AQP0-LTR of the Cat\(^{Fr}\) mouse alters water permeability and calcium regulation of wild type AQP0

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Received 13 October 2005; received in revised form 6 December 2005; accepted 9 January 2006

Available online 13 February 2006

Abstract

Aquaporin 0 (AQP0) is the major intrinsic protein of the lens and its water permeability can be modulated by changes in pH and Ca\(^{2+}\). The Cataract Fraser (Cat\(^{Fr}\)) mouse accumulates an aberrant AQP0 (AQP0-LTR) in sub-cellular compartments resulting in a congenital cataract. We investigated the interference of AQP0-LTR with normal function of AQP0 in three systems. First, we created a transgenic mouse expressing AQP0 and AQP0-LTR in the lens. Expression of AQP0 did not prevent the congenital cataract but improved the size and transparency of the lens. Second, we measured water permeability of AQP0 co-expressed with AQP0-LTR in *Xenopus* oocytes. A low expression level of AQP0-LTR decreased the water permeability of AQP0, and a high expression level eliminated its calcium regulation. Third, we studied trafficking of AQP0 and AQP0-LTR in transfected lens epithelial cells. At low expression level, AQP0-LTR migrated with AQP0 toward the cell membrane, but at high expression level, it accumulated in sub-cellular compartments. The deleterious effect of AQP0-LTR on lens development may be explained by lowering water permeability and abolishing calcium regulation of AQP0. This study provides the first evidence that calcium regulation of AQP0 water permeability may be crucial for maintaining normal lens homeostasis and development.

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Keywords: Aquaporin; Cataract; LTR; *Xenopus* oocyte; Transgenic mouse; Lens epithelial cell

1. Introduction

Aquaporin 0 (AQP0), also known as MIP26, is the founding member of the aquaporin superfamily of proteins that facilitate the bi-directional transport of water and small neutral solutes, such as glycerol and urea, across the plasma membrane of microbial, plant and animal cells [1]. Aquaporins assemble as tetramers in which the monomer serves as the water pore [2–4]. AQP0 is expressed primarily in the lens and in trace amounts in liver and testis [5–7]. In the lens, AQP0 is expressed exclusively in the fiber cells where it comprises more than 60% of the total membrane protein content. AQP0 water permeability is regulated by pH and calcium in both *Xenopus* oocytes [8,9] and lens vesicles [10].

Genetic mutations, metabolic diseases and aging can lead to cataract, the principal cause of blindness in the world. In humans congenital cataracts have been mapped to at least nine loci including AQP0. Mouse models of cataractogenesis have proven to be valuable tools in the study of the role of AQP0 in lens development and physiology under both normal and pathological conditions [11,12].

AQP0 is essential for developing a normal lens structure and maintaining lens transparency [13,14]. Lenses of AQP0 knockout mice (AQP0 \(^{-/-}\)) present a congenital cataract, fail to develop normal structure, and lose 80% of their water permeability [15,16]. The Cat\(^{Fr}\) mouse (Cat Fr/Fr) synthesizes a mutated AQP0 (AQP0-LTR), which is the result of the insertion of an Etn element into AQP0 gene. Etn elements have been shown to disrupt several mouse genes but the CatFr mutant appears to be the only case where an LTR sequence has been translated into a peptide [17–19]. This peptide sequence shows no similarity to AQP0, and AQP0-LTR is retained in the sub-cellular compartments [20]. The lack of functional AQP0 in the plasma membrane produces a congenital cataract with aberrant lens structure [15,20]. The Cat\(^{Fr}\) lens loses 70% of its water permeability...
permeability, as (AQP0 −/−) lenses do [21]. Interestingly, Cat Fr/+ heterozygous mice present an earlier onset of cataract than the AQP0 +/+ heterozygous mice, at 6 weeks and 24 weeks of age, respectively. The more severe phenotype presented by the Cat Fr/+ could be explained by a deleterious role of the defective protein AQP0-LTR and/or an interaction with AQP0.

In this paper, we investigate the effect of AQP0-LTR on AQP0 when expressed together in three different systems. We studied lens morphology in a transgenic mouse (Tg-AQP0), the effect of AQP0-LTR on AQP0 water permeability in Xenopus oocytes, and trafficking in co-transfected human lens epithelial cells.

