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AQUAPORIN-4 Mz ISOFORM: BRAIN EXPRESSION, SUPRAMOLECULAR ASSEMBLY AND NEUROMYELITIS OPTICA ANTIBODY BINDING

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Abstract

Water channel aquaporin-4 (AQP4) is expressed in astrocytes throughout brain and spinal cord. Two major AQP4 isoforms are expressed, M1 and M23, having different translation initiation sites. A longer isoform (Mz) has been reported in rat with translation initiation 126-bp upstream from that of M1. By immunoblot analysis of SDS and native gels probed with a C-terminus anti-AQP4 antibody, Mz was detected in rat brain as a distinct band of size ~39 kDa. Mz was absent in human and mouse brain because of in-frame stop codons. The ability of rat Mz to form orthogonal arrays of particles (OAPs) was investigated by single particle tracking and native gel electrophoresis. We found that Mz, like M1, diffused rapidly in the cell plasma membrane and did not form OAPs. However, when coexpressed with M23, Mz associated in OAPs by forming heterotetramers with M23. Unexpectedly, Mz-expressing cells bound neuromyelitis optica autoantibodies (NMO-IgG) poorly, <5-fold compared to M1-expressing cells. Truncation analysis suggested that the poor NMO-IgG binding to Mz involves residues 31–41 upstream of Met-1. We conclude that Mz AQP4 is: (a) present at low level in rat but not human or mouse brain; (b) unable to form OAPs on its own but able to associate with M23 AQP4 in heterotetramers; and (c) largely unable to bind NMO-IgG because of N-terminus effects on the structure of the AQP4 / NMO-IgG binding site.

Keywords

AQP4; water channel; astrocyte; NMO; orthogonal arrays of particles

INTRODUCTION

Aquaporin-4 (AQP4) is a water-selective channel expressed in astrocytes throughout the central nervous system, prominently at foot-processes at blood-brain and blood-cerebrospinal fluid interfaces (Nielsen et al., 1997; Rash et al., 1998). Studies in knockout mice lacking AQP4 have implicated the involvement of AQP4 in brain water balance, astrocyte migration, neuroexcitation and neuroinflammation (reviewed in Verkman et al., 2006). AQP4 knockout mice show reduced cytotoxic brain edema and increased vasogenic brain edema in response to various stresses (Manley et al., 2000; Papadopoulos et al., 2004; Bloch et al., 2006; Yang et al., 2008), impaired glial scar formation (Saadoun et al., 2005; Auguste et al., 2007), increased seizure duration (Binder et al., 2006), and reduced inflammation in experimental autoimmune encephalomyelitis (Li et al., 2011). AQP4 is also

the target of pathogenic autoantibodies in the neuroinflammatory demyelinating disease neuromyelitis optica (NMO) (Lennon et al., 2005).

AQP4 is present in two major isoforms, a relatively long (M1) isoform with translation initiation at Met-1, and a relatively short (M23) isoform with translation initiation at Met-23 (Jung et al., 1994; Yang et al., 1995; Lu et al., 1996). The M23 isoform is able to assemble in supramolecular structures called orthogonal arrays of particles (OAPs), which are cobblestone-like square arrays of particles seen originally in brain and other tissues by freeze-fracture electron microscopy (Landis and Reese, 1974; Rash et al., 1974; Orci et al., 1981; Verbavatz et al., 1997). We reported previously that OAP formation by M23 involves specific intermolecular N-terminus interactions, while M1, which does not itself form OAPs, contains residues upstream of Met-23 that block the N-terminus association (Crane and Verkman, 2009). We found by 2-color single particle tracking, single-spot photobleaching, and blue-native (BN) gel electrophoresis that M1 and M23 can associate as heterotetramers (Crane et al., 2009; Tajima et al., 2010), allowing M1 to associate with M23 in OAPs, albeit of smaller size than OAPs composed of M23 alone. The biological significance of OAP formation by AQP4 remains unknown, though it has been proposed that OAPs might facilitate AQP4 water transport, polarization to astrocyte foot-processes, and cell-cell adhesion (Yang et al., 1997; Van Hoek et al., 2000; Amiry-Moghaddam et al., 2004; Silberstein et al., 2004; Hiroaki et al., 2006). AQP4 OAPs have also been proposed to be the target of NMO autoantibodies (NMO-IgG) (Nicchia et al., 2009).

