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An *MIP*/AQP0 mutation with impaired trafficking and function underlies an autosomal dominant congenital lamellar cataract

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Abstract

Autosomal dominant congenital cataracts have been associated with mutations of genes encoding several soluble and membrane proteins. By candidate gene screening, we identified a novel mutation in *MIP* (c.494 G>A) that segregates with a congenital lamellar cataract within a South Indian family and causes the replacement of a highly conserved glycine by aspartate (G165D) within aquaporin0 (AQP0). Unlike wild type AQP0, expression of AQP0-G165D in *Xenopus* oocytes did not facilitate swelling in hypotonic medium. In transfected HeLa cells, wild type AQP0 localized at the plasma membrane while AQP0-G165D was retained within the secretory pathway, and localized mainly within the endoplasmic reticulum. These results suggest that mutation of this conserved glycine residue leads to improper trafficking of AQP0-G165D and loss of water channel function. They emphasize the importance of AQP0 for maintenance of lens transparency and identify a critical residue that is conserved among aquaporins, but has not previously been associated with disease-associated replacement.

Keywords

Aquaporin0; cataract; lens; *MIP*

1. Introduction

Childhood blindness affects approximately 1.5 million children worldwide among whom 90% are from developing countries (Apple et al., 2000). Congenital cataracts (visible at birth or during the first year of life) are the leading cause of irreversible blindness in childhood. Most familial, non-syndromic congenital cataracts are inherited as autosomal dominant traits with complete penetrance. They have been mapped to many different loci (≥39). Mutations have been identified in genes encoding crystallins (including *CRYAA, CRYBB2, CRYGC,*

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CRYGD), other soluble proteins (such as the transcription factors *HSF4, PITX3*, and *BFSP2*), and membrane proteins (including *GJA3, GJA8*, and *MIP*) (Hejtmancik, 2008).

While conducting a prospective study of cases of childhood cataract at the Regional Institute of Ophthalmology, Chennai, India, we identified a family with autosomal dominant congenital cataracts of similar phenotype. This manuscript describes the association of this cataract with a novel mutation of *MIP* (which encodes the water channel, AQP0) and its cellular and physiological characterization.

2. Materials and methods

2.1. Patient ascertainment and collection of genetic material

A prospective study of cases of childhood cataract registered at the Regional Insitute of Ophthalmology, Egmore, Chennai, India was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Ethical Committee of Dr. ALM - Post Graduate Institute of Basic Medical Sciences, University of Madras, Chennai, South India. Clinical and ophthalmological examinations were performed by a senior ophthalmologist. Case histories were recorded using a questionnaire designed for the study. Blood samples (5–10 ml) were collected from available affected and unaffected family members.

2.2. Genomic DNA isolation, sequencing, and restriction fragment analysis

Genomic DNA was isolated from peripheral blood as previously described (Santhiya et al., 2010). PCR was employed to amplify all exons of *CRYAA, CRYBB2, CRYGC, CRYGD, GJA3, GJA8, PITX3* and *MIP* from the genomic DNA of the proband (Gu et al., 2007;Santhiya et al., 2010). PCR conditions for exons of the *MIP* gene were: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 63°C – 65°C for 45 sec, and extension at 72°C for 45 sec, and a final extension at 72°C for 7 min in a PTC 200 DNA Engine (BioRad, USA). PCR products were separated on 2% agarose gels. Amplicons were purified and subsequently sent to be sequenced at 1st Base Pvt. Ltd. (Singapore).

To analyze the *MIP* gene for the c.494 G>A substitution which generates a HincII restriction site, 10 μl of PCR products were digested with HincII (New England Biolabs, Ipswich, MA) at 37°C for 1 hr as per the manufacturer's guidelines and separated on 2% agarose gels.

2.3. Generation of AQP0 constructs

DNA encoding AQP0-G165D was obtained by polymerase chain reaction using oligonucleotide primers encoding the nucleotide substitution, Phusion High-Fidelity DNA polymerase (New England Biolabs) and a plasmid template containing wild type human AQP0 with GFP appended in frame to its C-terminus in pcDNA3.1/*myc*-His (a kind gift from Dr. R. Varadaraj, State University of New York, Stony Brook, NY, USA) (Varadaraj et al., 2008) according to the strategy we have used previously (Minogue et al., 2005). Then, the coding regions of wild type and mutant AQP0 (without the C-terminal GFP) were amplified by PCR and subcloned into the eukaryotic expression vector, pcDNA3.1 (Invitrogen, Carlsbad, CA), and into pBSSK-XG which contains 5'- and 3'-flanking sequences from the *Xenopus* β-globin gene to facilitate expression in oocytes (Satin et al., 1992).

