFM as well as the size of the AQP4 pools increased in parallel with the
increased amount of the AQP4-M23 isoform and were the largest in cells transfected with AQP4-M23. All these data further confirm that AQP4-M23 can be translated from AQP4-M1 transcript by a leaky scanning mechanism and highlight the presence of OAPs in transiently transfected HeLa cells.

NMO-IgG antibodies recognize AQP4-M1 transiently transfected HeLa cells. To further demonstrate the presence of OAPs in AQP4-M1 transiently transfected HeLa cells and in order to give additional support to the leaky scanning as a possible mechanism of regulation of the AQP4-M1/M23 expression ratio, we used NMO-IgG antibodies which recognize AQP4 only when assembled into OAPs (34). As shown in Fig 4, the NMO-IgG antibody recognized AQP4 only in cells (Fig 4, inset) transfected with AQP4-M1, and not in AQP4-M1M23I expressing cells, even though AQP4 expression, visualized using the AQP4 commercial antibody, was not significantly different in the two conditions. To promote formation of OAPs, we co-expressed AQP4-M23 with AQP4-M1M23I, or AQP4-M1, and determined whether NMO-IgG now recognized AQP4. As shown in Fig 4, the NMO-IgG serum recognized AQP4 in all cells expressing AQP4-M23. All these results were also confirmed modifying the Kozak nucleotide context of the second TIS but these results were also confirmed modifying the Kozak nucleotide context of the second TIS but without altering the codon sequence for the M23. (see supplementary Fig 1)

OAPs are made of both M23 and M1 isoforms and M23 reduces M1 plasma membrane lateral diffusion. In order to study if OAPs are exclusively made of AQP4-M23 or they are a mixture of AQP4-M1 and AQP4-M23, we used HeLa cells stably transfected with fluorescent AQP4-M1 (AQP4-M1-GFP) using a construct expressing AQP4-M1 fused at its N-terminus with GFP. In this case, being the GFP TIS the strongest expressing AQP4-M1 fused at its N-terminus with AQP4-M1 (AQP4-M1-GFP) using a construct HeLa cells stably transfected with fluorescent mixture of AQP4-M1 and AQP4-M23, we used exclusively made of AQP4-M23 or they are a diffusion

M23 reduces M1 plasma membrane lateral diffusion whereas the AQP4-M1-GFP + AQP4-M23 cells show a typical dot-like plasma membrane staining. After the laser shot at 0 min, the fluorescence was reduced in the bleached regions in both cell types, and after 6 minutes AQP4-M1-GFP expressing cells show a 80% of fluorescence recovery in the bleached region (Fig 5B, top). Interestingly, the fluorescence recovery was remarkably slowed in cells expressing both AQP4-M1-GFP and AQP4-M23 (Fig 5B, bottom) with a recovery of approximately 30% of the fluorescence level over 25 min. The reciprocal of the half-time (1/t_1/2) recovery was about 6 times lower in OAP expressing cells compared to AQP4-M1 expressing cells (Fig 5C) demonstrating that AQP4 is much less mobile in the plasma membrane when organized into OAPs.