2. Materials and methods

2.1. Generation of a transgenic mouse expressing AQP0 and AQP0-LTR

Transgenic CatFr mice expressing AQP0 were produced at the UCI Transgenic Facility using standard methods of pronuclear microinjection for random insertion [22]. Briefly, blastocyst embryos from CatFr mice were injected with bovine AQP0 cDNA under the lens-specific mouse α-crystallin promoter [23]. We used bovine AQP0 that has 95% amino acid sequence identity with mouse AQP0. Furthermore, it has been shown that mouse AQP0 exhibits similar sensitivity to pH and calcium as bovine AQP0 [10]. Fig. 1A shows a schematic diagram of the transgene we constructed (PaA-Cryst-AQP0). The BglII–BamHI fragment of the lens specific mouse α-crystallin promoter (PaA-Cryst) (a gift from Joram Piatigorsky, NEI, Bethesda) was inserted into the BglII site of pRES-EGFP-C1 expression vector (Clontech). The bovine AQP0 (AQP0) cDNA was inserted into the vector as a XhoI–BamHI fragment. Transgenic animals were identified by PCR analysis of tail DNA. Seven-day- or 9-month-old animals were sacrificed, the eyes were enucleated and the lenses isolated for Western blot, dark-field photography, or histology.

All mice were cared for in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animals. We used BALB/c mice as the control to provide normal expression and localization of AQP0 in the cortical cell membranes from Tg-AQP0 lenses (Fig. 1C).

2.2. Western blots of lens membranes

Lens membrane proteins were prepared as described [24] and solubilized in 10% SDS. Membrane proteins were analyzed by SDS-PAGE gel electrophoresis and Western blotting. AQP0 immunodetection was performed using a rabbit polyclonal antibody raised against the peptide PPRKSVSLRSILKGSRPSE (C-terminus amino acids 224–245 of bovine AQP0) (GeneMed Synthesis Inc). The double band indicates strong proteolytic activity in the lens. Antibody staining shows normal expression and localization of AQP0 in the cortical cell membranes from Tg-AQP0 lenses (Fig. 1C).

2.3. Immunohistochemistry on lens sections

Paraffin tissue sections were prepared by the UCI Histology Lab. The tissue sections were incubated with the rabbit anti AQP0 antibody described above. The slides were incubated with Alexa-Fluor-488 labeled goat anti-rabbit IgG ( Molecular Probes) for visualization with a confocal microscope (MRC-1024 BioRad).

2.4. Lens histology, dark field photography, weight and diameter

Lens sections were stained with hematoxylin and eosin (H&E). Isolated lenses were illuminated with a focused light beam in a darkened room for photography (Nikon Diaphot 300 microscope). Lens weight was measured on an analytical balance (Mettler, A6245). Lens diameter was measured under a dissecting microscope.

2.5. AQP0-LTR construct and cRNA preparation for Xenopus oocytes

The expression construct for bovine AQP0 was a gift from Peter Agre (Duke University) [25]. The LTR sequence (I.M.A.G.E. 3168575 ) was amplified by PCR and inserted at position 203 in AQP0 protein to re-create the CatFr mutation [13]. cRNAs were transcribed in vitro using T3 RNA polymerase (mMESSAGE mMACHINE kit; Ambion).

2.6. Xenopus oocyte swelling assay

Female Xenopus laevis were anesthetized, and stage V and VI oocytes were injected as previously described [8]. Two days post-injection, swelling assays were performed at room temperature (20–21 °C) by transferring oocytes from 200 mosM (100% ND96) to 70 mosM (30% ND96) solution. Before the swelling assay was performed in hypotonic solutions of ND96 (30%) with the appropriate Ca2+ concentration and pH, the oocytes were incubated in 100% ND96 with the desired test Ca2+ concentration and pH for 5 min.

2.7. Expression of AQP0 and AQP0-LTR in transfected lens epithelial cells

Human lens epithelial cells (ATCC: CRL-11421) were cultured in complete EMEM medium. AQP0 and AQP0-LTR cDNAs were inserted into the pEGFP-C1 vector XhoI–BamHI sites (Clontech) creating GFP-AQP0 and GFP-AQP0-LTR expression constructs. Unlabeled AQP0 construct was made by deleting GFP from the pEGFP-C1 vector. Cells were transfected with 0.25 to 1 μg of DNA using the FuGENE 6 Transfection Reagent (Roche). After 2 days, the transfected cells were imaged using a confocal microscope (Zeiss LSM 510 Meta confocal system). All products for cell culture were purchased from GIBCO BRL.