Rapid amplification of cDNA ends (RACE) analysis has indicated the presence, in rat, of at least six AQP4 isoforms generated by alternative splicing, as well as a very long (called Mz) AQP4 isoform with translational initiation 126 bp upstream of Met 1 (Moe et al., 2008). However, only the Mz isoform is water permeable and potentially generated *in vivo*. Here, using biophysical and biochemical methods, we investigated the expression Mz AQP4 in rodent and human brain, and its involvement in OAP assembly and NMO autoantibody binding. Unexpectedly, we found that Mz AQP4 bound NMO-IgG very poorly compared to M1 AQP4, and investigated potential mechanisms for this phenomenon.

EXPERIMENTAL PROCEDURES

DNA constructs, cell culture, and transfections

cDNA constructs encoding rat M1, M23, Mz, mouse M1 with 5' UTR, and human M1 with 5' UTR were PCR-amplified using whole-brain cDNA as template. The sequence of human M1 with 5' UTR was deposited in GenBank (HQ901095). The sequence of mouse M1 with 5' UTR was obtained from NCBI, AF469168 (Zelenin et al., 2000). PCR fragments were ligated into mammalian expression vector pcDNA3.1. cDNA constructs encoding rat M1, M23 and Mz containing a 10-residue Myc epitope (NH₂-EQKLISEEDL-COOH) in the second extracellular loop were generated as described (Crane et al., 2008). Mz AQP4 truncation mutants were generated by PCR using the above plasmids as templates, with constant, strong Kozak sequence (ACCATGGTG) (Kozak et al., 2002). All constructs were fully sequenced.

U87MG (human glioblastoma-astrocytoma, ATCC HTB-14) cell cultures were maintained at 37 °C in 5% CO₂, 95% air in appropriate medium containing 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were transfected with cDNAs in antibiotic-free medium 12–24 h before the experiments using FuGene HD (Roche, Switzerland) according to the manufacturer's protocol.

NMO patient sera and IgG purification

NMO serum was obtained from four NMO-IgG seropositive patients. Control (non-NMO) human serum was obtained from the UCSF cell culture facility. Purified IgG from NMO and control sera were isolated using a Melon Gel IgG Spin Purification Kit (Pierce, Rockford, IL) (Awai et al., 2006).

Quantitative immunofluorescence

Live U87MG cells were incubated in blocking buffer (PBS containing 6 mM glucose, 1 mM pyruvate, 1 % bovine serum albumin, 2% goat serum) for 20 min followed by 30 min with NMO (or control) serum in blocking buffer. Cells were then rinsed with PBS, fixed in 4% paraformaldehyde, and permeabilized with 0.25% Triton X-100. Cells were blocked again and incubated for 20 min with 0.4 µg/ml rabbit anti-AQP4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), then rinsed with PBS. Finally, cells were incubated for 30 min with 4 µg/ml goat anti-human IgG-conjugated Alexa Fluor 555 and goat anti-rabbit IgG-conjugated Alexa Fluor 488 (Invitrogen) in blocking buffer. Quantitative analysis of AQP4-antibody binding was done as described (Crane et al., 2011).

Quantum dot labeling and single particle tracking

Cells were washed with 3 ml PBS containing 6 mM glucose and 1 mM pyruvate (GP buffer) and incubated for 5 min in blocking buffer (GP buffer containing 1% bovine serum albumin), followed by 5 min with 70 ng/ml mouse anti-Myc antibody (Covance, Emeryville, CA) in blocking buffer. Cells were then rinsed with GP buffer, incubated for 5 min with 0.1 nM goat F(ab')₂ anti-mouse IgG-conjugated Qdot 655 (Invitrogen) in blocking buffer, and then rinsed again with GP buffer. Coverslips were transferred to a perfusion chamber and maintained in GP buffer for single particle tracking (SPT). SPT measurements were done and analyzed as described (Crane et al., 2008).