**DISCUSSION**

The different ratios of M1 and M23 isoforms found in different tissues where AQP4 is expressed indicates that cells can regulate this ratio and therefore OAP size, with possible functional implications. Although in a recent paper it has been demonstrated that hydrophobic intermolecular interactions at the N-terminus of AQP4 are necessary for OAP formation (50), the molecular mechanisms controlling the expression of the two isoforms of AQP4 to generate OAPs of different sizes (40) still remains unclear as well as the mechanism by which AQP4-M23 is expressed from the AQP4 gene. Interestingly, even though in human brain it has been shown a much higher relative abundance of M1 versus M23 mRNA (42), this ratio is inverted when it comes to AQP4 protein expression levels being the M23 always the most abundant compared to M1 isoform. In this study we attempted to get an insight into the molecular basis that controls the expression of M1 and M23 isoforms. We used mammalian cells transiently transfected with M1- and/or M23-AQP4 coding sequence and with a mutated M1 isoform in which the methionine 23 was substitute with isoleucine to abolish the TIS-2 of AQP4, to analyze the source of the M23 isoform and verify the hypothesis that the translation of M23 isoform can be obtained from M1 mRNA. Given the absence of the intron sequences and the non-coding sequences in the transfected plasmids, transcriptional and M1/M23 alternative splicing mechanisms that have been previously reported to explain the presence of different isoforms in rat,
human, and mouse AQP4 (51,6,52,42,7,8) are excluded. Based on the obtained results, we propose that the synthesis of the M23 isoform possibly occurs via leaky scanning mechanism. The first direct evidence of this mechanism is the appearance of a 30 kDa isoform (M23) in cells transfected with the M1 coding sequence associated with the formation of OAPs, as observable by TIRF and BN-SDS/PAGE. In addition, replacement of the methionine at position 23 with a isoleucine (AQP4-M1_{M23I}) prevented both translation of the 30 kDa isoform and the formation of OAPs. We conclude that the formation of OAPs can occur even in the absence of M23-AQP4 mRNA. Our results provide the possibility that the size and formation of OAPs in different cells depends on the efficacy of the leaky scanning mechanism. Some hypothesis are so far available on the basic tetrameric composition of AQP4 and therefore on the composition of the OAPs. Neely (53) demonstrated that the two AQP4-M1 and -M23 isoforms are able to form heterotetramers supposed to aggregate to form OAPs whose dimension is depending on M1/M23 ratio. Crane (39) however, using a quantum dot based approach recently reported that AQP4-M1 does not incorporate into OAPs, but it modulates array size by competing for AQP4-M23 monomers at the OAP edges (39,50) and by using TIRF microscopy revealed that OAPs are relatively immobile in the plasma membrane. In our approach we used cells stably expressing GFP tagged tetramers of AQP4-M1 isoform. In experiments in which this cell line was transiently transfected with the untagged M23-AQP4 isoform we observed the appearance of fluorescent GFP-OAPs that were clearly distinguishable by TIRFM, indicating that the fluorescent M1 was inserted and not excluded into the OAP structure and that AQP4-M1 is also an important determinant of OAPs structure. Importantly, while AQP4-M1-GFP appears to be relatively mobile in the plasma membrane, the coexpression of the untagged AQP4-M23 induced the appearance of almost immobile GFP-OAPs. This is in agreement with what reported by Crane (39), indicating that OAPs as a stationary structure whose mobility is only moderately depending on other components such as the cytoskeleton or DAPs proteins (39). This may have physiological and pathological consequences. For example, immobile AQP4-OAPs such those present in astrocyte endfeet close to BBB may have a role in sustaining the polarized expression of AQP4 for a highly confined osmotically driven water flux. Otherwise large OAPs could be useful to crowd together DAPs, ion channels (i.e. KIR4.1) and other membrane proteins expressed (41). In fact, the finding that alfa-syntrophin (38,9) or dystrophin deletion (25,42) results in a loss of perivascular accumulation of AQP4 in astrocytes and muscle suggest that other components are required for OAPs membrane stability. Smaller and more mobile AQP4-OAPs may have a role in cell migration and glial scar formation (54,55).

In conclusion our present data demonstrate that the leaky scanning mechanism exists as translational mechanism for the expression of AQP4 protein isoforms. We speculate that this mechanism can have important pathophysiological implications for the cell regulation of the M1/M23 ratio and therefore of OAPs size. We also confirm that OAPs are made of both M1 and M23 isoform and that the ratio is important in determining the size of the OAPs.

REFERENCES

1. Frigeri, A., Gropper, M. A., Turck, C. W., and Verkman, A. S. (1995) Immunolocalization of the mercurial-insensitive water channel and glycerol intrinsic protein in epithelial cell plasma membranes. Proc Natl Acad Sci USA 92, 4328-4331

2. Frigeri, A., Gropper, M. A., Umenishi, F., Kawashima, M., Brown, D., and Verkman, A. S. (1995) Localization of MIWC and GLIP water channel homologs in neuromuscular, epithelial and glandular tissues. J Cell Sci. 108, 2993-3002

3. Ma, T., Yang, B., Gillespie, A., Carlson, E. J., Epstein, C. J., and Verkman, A. S. (1997) Generation and phenotype of a transgenic knockout mouse lacking the mercurial-insensitive water channel aquaporin-4. J Clin Invest. 100, 957-962

4. Verkman, A. S., Binder, D. K., Bloch, O., Auguste, K., and Papadopoulos, M. C. (2006) Three distinct roles of aquaporin-4 in brain function revealed by knockout mice. Biochim. Biophys. Acta 1758, 1085–1093

5. Amiry-Moghaddam, M., Ottersen. O. P. (2004). The molecular basis of water transport in the brain. Nat Rev Neurosci. 4:991-1001

