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Localization of AQP5/AQP8 chimeras in MDCK-II cells: Exchange of the N- and C-termini

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Abstract

AQP5 and AQP8 possess targeting/retention motifs which mediate their localization to the apical and basolateral membranes, respectively, of polarized MDCK-II cells. As targeting/retention motifs have been localized to the N- or C-termini of other AQPs, we sought the location of such motifs in AQPs 5 and 8 by exchanging their corresponding N- or C-termini and examining the expression, localization, and function of the resultant chimeras. We did not detect the expression of constructs in which the C-terminus of AQP5 was replaced by the C-terminus of AQP8. Substitution of the N-terminus of AQP8 for the N-terminus of AQP5 generated a construct which was trapped intracellularly and did not significantly facilitate transepithelial fluid movement. In contrast, modifications of the N- and C-termini of AQP8 were better tolerated. Substitution of either AQP8 terminus by the corresponding AQP5 terminus generated constructs which localized to basolateral membranes and facilitated transepithelial fluid movement. Our results suggest that, unlike the other AQP targeting/retention signals reported thus far, an AQP8 basolateral targeting/retention motif might reside between the two cytosolic termini.
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The aquaporins (AQPs) are a family of polytopic membrane proteins that facilitate the movement of water and, in some cases, select small molecules (e.g., glycerol, urea) or ions [1] across membrane barriers in response to an osmotic gradient (for general reviews on AQPs see [2–4]). In membranes, the AQPs exist as homotetramers, with each monomer containing a selective pore. The monomers are composed of six membrane-spanning domains linked by five loops. The N- and C-termini reside in the cytoplasm. An hourglass model depicting AQP1 topology has been described [2].

AQPs localize to apical, basolateral, or both domains of epithelial plasma membranes. The localization of AQPs to specific plasma membrane domains involves the recognition of targeting/retention signals embedded in AQP structures by components of the cellular protein sorting machinery [5,6]. Knowledge of targeting/retention signals is important as these signals can be used to help identify interacting components of the protein localization machinery, and to direct proteins to appropriate membrane domains for therapeutic purposes. Aberrant localization of membrane proteins has been linked to human disease [7–10].

Previously we reported that AQP5 and AQP8 localize to apical and basolateral membranes, respectively, in polarized MDCK-II cells grown on polycarbonate

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membranes [11]. We have been interested in identifying the regions of AQP5 and AQP8 encoding apical and basolateral localization information, respectively. Because localization signals have been reported to occur in the cytosolic tails of AQP2 [12], AQP4 [13], and other polytopic membrane proteins [5], we exchanged corresponding N- and C-termini between AQP5 and AQP8, and examined the expression, function, and localization of the resultant chimeras.

Materials and methods

Expression constructs. The pTRE.AQP5 [11], pTRE.AQP8 [11], and pACCMVrAQP8 [14] constructs were described previously. All modifications of AQP5 and AQP8 cDNA reported here were made using these plasmid constructs and an Advantage HF PCR Kit from Clontech. Predictions of parental and modified AQP membrane topologies and molecular weights were obtained using: (i) GeneTool (ver. 1.0) and PepTool (ver. 1.1), both from Biotools, and (ii) the Swiss-Prot Protein Knowledge Base (http:us.expasy.org/psrot/). Predicted topological structures of parental and modified AQP structures (PepTool) are given in Fig. 1. Exchange of corresponding N- or C-termini between AQP5 and AQP8 was accomplished using PCR-mediated gene splicing by overlap extension [15]. The final PCR products were inserted into the *Eco*RI and *Bam*HI restriction sites of the pTRE plasmid multiple cloning site. Table 1 lists cDNA fusion sites and specific deleted and replacement amino acid residues for each construct.

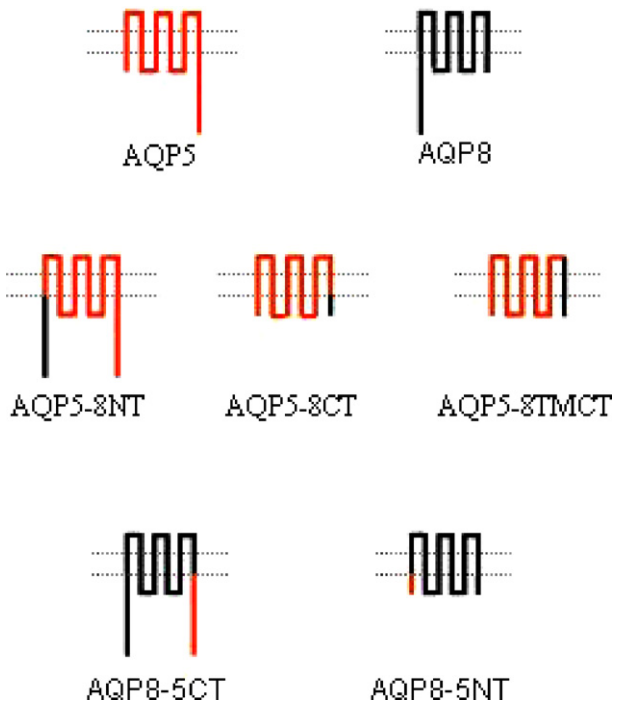

Fig. 1. Predicted membrane topologies of parental and chimeric AQPs. Parental AQPs 5 (red) and 8 (black) were used to construct chimeric AQPs as described in Materials and methods. PEPTOOL (ver. 1.1) was used to predict membrane topologies of the parental and chimeric AQPs. For each chimera, deleted and replacement amino acids are given in Table 1.