Electrophoresis and immunoblot analysis

Polyacrylamide native gradient gels (3–9 %) were prepared as described (Wittig et al., 2006). Samples (20 µg protein) were mixed with 5% Coomassie Blue G-250 and loaded in each lane. Ferritin was used as the molecular mass standard (440 and 880 kDa). Proteins were blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) for immunoblot analysis. For the second dimension, lanes from the first dimension were cut into individual strips and equilibrated in denaturation buffer (1% SDS, 1% β-mercaptoethanol) for 1–2 h at room temperature. A single strip was then placed into a second dimension gel of the same thickness and subjected to Tricine SDS-PAGE using standard protocol as described (Shagger et al., 2006). Proteins were blotted as above. One-dimensional Tricine SDS-PAGE was performed as described (Shagger et al., 2006). Immunoblot analysis was performed as described (Crane et al., 2009). Antibody-based gel shift assay was performed as described (Camacho-Carvajal et al., 2004). Briefly, 20 µg of protein samples were preincubated with 3 µg purified IgG from an NMO seropositive patient (or control human IgG) for 30 min on ice. Coomassie Blue G-250 was added prior to loading onto the native gel.

RESULTS

Fig. 1A compares the aligned upstream DNA sequences of human, rat and mouse AQP4. The long, Mz isoform of rat AQP4 contains an ATG translation initiation site 126 bp upstream of the ATG encoding Met-1 of M1 AQP4. ATG translation initiation sites are also present in the human and mouse AQP4 sequences. However, the human and mouse transcripts are not predicted to be translated into full-length Mz AQP4 protein because of an

in-frame stop codon (TAA) at 60 bp downstream of the first ATG start codon in the human sequence, and an out-of-frame first ATG codon in the mouse sequence. Hydropathy and sequence analysis indicate that the 41-amino acids upstream of Met-1 in rat Mz are modestly polar (Fig. 1B).

To verify the sequence predictions, full-length human, rat and mouse AQP4 Mz sequences were cloned in mammalian expression plasmid pcDNA3.1, each with a strong Kozak sequence. We used the U87MG cell line because it is a human astrocyte-derived cell line showing efficient plasma membrane targeting of AQP4 isoforms, rapid growth and strong adherence to plastic or glass supports. Non-transfected U87MG cells do not express AQP4. Immunoblot analysis showed a band of molecular size of ~39 kDa for the rat AQP4 sequence, corresponding to Mz AQP4, but not for the human or mouse AQP4 sequences, where only bands at 32 and 34 kDa were seen, corresponding to M1 and M23 AQP4 isoforms (Fig. 1C). Only Mz AQP4 was expressed for the rat sequence because of the strong Kozak sequence in the construct.

SDS-PAGE and AQP4 immunoblot of homogenates of brain cortex showed strong bands corresponding to M23 AQP4, and weaker bands for M1 AQP4 (Fig. 2A). A faint band corresponding to Mz AQP4 was seen in rat but not human or mouse brain. These results support the predicted presence, based on sequence analysis, of Mz in rat but not in human or mouse brain. The relatively low Mz expression in rat brain is consistent with the unfavorable Kozak's sequence CACATGGTG (Moe et al., 2008).

BN/SDS-PAGE of brain cortex homogenates was done to assess AQP4 supramolecular assembly. The 2-dimensional immunoblot in Fig. 2B shows multiple pools of AQP4 (horizontal dimension), with the smaller AQP4 pools on the right side of the blot showing relatively high M1-to-M23 ratios compared to the larger pools on the left. A similar pattern of AQP4 pools and M1/M23 expression was seen in rat brain, but, in addition, a band corresponding to Mz was seen best in the large AQP4 pools containing relatively high amounts of protein. The presence of Mz in the large AQP4 pools suggests that it can assemble in OAPs together with M23 and M1 AQP4, as previously shown (Strand et al., 2009). The similar pattern of AQP4 pools in rat vs. human and mouse brain suggests that rat Mz has minimal influence on AQP4 supramolecular assembly in rat.

The possibility that Mz can form OAPs on its own was investigated by single particle tracking of quantum dot-labeled Mz. For these studies a Myc epitope was inserted into the second extracellular loop of Mz (Fig. 3A), as was done previously for M1 and M23, where the epitope insertion was shown not to affect AQP4 function, targeting or assembly (Crane et al., 2008). The diffusion of OAP-associated AQP4 is slow and confined, whereas individual AQP4 tetramers or small aggregates diffuse more rapidly and freely. For SPT analysis, epitope-tagged Mz, M1 and M23 AQP4 were transiently transfected (separately) in U87MG cells. Representative single particle trajectories (Fig. 3B) show that Mz and M1 diffused over μm -scale distances in 6 s, whereas M23 was nearly immobile, confined to nm-scale spots. Fig. 3C (top) shows cumulative probability distributions of particle ranges at 1 s, which include data from many cells and trajectories. While median M23 range was less than 40 nm, Mz and M1 diffused over 100 nm in 1 s. We conclude that like M1, Mz by itself does not assemble in OAPs.