2.4. Xenopus Oocyte Expression

cRNA transcripts and *Xenopus laevis* oocytes were prepared as previously described (Puljung et al., 2004;Kyle et al., 2008). The DNA plasmids were linearized with SpeI, and

the cDNAs transcribed with T7 RNA polymerase using the T7 mMessage mMachine kit (Applied Biosystems/Ambion, Austin, TX). Adult female *Xenopus laevis* were anesthetized with 0.2% tricaine methanesulfonate. Ovaries were isolated, placed in 0 Ca^{2+} OR2 (90 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.6) and treated with 3 mg/ml collagenase (Worthington Biochemical Corporation, Lakewood, NJ) for 1 hr. Oocytes were manually defolliculated and placed in normal OR2 supplemented with sodium pyruvate, glucose and antibiotics (90 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.27 g/L sodium pyruvate, 2 mg/L gentamicin, 5 mM glucose and 5 mM HEPES, pH 7.6). For swelling experiments, oocytes were injected with 50 nl of water or 100 ng of cRNA in a total volume of 50 nl.

To assess expression of the AQP0 constructs by immunoblot or epifluorescence, oocytes were injected with 25 ng of cRNA. Immunoblot detection of expressed AQP0 or G165D was performed by a modification of the procedure described by Mulders et al. (1995). Four oocytes were homogenized in 500 μl of 5 mM Tris pH 8.0 containing 1 mM EDTA, 1 mM EGTA, 2 mM PMSF and a cocktail of proteases inhibitors (cOmplete Mini tablet; Roche Applied Science, Indianapolis, IN) by 10 passages through a 20 gauge needle followed by 10 passages through a 27 gauge needle. Then, the homogenates were centrifuged at 700 g for 5 minutes to remove the egg yolk. An aliquot of the resulting supernatant (or cleared homogenate) was then centrifuged at 100,000 g for 1 hour. The resulting membrane-enriched pellet was resuspended in 30 μl of the homogenization buffer. Equal volumes of cleared homogenates and all of the resuspended membrane-enriched pellets were loaded in individual gel lanes. The samples were resolved on 12% SDS-containing polyacrylamide gels, transferred to Immobilon-P membranes and immunoblotted with rabbit polyclonal anti-AQP0 antibodies (Alpha Diagnostic International, San Antonio, TX). In some experiments, AQP0 protein expression in oocytes was confirmed by visualization of GFP-tagged constructs by epifluorescence microscopy using an Olympus stereomicroscope (Olympus America Inc., Center Valley, PA) equipped with a Zeiss Axiocam digital camera (Carl Zeiss, Munich, Germany).

2.5. Water channel (swelling) assays

Water channel activity was assessed by a modification of established swelling assays (Mulders et al., 1995;Németh-Cahalan and Hall, 2000). *Xenopus* oocytes microinjected with cRNAs encoding wild type or mutant AQP0 (without a GFP tag) were placed in hypotonic medium three days after microinjection. Water-injected oocytes were used as negative controls. Because of the increased permeability of AQP0 channels in low Ca^{2+} and at pH 6.5 (Németh-Cahalan and Hall, 2000;Németh-Cahalan et al., 2004), Ca^{2+} was omitted from normal OR2 and the pH was adjusted to 6.5. Oocytes were pre-incubated in 100% OR2, containing 0 Ca^{2+} (~200 mOsM; 90 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 6.5) for more than 5 min at room temperature. Then, oocytes were transferred to 10% 0 Ca^{2+} OR2 (~20 mOsM; 9 mM NaCl, 0.25 mM KCl, 0.1 mM $MgCl_2$, and 0.5 mM HEPES, pH 6.5). Oocyte swelling was monitored using an Olympus IX81 TIRF inverted microscope equipped with a 2X objective (n.a. 0.06) and a Retiga camera. Digital images were recorded every 3s for 3.5 min. Oocyte images were binarized using Otsu threshold, processed to remove any background objects and measured by a macro script in ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, http://imagej.nih.gov/ij/, 1997–2012). The rates of change in oocyte area (pixels vs. time) were analyzed using Origin 8 software. The numbers of oocytes studied (n) are indicated in the figure legend.

