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New isoforms of rat Aquaporin-4

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Abstract

Aquaporin-4 (AQP4) is a brain aquaporin implicated in the pathophysiology of numerous clinical conditions including brain edema. Here we show that rat AQP4 has six cDNA isoforms, formed by alternative splicing. These are named AQP4a–f, where AQP4a and AQP4c correspond to the two classical M1 and M23 isoforms, respectively. The various isoforms are differentially expressed in kidney and brain, and their prevalence does not correspond to the level of the respective mRNAs, pointing to posttranscriptional regulation. The three isoforms lacking exon 2, AQP4b, AQP4d, and AQP4f, have an intracellular localization when expressed in cell lines and do not transport water when expressed in *Xenopus* oocytes. In contrast, the largest of the new isoforms, AQP4e, which contains a novel N-terminal domain, is localized at the plasma membrane in cell lines and functions as a water transporter in *Xenopus* oocytes.

Keywords: AQP4; Aquaporin; Alternative splicing; Isoforms

Aquaporin-4 (AQP4), originally isolated from rat fetal lung and rat brain [1,2], is the aquaporin most strongly expressed in brain. It is localized mainly in astrocytic endfeet around blood vessels and bordering the subarachnoidal space, in ependymal cells lining the ventricles, and in glial lamellae in the supraoptic nucleus and other circumventricular organs [3]. This pattern of expression suggests that AQP4 is involved in transport of water at the brain/blood and brain/cerebrospinal fluid interfaces and indicates a role in osmotransduction [3,4]. In agreement, studies of AQP4-/- mice and mice with a disruption of AQP4 anchoring (mdx and alpha-syntrophin-/- mice) have implicated AQP4 in a long list of physiological processes [5,6]. The most striking effect of AQP4 knock-out is reduced brain edema after acute water intoxication and ischemic stroke [7].

AQP4 has two known isoforms, M1 and M23 [2], which differ in their ability to form square arrays in cell culture [8] and in their ability to form 2D-crystals when reconstituted in lipid bilayers [9]. The water transport capacity of the two isoforms has been found to be similar in *Xenopus* oocytes [10] but in epithelial cells the M23 variant has been reported to have increased single-channel osmotic water permeability by one order of magnitude [11].

The aim of this study was to provide a thorough mapping of the rat AQP4 gene. By rapid amplification of cDNA ends (RACE) analysis we find six cDNA isoforms: here named AQP4a, AQP4b, AQP4c, AQP4d, AQP4e, and AQP4f. AQP4a and AQP4c correspond to the two classic isoforms, M1 and M23. We find no evidence for M23 having a transcription start in intron 1. By designing TaqMan assays against the new exon–exon boundaries, we assessed the abundance of the new isoforms in different tissues and demonstrated a mismatch on the mRNA and protein levels, a mismatch that indicates significant posttranscriptional regulation of AQP4 gene expression.

To investigate protein localization, we expressed the new isoforms in cell lines and found that three of the new isoforms were intracellular, whereas AQP4e, the largest new isoform, was transported to the cell membrane. In *Xenopus* oocytes we demonstrate that AQP4e transports water, whereas the intracellular

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isoforms do not. Since the rat has been the main animal model for water transport studies of the brain, the newfound complexity of the rat AQP4 gene must be taken into account in further studies of the physiological and pathophysiological roles of AQP4.

Results

We have recharacterized the rat AQP4 gene and found six cDNA isoforms instead of the two classical isoforms M1 and M23. The increased complexity necessitates a simple nomenclature; thus we have named the isoforms AQP4a, AQP4b, AQP4c, AQP4d, AQP4e, and AQP4f, where AQP4a corresponds to the classical M1 isoform and AQP4c corresponds to M23. Fig. 1A. summarizes our results, with the novel exons, mRNAs, and predicted proteins and protein sizes indicated.

Our new model implies three transcription starts, at the 5' end of exon Z, exon 0, and exon X. Exon Z has a new open reading frame coding for the new, large AQP4e isoform with a novel N-terminal domain (Fig. 1B). The alternative splicing of exon 2, from the basic forms AQP4a, AQP4c, and AQP4e, produces AQP4b, AQP4d, and AQP4f (Fig. 1B), resulting in a total of six cDNA isoforms in the new model.

Our data (see below) are consistent with the initial characterization of AQP4 by Verkman and co-workers [1] and with a later revision [2]. The exception is that we find a T-to-C polymorphism in exon 1 (Fig. 1B, purple C, DNA position 468). This polymorphism, not previously described, occurs in several clones from three different tissues, indicating a genome polymorphism, but does not cause an amino acid substitution in the AQP4 protein.