Cell culture and transfection. The culture of MDCK-II cells and the generation of stable transfectants (hygromycin-resistant) were carried out as described previously [11]. Stable transfectants were maintained in normal growth medium (hygromycin-free), and studies were conducted no sooner than one week after hygromycin removal [16]. To obtain transient transfectants for confocal microscopy, 3.4×10^5 cells/well were seeded onto Costar 24 mm polycarbonate filters (Quality Biologics), and transfections were performed 24 h later in 235 μl Optimem containing 1.9 μg of a pTRE plasmid encoding a normal or modified AQP transgene and 4.7 μl of Lipofectamine 2000 (prepared as described by Invitrogen). To obtain transient MDCK-II transfectants for Western blot analysis, transfections were performed on 10-cm dishes using 3.8×10^6 cells/plate seeded 24 h prior to transfection, and 3.3 ml/plate Optimem transfecting medium containing 26.6 μg DNA and 65.8 μl Lipofectamine 2000.

Confocal microscopy. MDCK-II parental cells or transfectants were seeded on Corning 24 mm polycarbonate filters, fixed, immunostained, and analyzed using a BioRad MRC 1024 laser scanning confocal imaging system as described previously [11]. Primary and secondary antibodies were affinity purified and used at 1:50 dilutions. Initial concentrations of primary antibodies were as follows: rabbit-anti-$AQP5_{CT}$ (0.7 mg/ml); rabbit-anti-$AQP8_{NT}$ (0.45 mg/ml); and rabbit-anti-$AQP8_{CT}$ (1 mg/ml). Rabbit-anti-$AQP5_{CT}$ (recognizes the COOH terminus of rat AQP5) and rabbit-anti-$AQP8_{NT}$ (recognizes the NH_2-terminus of rat AQP8) have been described previously [14]. Rabbit-anti-$AQP8_{CT}$ (recognizes the COOH-terminus of rAQP8) was obtained from Alpha Diagnostics. The secondary antibody (FITC)-conjugated Affinipure goat anti-rabbit IgG (H + L) (1.5 mg/ml) was purchased from Jackson ImmunoResearch Laboratories. Propidium iodide was used to detect nuclei (red).

Western blot. For each transfected construct as well as non-transfected MDCK-II cells, two 100 mm dishes of confluent cells were harvested by scraping in PBS. Crude membranes were prepared by centrifuging the cells at 3000*g* for 5 min, resuspending the pellet in lysis buffer (100 μl) containing (in mM): 100 Tris–HCl (pH 8.0), 1 $MgCl_2$, 0.5 Complete protease inhibitor containing EDTA (Roche), and 0.1 phenylmethylsulfonyl fluoride (Calbiochem), and freezing the lysate at −80 °C. The lysate was then thawed on ice, homogenized with a syringe and needle, and diluted to 1 ml with a buffer containing 0.25 M sucrose, 10 mM Tris–Hepes (pH 7.4), 1%(v/v) aprotinin, 1 mM dithiothreitol, 0.5 mM AEBSF (Roche), 0.167 mM pepstatin A (ICN Biomedicals), and 0.167 mM leupeptin (ICN Biomedicals). The lysate was centrifuged at 3000*g* for 20 min, and the resulting supernatant was centrifuged at 50,000*g* for 1 h. The resulting pellet (crude membrane fraction) was suspended in 20–30 μl water containing Complete protease inhibitor (Roche) and stored at −20 °C. The BioRad protein assay was used to measure protein concentrations. Western blots were carried out using Novex 12% Tris–glycine gels and PVDF membranes. Primary antibodies (described above) were used at a 1:1000 dilution. The secondary antibody was used as described in an Amersham ECL kit.

Transepithelial fluid movement. Measurement of osmotically obliged transepithelial fluid movement was carried as described previously [11]. Monolayer cultures grown on 24-mm polycarbonate filters were incubated in the presence of hypertonic apical medium (440 mOsm) for 30 min in a 5% CO_2 incubator at 37 °C.

Results and discussion

Here we report the effect of exchanging N- and C-termini on the localization of AQPs 5 and 8. Such exchanges might involve the gain or loss of apical or basolateral sorting determinants, potentially altering AQP localization. Results of previous studies have