2.6. Cell Culture and Transfections

All cell culture media and supplements were obtained from Life Technologies (Grand Island, NY) unless otherwise noted. HeLa cells were grown in minimal essential medium supplemented with 0.1 mM non-essential amino acids, 10% fetal bovine serum (U. S. Bio-Technologies Inc., Pottstown, PA), 2 mM glutamine, 10 units/ml penicillin G, and 10 μg/ml streptomycin sulfate. Transient transfections were carried out using Lipofectamine Transfection Reagent and PLUS Reagent following similar protocols to our studies of expressed human connexins (Minogue et al., 2005; 2009).

2.7. Fluorescence Microscopy

Transfected cells cultured on glass coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 30 min at room temperature. After rinsing cells with phosphate-buffered saline, cells were subjected to immunofluorescence using rabbit polyclonal anti-AQP0 antibodies and mouse monoclonal anti-protein disulfide isomerase antibody (Thermo Fisher Scientific, Rockford, IL) and Cy3-conjugated goat anti-rabbit and AlexaFluo®488-conjugated goat anti-mouse IgG antibodies (Jackson ImmunoResearch, West Grove, PA) as previously described (Minogue et al., 2005).

Specimens were observed and images were acquired using an Olympus FluoView 1000 confocal microscope using a 60X (n.a. 1.42) oil immersion objective. Composite figures were assembled using Adobe Photoshop software (Adobe Systems, San Jose, CA).

3. Results

The proband was a 10 month old boy who presented in 2007 with a complaint of nystagmus in both eyes. Although slit lamp examination of this young child was not possible, lamellar cataracts were detected in both eyes upon dilation of the pupils (Fig. 1A). No other abnormalities were detected by ophthalmologic or general physical examination (specifically, ocular movements were full, conjunctivae were clear, and the anterior chambers looked normal). The child underwent surgery shortly afterwards for removal of the cataractous lenses and insertion of prostheses.

Family history (Fig. 1B) revealed that the proband (IV.1) had several close relatives with similar congenital cataracts and suggested an autosomal dominant inheritance pattern for them. His father (III.4) underwent surgery for cataract removal at 3 years of age and had a current bilateral visual acuity of 6/60. The paternal grandfather (II.2) was also affected; his visual acuity in the right eye was 2/60 (not improving with pinhole) and 2/60 in the left eye (6/60 with pinhole). The proband's mother (III.5) and grandmother (II.1) had normal visual acuities and examinations. There was no history of other ocular or systemic disorders within the family. Although not available for examination, a male cousin of the father was also reported with a history of congenital cataracts (III.7).

Genomic DNA was isolated from the proband, and exons from candidate genes that have been previously identified as mutated in patients with nonsyndromic autosomal dominant congenital cataract (ADCC) were amplified by PCR and sequenced (as described in Materials and Methods). Although some SNPs were found in some of the candidate genes (data not shown), no variations consistent with either known or unknown mutations were identified in *CRYAA, CRYBB2, CRYGC, CRYGD, GJA3, GJA8*, or *PITX3*. In contrast, bidirectional sequencing of the coding region of the *MIP* gene revealed a heterozygosity at position c.494G>A in exon 2 that created a restriction site for HincII. This substitution resulted in the replacement of glycine-165 by aspartic acid (G165D) within aquaporin0 (AQP0, also known as lens major intrinsic protein, MIP, or MP26), an amino acid residue within the 5th transmembrane helix of AQP0 (Fig. 1D). DNA sequencing and restriction

digestion demonstrated that this nucleotide substitution co-segregated with the disease phenotype, since it was present in affected family members (IV.1, III.4 and II.2), but absent in unaffected ones (II.1, III.5) (Fig. 1C and data not shown). Restriction fragment screening showed that the mutation was also absent in 109 unrelated members of a control population of the same ethnicity (data not shown). Since a polymorphism (c.-4T>C) in the 5'-untranslated region of the *MIP* gene was recently associated with age-related cataracts in a Chinese population (Zhou et al., 2011), we tested for its presence in the current pedigree. Our sequencing data showed no evidence for that substitution in the proband or two other affected individuals (father, III.4, and grandfather, II.2); they all had the TT genotype (data not shown).