No PCR evidence for classical M23 transcription start in intron 1

The classical structure of the AQP4 locus involved two transcription starts [1,2,17]. The larger isoform is transcribed from exon 0 (Fig. 2A, open arrow), whereas the shorter M23 isoform is supposed to have a transcription start in intron 1 (Fig. 2A, closed arrow).

This model was supported by an analysis of the 5' end of the M23 mRNA in the rat [18]. Furthermore, a cDNA clone released from Amgen also contained sequences upstream of exon 1 (Accession No. CB614668). In mouse, a similar model was proposed by Zelenin et al. [19], although the confounding effect of genomic DNA contamination could not be excluded.

A careful bioinformatic analysis revealed several inconsistencies in the sequence databases in regard to the 5' end of the rat AQP4 mRNA (data not shown, and Supplementary Fig. 1). To test the model, we synthesized a series of PCR primers located just upstream of exon 1 (Fig. 2B, red primers), but no sign of exon 1–extended transcripts were found by PCR (Fig. 2C). We synthesized a second series of primers (Fig. 2B, light blue primers) and still found no sign of upstream extension of exon 1 (Fig. 2C, lanes 7–12).

On the other hand, successful amplification of the M1 transcript was easily obtained by designing a single primer designed against exon 0 (Fig. 2C, lane 13). Also a control primer in exon 1 was successfully designed and tested (Fig. 2C lanes 5 and 6). Thus our PCR experiments did not support the hypothesis that M23 is coded from an extension of exon 1.

RACE characterization of the 5'end of rat AQP4 mRNA finds new exons

In their revision of the rat AQP4 characterization, Agre and co-workers [2] performed a limited RACE screen, finding exon 0 and noting that there could be additional exons. To map *de novo* the 5' end of the rat AQP4 mRNA, we used a PCR – based 5' RACE technique combined with Topo-cloning. We aimed to isolate multiple transcript clones from multiple tissues, to test the classical model of the AQP4 locus, to identify possible new exons, and to obtain a semiquantitative measure of the ratio of M1 and M23 transcripts in the rat.

A pair of RACE primers was designed in exon 2. Of 38 unique AQP4 clones isolated from kidney, hippocampus, and cerebellum RNA, none demonstrated an extension of exon 1 (Supplementary Fig. 2; cDNA sequences submitted to GenBank). This again failed to support the idea that the classical M23 isoform is encoded by a transcription start in intron 1.

However, we did find evidence of two new exons. The RACE transcripts fell into three distinct groups (Supplementary Fig. 2). One group of transcripts aligned perfectly with exon 2 and exon 1 and then skipped to a region of the genome between the previously mapped exon 1 and exon 0. We have named this new region exon X, because this exon has sequence similarity to exon X found by Zelenin and co-workers [19] in mouse. We consider exon X a strong candidate for coding the 5' UTR of the AQP4c mRNA isoform (corresponding to the classical isoform M23).

The majority of transcripts aligned with exon 2 and exon 1 and then skipped to the classical exon 0 (Supplementary Fig. 2, middle group). Here the transcripts aligned perfectly with the reference sequence of the M1 form of AQP4 (NM_012825). However, exon 0 was extended in almost all transcripts another 19 to 20 nucleotides. In this extension, 9 nucleotides were known from a previous study. [2]. The revised exon 0 is indicated in blue in Fig. 1B. In total, 21 independent clones of ~600 bp sequence came to an end within 1 or 2 nucleotides, thus constituting a probable transcription start point of AQP4a (the classical isoform M1).

The preponderance of exon 0 clones over exon X clones should imply a stronger expression of the larger, classical M1 protein isoform. However, on immunoblots, the shorter classical M23 isoform is the predominant isoform [10]. This discrepancy may indicate significant regulation at the level of translation. Another possible explanation is that the M1 and the M23 protein isoforms differ in regard to their stability.

A third group of RACE transcripts progressed even further upstream from the extended exon 0 (Supplementary Fig. 2, lower group). The longest clone isolated, R90_185 (GenBank Accession No EV243422), stretched 185 bp upstream of the revised exon 0 and contained a new start codon (Fig. 1B). This start codon was in reading frame with the rest of AQP4, making this a candidate for encoding the faint, minor AQP4 protein isoforms seen on immunoblots [10]. We named the longer exon 0-extension exon Z and named the new putative protein isoform