6. Lu, M., Lee, M. D., Smith, B. L., Jung, J. S., Agre, P., Verdijk, M. A., Merkx, G., Rijss, J. P., and Deen, P. M. (1996) The
human AQP4 gene: definition of the locus encoding two water channel polypeptides in brain. *Proc Natl Acad Sci U S A*. **93**, 10908-10912

7. Zelenin, S., Gunnarson, E., Alikina, T., Bondar, A., and Aperia, A. (2000). Identification of a new form of AQP4 mRNA that is developmentally expressed in mouse brain. *Pediatr Res.* **48**, 335-339

8. Moe, S. E., Sorbo, J. G., Sogaard, R., Zeuthen, T., Ottersen P. O., and Holen, T. New isoforms of rat Aquaporin-4 (2008) *Genomics*. **91**, 367-377

9. Neely, J. D., Amiry-Moghaddam, M., Ottersen, O. P., Froehner, S. C., Agre, P., and Adams, M. E. (2001) Syntrophin-dependent expression and localization of Aquaporin-4 water channel protein. *Proc Natl Acad Sci U S A*. **98**, 14108-14113

10. Jung, J. S., Bhat, R. V., Preston, G. M., Guggino, W. B., Baraban, J. M., and Agre, P. (1994) Molecular characterization of an aquaporin cDNA from brain: candidate osmoreceptor and regulator of water balance. *Proc Natl Acad Sci U S A*. **91**, 13052-13056

11. Rash, J. E., Davidson, K. G., Yasumura, T., and Furman, C. S. (1996) Freeze-fracture and immunogold analysis of aquaporin-4 (AQP4) square arrays, with models of AQP4 lattice assembly. *Neuroscience*. **129**, 915-934

12. Rash, J. E., Yasumura, T., Hudson, C. S., Agre, P., and Nielsen, S. (1998) Immunogold labeling of aquaporin-4 in square arrays of astrocyte and ependymocyte plasma membranes in rat brain and spinal cord. *Proc Natl Acad Sci U S A*. **95**, 11981-11986

13. Verbavatz, J. M., Ma, T., Gobin, R., and Verkman A. S. (1997) Absence of orthogonal arrays in kidney, brain and muscle from transgenic knockout mice lacking water channel aquaporin-4. *J Cell Sci*. **110**, 2855-2860

14. Furman, C. S., Gorelick-Feldman, D. A., Davidson, K. G., Yasumura, T., Neely, J. D., Agre, P., and Rash, J. E. (2003) Aquaporin-4 square array assembly: opposing actions of M1 and M23 isoforms. *Proc Natl Acad Sci U S A*. **100**, 13609-13614

15. Yang, B., Brown, D., and Verkman, A. S. (1996) The mercurial insensitive water channel (AQP-4) forms orthogonal arrays in stably transfected Chinese hamster ovary cells. *J Biol Chem*. **271**, 4577-4580

16. Bloch, O., Papadopoulos, M. C., Manley, G. T., Verkman, A. S. (2005) Aquaporin-4 gene deletion in mice increases focal edema associated with staphylococcal brain abscess. *J Neurochem*. **95**, 254-262

17. Manley, G. T., Fujimura, M., Ma, T., Noshita, N., Filiz, F., Bollen, A. W., Chan, P., and, Verkman, A. S. (2000) Aquaporin-4 deletion in mice reduces brain edema after acute water intoxication and ischemic stroke. *Nat Med.* **6**, 159-163.

18. Papadopoulos, M. C., Manley, G. T., Krishna, S., and Verkman, A. S. (2004) Aquaporin-4 facilitates reabsorption of excess fluid in vasogenic brain edema. *FASEB J*. **18**, 1291-1293

19. Papadopoulos, M. C., and Verkman, A. S. (2005) Aquaporin-4 gene disruption in mice reduces brain swelling and mortality in pneumococcal meningitis. *J Biol Chem*. **280**, 13906-13912

20. Saadoun, S., Papadopoulos, M. C., Davies, D. C., Krishna, S., and Bell, B. A. (2002) Aquaporin-4 expression is increased in oedematous human brain tumours. *J Neurol Neurosurg Psychiatry*. **72**, 262-265

21. Saadoun, S., Papadopoulos, M. C., and Krishna, S. (2003) Water transport becomes uncoupled from K+ siphoning in brain contusion, bacterial meningitis, and brain tumours: immunohistochemical case review. *J Clin Pathol.* **56**, 972-975