3. Results

3.1. Generation of a transgenic mouse expressing AQP0 and AQP0-LTR in the lens

To study the effect of co-expressed AQP0 and AQP0-LTR on lens morphology, we produced a transgenic mouse (Tg-AQP0) by inserting AQP0 into the CatFr mouse genome that already expresses AQP0-LTR. AQP0 was expressed under the lens-specific αA-crystallin promoter [23] (Fig. 1A). We showed by Western blot (Fig. 1B) that AQP0 was expressed in the transgenic lens membranes (lane C). The double band indicates strong proteolytic activity in the lens as previously reported in cataractous lenses [26]. The AQP0 antibody recognizes the C-terminus of AQP0 (lane A), and there is no cross reactivity with the mutant AQP0-LTR (lane B). Antibody staining shows normal expression and localization of AQP0 in the cortical cell membranes from Tg-AQP0 lenses (Fig. 1C).

3.2. Characterization of the transgenic lens

Fig. 2A shows representative dark-field photographs of CatFr and Tg-AQP0 lenses at 7 days and 9 months of age. Tg-AQP0 lens presented a central congenital cataract similar to that of CatFr lens, but there was a clear region in the lens
periphery even at 9 months of age, suggesting the continuous growth and maintenance of fiber cell structure in the cortex of the lens. Also, the cataract in Tg-AQP0 showed a less condensed opacity. Tg-AQP0 lenses retained normal lens shape while CatFr lenses were wrinkled with age. This structural improvement was paralleled by an increase in the weight (146%) and diameter (120%) of transgenic lenses compared to CatFr lenses (Fig. 2B). We examined the lenses from 7-day-old mice (Fig. 3). In the control lens, we observed an essential structure, the bow region, formed by the nuclei of epithelial cells differentiating into fiber cells arranged in concentric layers (Fig. 3A). In the cortex region of Tg-AQP0 lens, we observed fiber cell differentiation, elongation and stratification similar to the control lens. However, amorphous cells replaced the mature fiber cells in the nucleus of the lens (Fig. 3B). Although the bow region was present in CatFr (Fig. 3C), lens fiber cell did not elongate and stratify as observed in Tg-AQP0 lens. In summary, despite the presence of AQP0-LTR, AQP0 promoted fiber-cell growth and stratification in the cortex that resulted in a clear periphery and increased lens

Fig. 2. Comparison of control, Tg-AQP0 and CatFr lenses at 7 days and 9 months of age. (A) Representative dark-field photographs of CatFr and transgenic lenses. (B) Weights and diameters of control, Tg-AQP0, and CatFr lenses. Tg-AQP0 lenses maintained a clearer periphery than CatFr lenses at both 7 days and 9 months of age. The weights and diameters of Tg-AQP0 lenses were intermediate between those of control and CatFr lenses at both 7 days and 9 months of age.
size. Nevertheless, Tg-AQP0 failed to complete lens organization, and this failure could be attributed to the presence of AQP0-LTR interfering with AQP0. Consequently we examined the interaction between AQP0 and AQP0-LTR in two in vitro expression system: *Xenopus* oocytes and cultured human lens epithelial cells.

### 3.3. Expression of AQP0 and AQP0-LTR in *Xenopus* oocytes

When expressed in *Xenopus* oocytes, AQP0 induces a water permeability ($P_f$) which can be increased by removing external calcium or acidifying the external medium [8,9]. We co-injected 10 ng of AQP0 cRNA with different amounts of AQP0-LTR cRNA in *Xenopus* oocytes and measured the resulting $P_f$. Oocytes injected with 10 ng of AQP0 alone were used as control (Fig. 4). Injection of AQP0-LTR alone had no effect on the $P_f$ of *Xenopus* oocytes (data not shown). When 10 ng of AQP0 and 2.5 ng of AQP0-LTR were injected into oocytes, $P_f$ was reduced to half compared with control oocytes (13.3 ± 1.6 μm/s and 28.95 ± 1.3 μm/s, respectively). Regulation of $P_f$ by Ca$^{2+}$ remained intact. However, when more than 2.5 ng of AQP0-LTR and 10 or 20 ng of AQP0 were injected into oocytes, $P_f$ was similar to that of control oocytes, but Ca$^{2+}$ regulation of $P_f$ was abolished. Interestingly, pH regulation of AQP0 water permeability was never affected by the presence of AQP0-LTR (data not shown). These data support our previous conclusion that pH and calcium regulation are separable and show two effects of AQP0-LTR on AQP0 function: reduction of $P_f$ (when AQP0-LTR was in low quantity) and elimination of calcium sensitivity (when AQP0-LTR was in high quantity).