To investigate whether rat Mz is able to co-assemble with M23 in OAPs, SPT was done on U87MG cells co-expressing similar quantities of Mz and M23 AQP4. Cumulative probability analysis in Fig. 3C (bottom) showed a distribution intermediate to those of Mz and M23 alone. A similar distribution was found in cells co-transfected with M1 and M23. These results indicate that Mz, like M1, is able to associate with M23 in OAPs, probably by

forming heterotetramers, as previously shown (Neely et al., 1999; Crane et al., 2009; Tajima et al., 2010). Mz thus resembles M1 in terms of its supramolecular assembly properties.

BN-PAGE of the transfected U87MG cells independently confirmed the conclusion from SPT that rat Mz does not form OAPs on its own, as evidenced by a single spot, as found as well for M1 (Fig. 3D, first two lanes). In contrast, multiple bands were seen for rat M23, which corresponds to OAPs of different sizes (third lane). Co-expression of M23 with Mz or M1 (M23:M1 and M23:Mz ratios 3:1) also produced multiple bands, but of reduced average size compared to M23 alone (fourth and fifth lanes), supporting the conclusion that Mz, like M1, is able to associate with M23 in OAPs, albeit of smaller size than OAPs formed by M23 alone (Furman et al., 2003; Silberstein et al., 2004).

The ability NMO AQP4 autoantibody (NMO-IgG) to recognize Mz AQP4 was evaluated, as Mz has a quite distinct N-terminus from M1 or M23 AQP4, and because several NMO models have been generated in rats (Bradl et al., 2009; Bizzoco et al., 2009; Kinoshita et al., 2009; 2010). To confirm the efficiency of Mz plasma membrane targeting, cells were transfected with Mz or M1 or M23 AQP4, each containing an extracellular Myc epitope. Immunostaining of live cells with anti-Myc antibody show comparable plasma membrane expression of each AQP4 isoform (Fig. 4A, top). By TIRFM (Fig. 4A, bottom), Mz and M1 AQP4 showed a smooth pattern of fluorescence, confirming the absence of OAPs, whereas M23 AQP4, shown for comparison, had a characteristic punctate pattern of fluorescence indicative of large OAPs. SDS-PAGE in Fig. 4B showed the predicted bands of sizes ~39 kDa for Mz AQP4 and ~34 kDa for M1 AQP4, with both bands seen in cells cotransfected with Mz and M1.

U87MG cells were stained with NMO-IgG using NMO patient sera. For quantitative comparisons, after NMO-IgG labeling cells were permeabilized and costained for AQP4, such that the red-to-green (R/G) fluorescence ratio provides a quantitative, normalized measure of NMO-IgG binding. Fig. 4C shows data from one NMO serum specimen. While AQP4 immunofluorescence was comparable in cells expressing Mz and M1 AQP4, alone or together, little NMO-IgG binding was found in the cells expressing Mz alone. Similar results were found with sera from other three different seropositive NMO patients. Fig. 4D (left) shows NMO-IgG binding curves in which the fluorescence ratio was measured as a function of serum concentration. Remarkably less NMO-IgG binding was found in cells expressing Mz alone, compared with M1 alone or M1 and Mz together. Fig. 4D (right) shows data for three other NMO sera obtained at fixed concentration (10% serum). In each case there was remarkably more binding to M1 than to Mz AQP4.

To investigate the possible involvement of N-terminal residues in the relatively poor binding of NMO-IgG to Mz AQP4, we generated three Mz truncation mutants with deletions of 12, 22 and 32 residues from the Mz methionine translation start, each containing an identical strong Kozak sequence (Fig. 5A). Fig. 5B shows strong NMO-IgG staining of U87MG cells expressing the $\Delta 22$ and $\Delta 32$ Mz mutants, as found for M1 AQP4, but little staining of the $\Delta 12$ mutant, as found for full-length Mz AQP4. These data suggest that the poor NMO-IgG binding to Mz AQP4 may involve the ten-amino acid sequence 'PNQTSARNLI' located between the truncation sites in the $\Delta 12$ and $\Delta 22$ mutants. SDS-PAGE confirmed the expected molecular sizes of the Mz truncation mutants (Fig. 5C). BN/PAGE analysis (Fig. 5D) shows that each of the Mz truncation mutants is unable to form OAPs, as observed by single spots, as seen for full-length Mz and M1 AQP4, in contrast to multiple bands seen for M23 AQP4.