22. Schotland, D. L., Bonilla, E., and Wakayama, Y. (1981) Freeze fracture studies of muscle plasma membrane in human muscular dystrophy. *Acta Neuropathol.* **54**, 189-197

23. Wakayama, Y., Okayasu, H., Shibuya, S., and Kumagai, T. (1984) Duchenne dystrophy: reduced density of orthogonal
array subunit particles in muscle plasma membrane. *Neurology.* **34**, 1313-1317

24. Wakayama, Y., Kumagai, T., and Jimi, T. (1986) Small size of orthogonal array in muscle plasma membrane of Fukuyama type congenital muscular dystrophy. *Acta Neuropathol.* **72**, 130-133

25. Frigeri, A., Nicchia, G. P., Verbavatz, J. M., Valenti, G., and Svelto, M. (1998) Expression of aquaporin-4 in fast-twitch fibers of mammalian skeletal muscle. *J Clin Invest.* **102**, 695-703

26. Frigeri, A., Nicchia, G. P., Nico, B., Quondamatteo, F., Herken, R., Roncali, L., and Svelto, M. (2001) Aquaporin-4 deficiency in skeletal muscle and brain of dystrophic mdx mice. *FASEB J.* **15**, 90-98

27. Frydenlund, D. S., Bhardwaj, A., Otsuka, T., Mylonakou, M. N., Yasumura, T., Davidson, K. G., Zeynalov, E., Skare, O., Laake, P., Haug, F. M., Rash, J. E., Agre, P., Ottersen, O. P., and Amiry-Moghaddam, M. (2006) Temporary loss of perivascular aquaporin-4 in neocortex after transient middle cerebral artery occlusion in mice. *Proc Natl Acad Sci U S A.* **103**, 13532-13536

28. Lennon, V. A., Kryzer, T. J., Pittcock, S. J., Verkman, A. S., and Hinson, S. R. (2005) IgG marker of optic-spinal multiple sclerosis binds to the aquaporin-4 water channel. *J Exp Med.* **202**, 473-477

29. Lennon, V. A., Wingerchuk, D. M., Kryzer, T. J., Pittcock, S. J., Lucchinetti, C. F., Fujihara, K., Nakashima, I., and Weinshenker, B. G. (2004) A serum autoantibody marker of neuromyelitis optica: distinction from multiple sclerosis. *Lancet.* **364**, 2106-2112

30. Matsuoka, T., Matsushita, T., Kawano, Y., Osoegawa, M., Ochi, H., Ishizu, T., Minohara, M., Kikuchi, H., Mihara, F., Ohyagi, Y., and Kira, J. (2007) Heterogeneity of aquaporin-4 autoimmunity and spinal cord lesions in multiple sclerosis in Japanese. *Brain.* **130**, 1206-1223

31. Misu, T., Fujihara, K., Kakita, A., Konno, H., Nakamura, M., Watanabe, S., Takahashi, T., Nakashima, I., Takahashi, H., and Itoyama, Y. (2007) Loss of aquaporin 4 in lesions of neuromyelitis optica: distinction from multiple sclerosis. *Brain.* **130**, 1224-1234

32. Roemer, S. F., Parisi, J. E., Lennon, V. A., Benarroch, E. E., Lassmann, H., Bruck, W., Mandler, R. N., Weinshenker, B. G., Pittcock, S. J., Wingerchuk, D. M., and Lucchinetti, C. F. (2007) Pattern-specific loss of aquaporin-4 immunoreactivity distinguishes neuromyelitis optica from multiple sclerosis. *Brain.* **130**:1194-1205

33. Takahashi, T., Fujihara, K., Nakashima, I., Misu, T., Miyazawa, I., Nakamura, M., Watanabe, S., Shiga, Y., Kanaoka, C., Fujimori, J., Sato, S., and Itoyama, Y. (2007) Anti-aquaporin-4 antibody is involved in the pathogenesis of NMO: a study on antibody titre. *Brain.* **130**, 1235-1243

34. Nicchia, G. P., Mastrototaro, M., Rossi, A., Pisani, F., Tortorella, C., Ruggieri, M., Lia, A., Trojano, M., Frigeri, A., and Svelto, M. (2009) Aquaporin-4 orthogonal arrays of particles are the target for neuromyelitis optica autoantibodies. *Glia.* **57**, 1363-1373

35. Yang, B., van Hoek, A. N., and Verkman, A. S. (1997) Very high single channel water permeability of aquaporin-4 in baculovirus-infected insect cells and liposomes reconstituted with purified aquaporin-4. *Biochemistry.* **36**, 7625-7632