Since AQP0 is a major lens plasma membrane protein that is a member of the aquaporin family of water channels, we tested for its function and behavior in heterologous expression systems. *In vitro* transcribed cRNAs encoding wild type AQP0 or AQP0-G165D were injected into *Xenopus* oocytes. To detect water channel activity, the oocytes were incubated in hypotonic medium and observed for swelling. The swelling rate of oocytes expressing AQP0-G165D did not differ significantly from that of control (water-injected) oocytes, but it was much slower than that of oocytes expressing wild type AQP0 (Fig. 2A, B). This suggested that expression of comparable amounts of mutant cRNA did not produce detectable water channel function.

To confirm production of both wild type and mutant proteins, oocytes were injected with constructs coding for AQP0-GFP fusion proteins. These oocytes showed green fluorescence by epifluorescence microscopy (data not shown), but the green fluorescence was more intense in oocytes injected with the wild type construct than in those injected with the G165D construct. Protein production was also assessed by immunobloting oocyte homogenates or enriched membrane preparations. Both wild type and G165D proteins were detected; but, the abundance of G165D was less than that of the wild type protein (Fig. 2C), and it had a slightly slower electrophoretic mobility (not shown).

We also tested the cellular localization of wild type and mutant AQP0 after transient transfection of HeLa cells. As expected, the expressed wild type AQP0 was detected by immunofluorescence at the plasma membrane and in cytoplasmic sites consistent with the synthetic/secretory pathway for a plasma membrane protein (Fig. 2D). In contrast, AQP0-G165D was not observed at the plasma membrane, but was detected in a cytoplasmic distribution that showed extensive overlap with that of protein disulfide isomerase (PDI), a protein that is resident within the endoplasmic reticulum (Fig. 2D). These results demonstrate impaired trafficking of AQP0-G165D which likely contributed to the absence of detectable water channel activity.

4. Discussion

We have presented genetic and functional evidence linking a new mutation of AQP0 (G165D) to autosomal dominant congenital cataracts.

AQP0/MIP is the most abundant membrane protein within lens fiber cells. It facilitates the movement of water into and across lens fiber cells, and it may have additional functions including acting as an adhesion molecule by forming "thin junctions" and minimizing the extracellular space between lens fiber cells (Engel et al., 2008). Mutations of *MIP* have previously been identified in families with non-syndromic congenital cataracts that are inherited as autosomal dominant traits. However, the functional consequences of these mutations have been analyzed only for E134G, T138R, and G213Vfs*46 (Francis et al., 2000;Varadaraj et al., 2008). These three mutants show loss of water permeability function

and impaired trafficking in expression systems. Similarly, cataract-linked mutant forms of mouse AQP0 have been associated with loss of water channel function and impaired trafficking (Shiels and Bassnett, 1996;Varadaraj et al., 1999;Shiels et al., 2000; Kalman et al., 2006). In most mouse models, the cataract is inherited according to an autosomal dominant pattern.

Our data from expression studies suggest that the c.494 G>A, G165D substitution has several consequences that may contribute to the pathophysiology. While wild type AQP0 acted as a water channel, we did not detect such function when a comparable amount of G165D cRNA was injected into the oocytes. Levels of G165D were lower than levels of wild type AQP0 in oocytes injected with the same amounts of cRNA (both in homogenates and in membrane-enriched pellets), similar to results previously obtained for a phosphorylation site-specific mutant of AQP0 (Mulders et al., 1995). The different electrophoretic mobility of G165D suggests that it might undergo different post-translational modification(s) than wild type AQP0. G165D was retained within the secretory pathway (mainly in the endoplasmic reticulum) in transfected cells, unlike wild type AQP0 which reached the plasma membrane. Retention or accumulation of proteins in the endoplasmic reticulum can cause ER stress and an unfolded protein response which have been implicated in the pathogenesis of several diseases (Reneker et al., 2011; Wang et al., 2012). The loss of water channel function could reflect RNA instability, impaired translation, protein degradation, or differential post-translational modification as well as impaired trafficking.

Analysis of the AQP0 channel structure may give insights into the importance of Glycine-165. The structure of AQP0 (Gonen et al., 2004;Harries et al., 2004) indicates that due to the tilt of the fifth transmembrane helix, glycine-165 does not face the channel pore (even though it is on the same helix face as histidine-172 which lies on the wall of the channel) and is embedded in a hydrophobic pocket. Glycine-165 is close to amino acid residues of the second transmembrane helix (within 3.5 Å of glycine-49 and alanine-53 and within 5 Å of leucine-50 and leucine-52, all aliphatic amino acids). Mutation of glycine-165 to aspartic acid results in replacement of a hydrogen with a carboxymethyl group in the amino acid side chain. The negative charge and larger size of the side chain of the aspartic acid will likely cause gross conformational changes by affecting packing and the hydrophobic environment that surrounds position 165, leading to misfolding of the mutant AQP0.