Last, to further investigate interaction of NMO-IgG with Mz and M1 AQP4, an antibody-shift assay (Yang et al., 2002; Camacho-Carvajal et al., 2004) was done in which samples

were treated with purified NMO-IgG or recombinant monoclonal NMO-IgG (rAb-53) prior to BN-PAGE. Fig. 5E shows little effect of NMO-IgG on the gel pattern of Mz AQP4, but a marked shift with M1 AQP4 to higher apparent molecular sizes, supporting the conclusion of much greater interaction of NMO-IgG with M1 vs. Mz AQP4.

DISCUSSION

Holen and colleagues reported the existence of multiple isoforms in rat AQP4, including one that formed a functional water channel, Mz (Moe et al., 2008; Strand et al., 2009; Fenton et al., 2010). We found here that the long, Mz isoform of AQP4 is expressed in rat but not human or mouse brain. Mz AQP4 is thus unlikely to be of biological relevance in human biology, though we cannot rule out possible significance of translation of the 60-bp open reading frame in the human AQP4 sequence, producing a 19-amino acid putative peptide, NH₂-MVQNLSTPNTPTQ TQSDKWP-COOH. However, this short peptide is predicted to be translated with low efficiency because of its poor Kozak sequence, CACATGGTG, and is likely to be degraded rapidly.

We further conclude that Mz AQP4 is unlikely to be of significance in rat biology because of its very low expression at the protein level, and because it co-assembles with M1 and M23 AQP4 in tetramers that do not have distinguishing features from M1–M23 heterotetramers. Single particle tracking of quantum dot-labeled Mz AQP4 showed that Mz does not by itself form OAPs, but can co-assemble with M23 AQP4 within OAPs. Native gel electrophoresis provided independent support for this conclusion. Mz thus resembles M1 in its supramolecular association characteristics. Notwithstanding our negative conclusion regarding the biological significance of Mz AQP4, the unexpected finding that NMO-IgG binds poorly to Mz AQP4 compared to M1 AQP4 is quite interesting in terms of the AQP4 structural determinants of NMO-IgG binding.

NMO-IgG binds to structural epitopes on the extracellular surface of AQP4. An initial study concluded that NMO-IgG binds exclusively to OAP-forming M23 AQP4 (Nicchia et al., 2009). However, we (Crane et al., 2009; 2011) and others (Hinson et al., 2007; 2008; Meder et al., 2010) reported contradictory data, showing that M1 AQP4 is able to bind NMO-IgG. While both polyclonal NMO patient sera, as well as monoclonal NMO-IgGs, were shown to bind to both M1 and M23 AQP4, there was considerable heterogeneity in relative M1-to-M23 binding affinity (Crane et al., 2011). However, for all NMO-IgGs examined, binding to M23 AQP4 had higher affinity than binding to M1 AQP4. Here, we again show that NMO-IgG binds to M1 AQP4, but, surprisingly, binds much less well to Mz. There are several possible explanations for substantially greater binding of NMO-IgGs to M1 vs. Mz AQP4. The extra N-terminal residues on Mz compared with M1 AQP4 may: (a) alter the extracellular binding epitopes for NMO-IgG; (b) reduce the possibility of tetramer aggregation; or (c) impair NMO-IgG-induced AQP4 dimerization. We found by analysis of Mz truncation mutants that residues located between 12–22 in Mz AQP4 are involved in the relatively poor binding of NMO-IgG to Mz AQP4. Our prior data indicated that differences in binding affinity of NMO-IgG for AQP4 isoforms is due to differences in the AQP4 epitope, not to bivalent binding or AQP4 crosslinking by NMO-IgG (Crane et al., 2011). Therefore, we speculate that the poor binding of NMO-IgG to Mz is likely due to structural differences in the extracellular domain of the AQP4 tetramer. Perhaps the added bulk of the Mz N-terminus prevents the normal monomer/monomer packing that occurs in M1 or M23 tetramers.

In conclusion, we found small amounts of Mz AQP4 in rat but not human or mouse brain, consistent with sequence predictions. The supramolecular assembly properties of rat Mz AQP4 were similar to those of M1 AQP4. The interesting and unanticipated observation of

poor NMO-IgG binding to Mz AQP4 appears to be best explained by structural alterations in the Mz AQP4 tetramer produced by the additional residues on its N-terminus.