36. van Hoek, A. N., Ma, T., Yang, B., Verkman, A. S., and Brown, D. (2000) Aquaporin-4 is expressed in basolateral membranes of proximal tubule S3 segments in mouse kidney. *Am J Physiol Renal Physiol.* **278**, 501-511

37. Silberstein, C., Bouley, R., Huang, Y., Fang, P., Pastor-Soler, N., Brown, D., and Van Hoek, A. N. (2004) Membrane organization and function of M1 and M23 isoforms of aquaporin-4 in epithelial cells. *Am J Physiol Renal Physiol.* **287**, S130-S136

38. Amiry-Moghaddam, M., Frydenlund, D. S., and Ottersen, O. P. (2004) Anchoring of aquaporin-4 in brain: molecular mechanisms and implications for the physiology and pathophysiology of water transport. *Neuroscience.* **129**, 999–1010
39. Crane, J. M., Van Hoek, A. N., Skach, W. R., and Verkman, A. S. (2008) Aquaporin-4 dynamics in orthogonal arrays in live cells visualized by quantum dot single particle tracking. *Mol Biol Cell*. 19, 3369-3378

40. Nicchia, G. P., Cogotzi, L., Rossi, A., Basco, D., Brancaccio, A., Svelto, M., and Frigeri, A. (2008) Expression of multiple AQ4 pools in the plasma membrane and their association with the dystrophin complex. *J Neurochem*. 105, 2156 - 2165

41. Nicchia, G. P., Rossi, A., Nudel, U., Svelto, M., and Frigeri, A. (2008) Dystrophin-dependent and -independent AQ4 pools are expressed in the mouse brain. *Glia*. 56, 869-876.

42. Umenishi, F., and Verkman, A. S. (1998) Isolation and functional analysis of alternative promoters in the human AQP4 water channel gene. *Genomics*. 50, 373-377

43. Kozak, M. (2007) Some thoughts about translation regulation: forward and backward glances. *J Cell Biochem*. 102, 280-290

44. Kozak, M. (1999) Initiation of translation in prokaryotes and eukaryotes. *Gene* 234, 187-208

45. Kozak, M. (2005) Regulation of translation via mRNA structure in prokaryotes and eukaryotes. *Gene* 361, 13-37

46. Nicchia, G. P., Frigeri, A., Liuzzi, G. M., and Svelto, M. (2003) Inhibition of aquaporin-4 expression in astrocytes by RNAi determines alteration in cell morphology, growth, and water transport and induces changes in ischemia-related genes. *FASEB J*. 17, 1508-1510

47. Nicchia, G. P., Srinivas, M., Li, W., Brosnan, C. F., Frigeri, A., and Spray, D. C. (2005) New possible roles for aquaporin-4 in astrocytes: cell cytoskeleton and functional relationship with connexin43. *FASEB J*. 19, 1674-1676

48. Wittig, I., Braun, H. P., and Schägger, H. (2006) Blue Native PAGE. *Nat Protoc*. 1, 418-428

49. Sorbo, J. G., Moe, S. E., Ottersen, O. P., and Holen, T. (2008) The molecular composition of square arrays. *Biochemistry*. 47:2631-2637

50. Crane, J. M., and Verkman, A. S. (2009) Determinants of aquaporin-4 assembly in orthogonal arrays revealed by live-cell single-molecule fluorescence imaging. *J Cell Sci*. 122:813-821

51. Hasegawa, H., Ma, T., Skach, W., Matthay, M. A., and Verkman, A. S. (1994) Molecular cloning of a mercurial-insensitive water channel expressed in selected water-transporting tissues. *J Biol Chem*. 269:5497-5500

52. Umenishi, F., Verkman, A. S., and Gropper, M. A. (1996) Quantitative analysis of aquaporin mRNA expression in rat tissues by RNase protection assay. *DNA Cell Biol*. 15, 475-480

53. Neely, J. D., Christensen, B. M., Nielsen, S., and Agre, P. (1999) Heterotetrameric composition of aquaporin-4 water channels. *Biochemistry*. 38:11156-11163

54. Saeidoun, S., Papadopoulos, M. C., Watanabe, H., Yan, D., Manley, G. T., and Verkman, A. S. (2005) Involvement of aquaporin-4 in astroglial cell migration and glial scar formation. *J Cell Sci*. 118, 5691-5698

55. Auguste, K. I., Jin, S., Uchida, K., Yan, D., Manley, G. T., Papadopoulos, M. C., and Verkman, A. S. (2007) Greatly impaired migration of implanted aquaporin-4-deficient astroglial cells in mouse brain toward a site of injury. *FASEB J*. 21, 108-116

**FOOTNOTES**

The authors would like to thank Miduturu Srinivas for his assistance in revising the English of the article. This work was supported by Progetto strategico regionale Neurobiotech, PS 124 Regione Puglia and Rete Nazionale di Proteomic, FIRB 2008, RBRN07BMCT.