Glycine-165 is highly conserved among members of the family of aquaglyceroporins. The amino acid at the corresponding position is identical in all of the human aquaporins except AQP11 and AQP12, and it is conserved in family members as distant as the E. coli AqpA and GlpF proteins (Gorelick et al., 2006). Mutations (at other positions) of other aquaporin family members have been identified in patients with a variety of diseases; the largest number of mutations has been identified in AQP2, and they are associated with nephrogenic diabetes insipidus (Loonen et al., 2008;Sorani et al., 2008). Like AQP0-G165D, many of the other identified mutations in the other aquaporins show loss of channel function and impaired trafficking. But, unlike AQP0-G165D (and the other *MIP* mutants) most of them are associated with diseases that are inherited as autosomal recessive traits.

The autosomal dominant inheritance pattern of cataracts associated with AQP0 mutations implies that a 50% reduction in AQP0 function is detrimental for the lens. This conclusion agrees with the observation that the lenses of heterozygous AQP0-null mice develop opacities (Shiels et al., 2001;Al Ghoul et al., 2003). However, the cataracts in people with the G165D mutation or other cataract-associated AQP0 mutations are detected in early childhood and are more severe than those observed in the heterozygous AQP0-null mice which do not show frank opacities until 24 weeks of age. This implies that either haplo-

insufficiency of AQP0 is more deleterious in humans than in mice or that the human mutants also carry a dominant-negative function. Since AQP0 is an oligomeric protein, it is possible that the mutant form interacts with and impairs trafficking of the co-expressed wild type AQP0, and thus, its function(s) at the plasma membrane.

This is the first report of a cataract-associated mutation in AQP0 in the Indian population. It is a novel missense mutation that affects a highly conserved amino acid in the aquaporin gene family; substitutions at this position have not previously been linked to disease. Since AQP0 may have several functions and interact with other integral or peripheral membrane proteins in the lens, it is likely that AQP0-G165D would be unable to exert either its channel or non-channel functions, since it does not traffic properly to the plasma membrane.

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Abbreviations

AQP0	Aquaporin0
MIP	lens fiber major intrinsic protein
PDI	protein disulfide isomerase

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Highlights

- A novel mutation of *MIP* (c.494 G>A) is associated with autosomal dominant congenital cataracts
- The mutation causes a substitution of aspartate for glycine at position 165 in the lens fiber protein Aquaporin0.
- The mutant protein shows reduced levels and improper trafficking to the plasma membrane.
- The mutant protein does not form functional water channels.