### 3.4. Co-expression of AQP0 and AQP0-LTR in lens epithelial cells

To further examine the localization of the interaction, we expressed unlabeled AQP0 and fluorescent AQP0-LTR in transfected human lens epithelial cells. We confirmed the finding of Shiels et al. [20] that AQP0-LTR was not present in the plasma membrane when cells were transfected with GFP-AQP0-LTR alone (0.25 μg and 1 μg DNA) (Fig. 5A and B). As a control, we transfected epithelial cells with GFP-AQP0 (1 μg), and observed fluorescence in the plasma membrane as well as the membranes of other organelles (Fig. 5C). When cells were co-transfected with 0.25 μg of GFP-AQP0-LTR and 1 μg of unlabeled-AQP0, we observed fluorescence at the plasma membrane indicating that GFP-AQP0-LTR is transported to the membrane in presence of AQP0 (Fig. 5D). When cells were co-transfected with 1 μg of GFP-AQP0-LTR and 1 μg of unlabeled-AQP0, we observed such a bright cellular fluorescence that the presence of plasma membrane fluorescence can be neither confirmed or ruled out (Fig. 5E).
In summary, AQP0-LTR was seen at the plasma membrane when it was in low quantity and AQP0 was present.

4. Discussion

Our study shows that AQP0-LTR interferes with the normal function of AQP0 and that this interaction aggravates cataract in CatFr heterozygotes and Tg-AQP0 mice. We demonstrate effects of co-expression of AQP0-LTR and wild-type AQP0 at three levels: in water permeability measurements using the *Xenopus* oocyte system; in transfected lens epithelial cells; and in transgenic mice. The results are mutually supportive of the hypothesis that AQP0-LTR exerts its deleterious effect by altering the function of wild type AQP0.

The interaction that produces distinct effects on function of AQP0 depends upon the quantity of AQP0-LTR. First, at low level of AQP0-LTR, AQP0-LTR localizes in the plasma membrane (in cell culture) and decreases AQP0 water permeability (in *Xenopus* oocytes). The reduction of water permeability at a low level of AQP0-LTR can be explained either by a reduced quantity of AQP0 in the plasma membrane or by formation of non-functional heterotetramers. In AQP0-LTR, the last transmembrane domain and the C-terminus are replaced by the LTR sequence, and therefore AQP0-LTR cannot form a functional channel. Thus, if AQP0 and AQP0-LTR were able to form heterotetramers, their structure would be abnormal and might alter the pore structure of AQP0 monomers or their subcellular localization; both effects would result in an overall decrease of water permeability. As a result, the lens internal circulation necessary to maintain homeostasis would be diminished.

Second, at high level of AQP0-LTR, AQP0-LTR accumulates in the subcellular compartments (in cell culture), and the calcium regulation of water permeability is abolished (in *Xenopus* oocytes). It is possible that AQP0-LTR has a strong affinity for itself and that above a critical concentration it homooligomerizes, excluding AQP0 and abolishing the trafficking of AQP0-LTR to the plasma membrane. At this stage we do not know if the altered Ca$^{2+}$ regulation is specific to AQP0 or extends to other proteins and Ca$^{2+}$-dependent mechanisms. Interestingly, the presence of AQP0-LTR mimics the effect of crippled calmodulin in water permeability assays performed in
Xenopus oocytes [9]. Therefore, the loss of Ca2+ sensitivity might be attributed to an action on a common cytoplasmic intermediate, for example, calmodulin. In the lens, calmodulin has been reported to regulate the activity of ion channels [27] and gap junction permeability [28]. We also noticed that the LTR peptide presents a potential CaM kinase II binding site, as does the AQP0 C-terminus (http://www.cbs.dtu.dk/services/netphosK). We can postulate that AQP0-LTR disrupts calcium regulation of AQP0 water permeability by competing with the AQP0 C-terminus for CaM kinase II. If AQP0-LTR binds to the location of the calcium stores in lens fiber cells and other cell types [30]. This study provides the first evidence that calcium regulation of AQP0 water permeability may be crucial for maintaining normal lens homeostasis and development and suggests a novel explanation for the gain of function of the AQP0-LTR mutation by interference with the water permeability of AQP0.

Acknowledgements

We thank Mary Hawley for expert technical assistance including oocyte preparation and mouse maintenance. We gratefully acknowledge many helpful discussions with Professor Michael D. Cahalan. We also thank the Transgenic Facility and the Histology Lab of UC Irvine for their essential assistance. This work was supported by EY5661 from the National Institutes of Health to J.E.H.

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