**FIGURE LEGENDS**

Figure 1. A possible mechanism responsible for AQP4-M23 synthesis from AQP4-M1 mRNA.
A) Western blot analysis of AQP4-M1 and AQP4-M23 in different tissues. A PVDF membrane containing rat tissues and transfected cells, immunobinned with anti-AQP4 antibodies after Glycine-SDS-PAGE. The AQP4-M1/M23 ratio is variable among the different tissues, in particular the AQP4-M23 isoform is abundant in the cerebrum, cerebellum and spinal cord. Note that in HeLa cells transiently transfected with the M1 isoform, two bands of 30 and 32 kDa are present. B) Analysis of AQP4 mRNA at the level of the translation initiation signals (TISs) surrounding M1 (TIS-1) and M23 (TIS-2). The 5’-proximal AUG corresponding to M1 is located in a sub-optimal context whereas the AUG corresponding to M23 is in an optimal context (42,43,44,45). C) A mutated form of AQP4-M1 mRNA (M23I) was generated by mutagenesis in which the substitution methionin in isoleucin at position 23 will destroy the TIS-2 avoiding the leaky scanning mechanism.

Figure 2. Expression of AQP4-M1, AQP4-M1M23I and AQP4-M23 in transiently transfected HeLa cells.
A) AQP4 immunoblot of membrane proteins prepared from HeLa cells transfected as indicated. Note that the 30 kDa band is present in cells transfected with the wild type AQP4-M1 and is absent in those transfected with AQP4-M1M23I. B) Epifluorescence micrographs of HeLa cells transfected with AQP4-M1, AQP4-M1M23I and AQP4-M23. Scale bar: 10 µm. Note the dot like plasma membrane staining when M23 is expressed.

Figure 3. Visualization of AQP4 OAPs by total internal reflection fluorescence (TIRF) microscopy and 2D BN-SDS/PAGE.
A) TIRF micrographs of Alexa488-labeled AQP4-M1M23I, AQP4-M1, AQP4-M1M23I + AQP4-M23, AQP4-M1 + AQP4-M23, and AQP4-M23 in HeLa cells. Scale bar: 10 µm. B) Immunodetection of AQP4 pools after a 3–9% gradient BN–PAGE (first dimension) and 12% SDS/PAGE (second dimension). Note that in BN-SDS/PAGE experiments the expression of AQP4-M1 isoform induced the immunodetection of 5 different pools due to the concomitant expression of AQP4-M23 and the consequent formation of small OAPs. In contrast, the parallel analysis of AQP4-M1M23I revealed the presence of the single smaller AQP4 pool corresponding to AQP4 tetramers.

Figure 4. OAPs visualization using NMO-IgG serum.
Double immunofluorescence experiments performed with rabbit polyclonal AQP4 antibodies (AQP4) and with NMO serum (NMO). Several cells transfected with AQP4-M1 were recognized by the NMO-IgG serum whereas no positive cells were found when the mutated AQP4-M1M23I isoform was used. Scale bar: 15 µm.

Figure 5. Fluorescence Recovery After Photobleaching (FRAP) experiments performed on AQP4-M1 and AQP4-M23 expressing HeLa cells.
A) Four serial images from a data set are shown. The images displayed were recorded before and 1 and 6 min after photo bleaching pulse. B) Representative AQP4-M1GFP and AQP4-M1GFP + AQP4-M23 individual FRAP curves. Note that AQP4 organization in OAPs strongly slowed its plasma membrane lateral diffusion. C) Data are shown as reciprocal half-times (t1/2) for fluorescence recovery (mean +/- SE of separate sets of measurements, n=3-7)
Figure 1
Figure 2
Figure 3
Figure 5
Evidences for a leaky scanning mechanism for the synthesis of the shorter M23-protein isoform of Aquaporin-4: implication in orthogonal array formation and neuromyelitis optica antibody interaction
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J. Biol. Chem. published online December 10, 2009

Access the most updated version of this article at doi: 10.1074/jbc.M109.069245

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