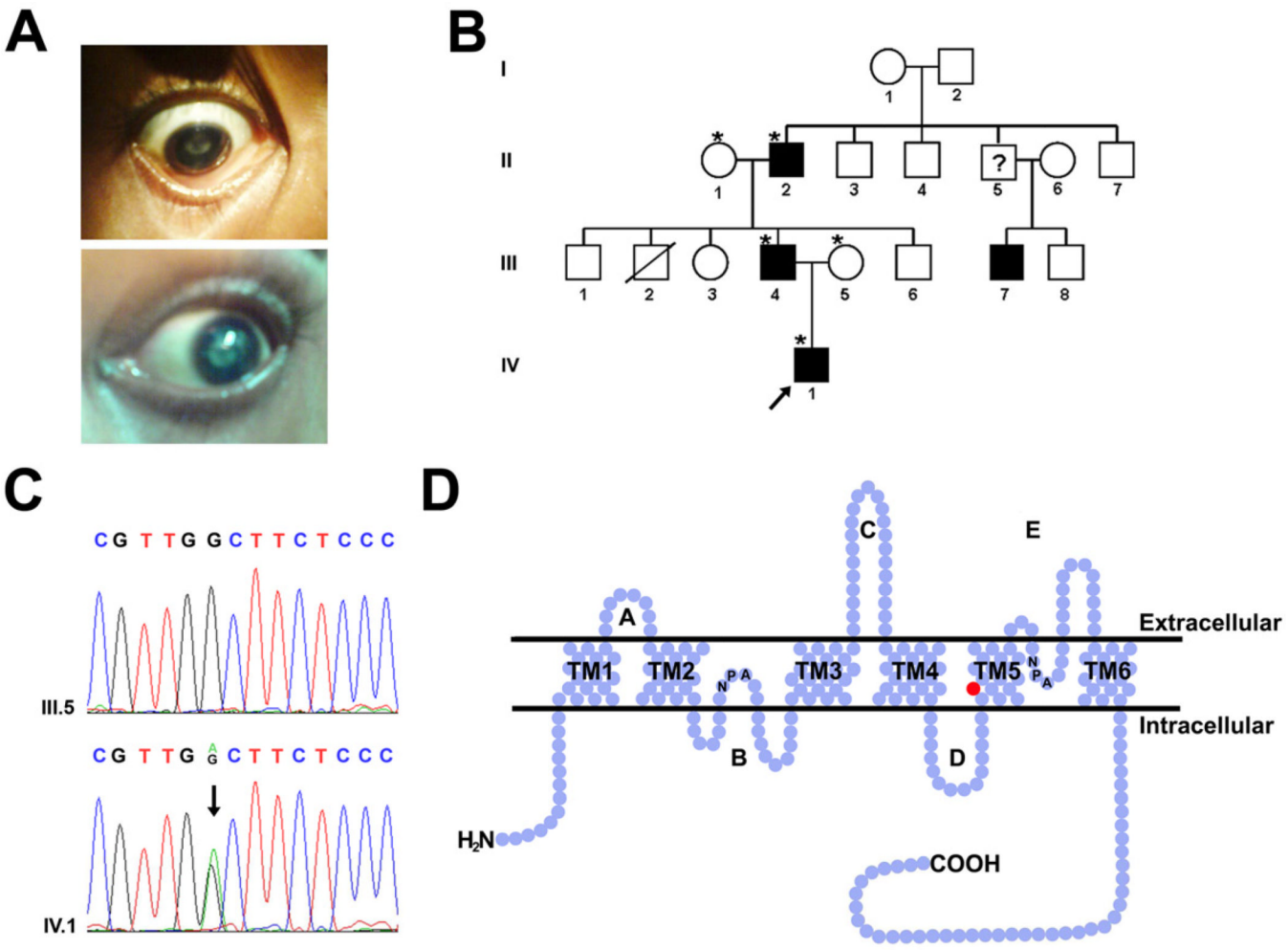

Figure 1.

A 494G>A nucleotide transition in the coding region of AQP0 segregates with the congenital lamellar cataract. A. Photographs show the congenital lamellar cataract in the proband's right (top) and left (bottom) eyes. B. Pedigree of the family with autosomal dominant lamellar cataract. Black symbols indicate affected individuals and open symbols indicate unaffected individuals. The diagonal line indicates a deceased family member. Family members whose DNA was analyzed by sequencing and restriction enzyme digestion are indicated by asterisks. The proband is indicated with an arrow. C. Chromatogram of the DNA sequence from an unaffected individual (III.5) shows only the wild type AQP0 allele which encodes glycine (GGC) at codon 165. The sequence chromatogram from the proband (IV.1) shows both G and A at position 494; thus, the mutant allele contains a G to A transition resulting in the substitution of glycine for aspartate at amino acid residue 165. D. Diagram of the topology of AQP0 in the plasma membrane. Transmembrane helices are numbered and intervening loops are indicated by letters. Amino acid residue 165 is located within the 5th transmembrane domain (red circle).