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Figure 1.

Sequence analysis of human, rat and mouse AQP4. A. Alignment of human, rat and mouse AQP4 sequences. Indicated in boxes are potential Mz, M1 and M23 translation initiation ATG sequences, and in-frame stop codons in the human (TAA) and mouse (TGA) sequences upstream of the M1 start codon. B. Kyte-Doolittle hydropathy plot of rat Mz AQP4 using a 5-residue running sum. Box at the bottom shows the amino acid sequence of Mz upstream from Met-1. C. AQP4 immunoblot of U87MG cell lysates following transfection with plasmids encoding rat, human and mouse AQP4 containing the Mz-initiation ATG.

Figure 2.

Expression of AQP4 isoforms and OAP composition in the brain cortex. A. SDS-PAGE and AQP4 immunoblot of rat, human and mouse brain cortex homogenates. B. Two-dimensional BN/SDS-PAGE and AQP4 immunoblot of rat (top), human (middle) and mouse (bottom) brain cortex homogenates. Molecular sizes are shown on the left, with decreasing apparent OAP size shown from left-to-right.

Figure 3.

OAP assembly and association by rat Mz AQP4. A. AQP4 schematic showing the positions of methionines corresponding to Mz, M1 and M23 in the cytoplasmic N-terminus, and the inserted Myc sequence (black) in the second extracellular loop. B. Representative single particle trajectories of AQP4 isoforms in U87MG cells transfected with Mz (left), M1 (middle) or M23 (right) AQP4. C. Cumulative probability distribution of ranges at 1 second [P(range)] of AQP4 isoforms in U87MG cells transfected with Mz, M1 or M23 alone (top), or in cells co-transfected with Mz+M23 (1:3) or M1+M23 (1:3) (bottom). P(range) for M23 and M1 AQP4 alone are shown for reference in the bottom panel. D. AQP4 immunoblot after BN-PAGE of lysates from U87MG cells transfected with Mz, M1 or M23 alone, or co-transfected with Mz+M23 (1:3) or M1+M23 (1:3) (left). BN-PAGE of lysates from cells co-transfected with Mz-myc+M23 (1:3) was probed with Myc and AQP4 antibodies (right).

Figure 4.

Binding of NMO-IgG to Mz AQP4. A. Immunofluorescence of U87MG cells transfected with Mz-myc, M1-myc and M23-myc and stained with anti-Myc antibody (top). Scale bar: 20 μ m. TIRF micrographs of Alexa488-labeled Mz, M1 or M23 AQP4 (bottom). Scale bar: 10 μ m. B. AQP4 immunoblot after SDS-PAGE of lysates from U87MG cells transfected with Mz alone, Mz+M1 (1:1) or M1 alone. C. Representative immunofluorescence of U87MG cells expressing Mz (top), M1 (middle) or equimolar Mz+M1 (bottom) and stained with 10% NMO patient serum (red), and with reference AQP4 antibody (green). Scale bar: 20 μ m. D. (left) Measured red-to-green fluorescence ratios (R/G) following immunostaining as shown in panel C for different NMO serum concentrations (mean $\pm$ S.E., n=4). U87MG cells were transfected with rat Mz (blue), M1 (red) or equimolar Mz+M1 (black). Curves

represent fits to a single-site binding model. D. (right) R/G for three other NMO sera, each at 10% concentration (mean $\pm$ S.E., n=4).

Figure 5.

Sequence dependence of poor NMO-IgG binding to Mz AQP4. **A**. N-terminal amino acid sequence of rat AQP4, with black arrows indicating sites of N-terminal truncations. **B**. Representative immunofluorescence from U87MG cells expressing AQP4 mutants Mz Δ12, Mz Δ22 and Mz Δ32. Cells were stained with 10% NMO patient serum (red) and reference AQP4 antibody (green). Scale bar: 20 μm. **C**. AQP4 immunoblot of lysates from U87MG cells transfected with Mz AQP4, and N-terminal deletion mutants Mz Δ12, Mz Δ22 and Mz Δ32. **D**. AQP4 immunoblot after BN-PAGE of lysates from U87MG cells transfected with full-length M23, M1 and Mz AQP4, and N-terminal Mz deletion mutants. **E**. Antibody-shift

assay showing AQP4 immunoblot of U87MG cells transfected with Mz or M1 and incubated with purified NMO-IgG before BN-PAGE.