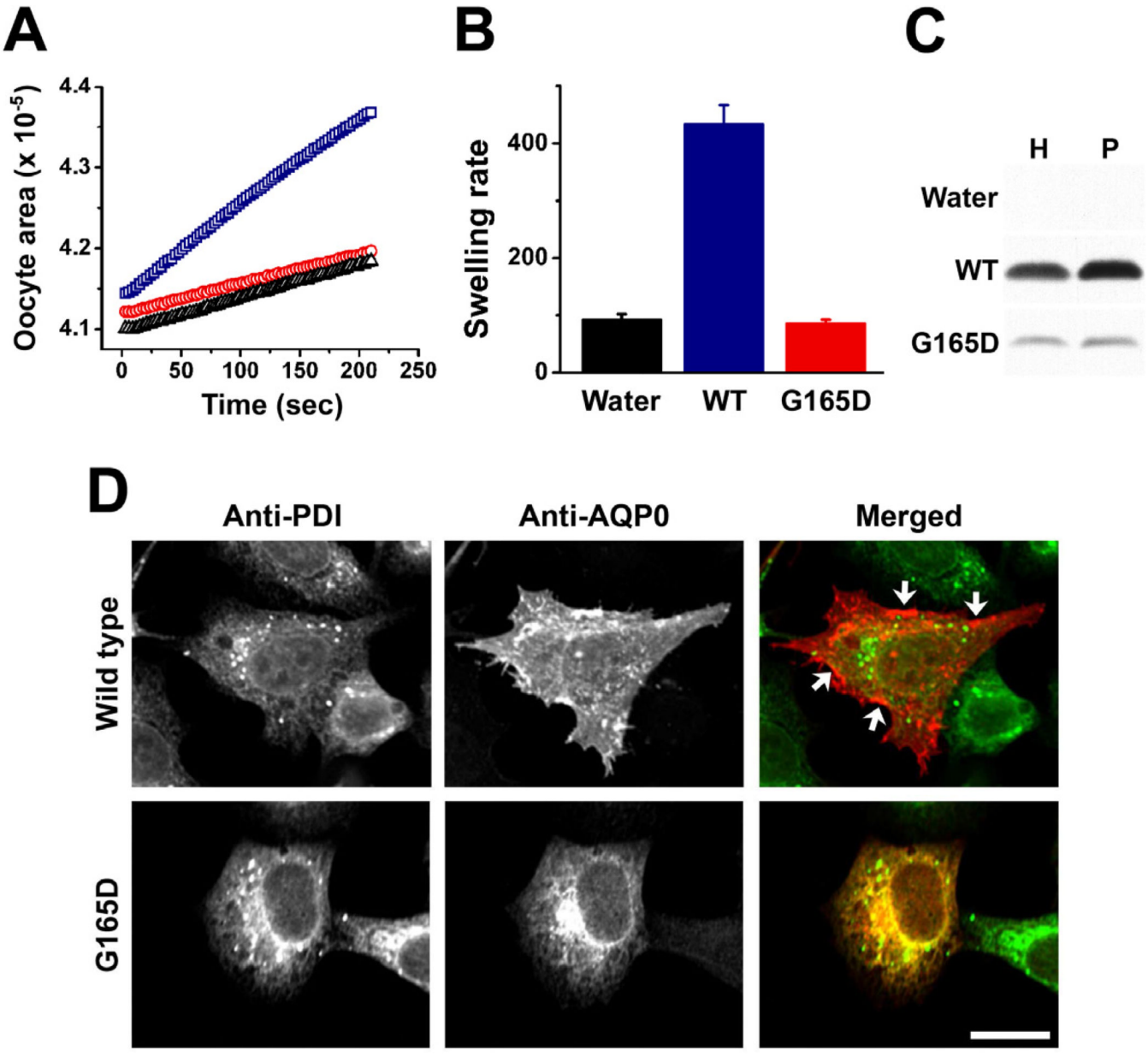

Figure 2.

The AQP0-G165D mutant does not exhibit water channel function and does not traffic properly to the plasma membrane. A. Graph shows representative curves of the time course of swelling (measured as oocyte area) of individual oocytes injected with water (black triangles), or cRNA for wild type AQP0 (blue squares) or AQP0-G165D (red circles) after they were transferred to a 10% hypotonic solution. B. Graph shows the swelling rates for oocytes injected with water, wild type AQP0 cRNA (WT) or AQP0-G165D cRNA (G165D). Values are presented as mean ± standard deviation (n = 10 for water, n = 15 for WT AQP0 and n = 18 for AQP0-G165D). C. Immunoblots show the levels of immunoreactive AQP0 in cleared homogenates (H) or membrane-enriched pellets (P) prepared from oocytes injected with water or with cRNAs encoding wild type AQP0 or the mutant G165D. D. Photomicrographs show the distributions of immunoreactive AQP0 and an ER-resident protein (PDI) in HeLa cells that were transiently transfected with wild type

AQP0 or AQP0-G165D. Twenty four hours later, cells were fixed and subjected to immunofluorescence using rabbit polyclonal anti-AQP0 and mouse monoclonal anti-PDI antibodies followed by Cy3-conjugated goat anti-rabbit and AlexaFluo®488-conjugated goat anti-mouse IgG antibodies. Arrows indicate immunoreactivity detected at the plasma membrane (only for wild type AQP0). Bar: 20 μm.