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Vasopressin regulated trafficking of a green fluorescent protein-aquaporin 2 chimera in LLC-PK1 cells

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Abstract Aquaporin 2 (AQP2) transfected into LLC-PK_1 cells functions as a vasopressin-regulated water channel that recycles between intracellular vesicles and the plasma membrane upon vasopressin stimulation. The green fluorescent protein (GFP) of the jellyfish, *Aequorea victoria*, was used as an autofluorescent tag to monitor AQP2 trafficking in transfected LLC-PK_1 cells. Two chimeras were constructed, one in which GFP was fused to the amino-terminus of AQP2 [GFP-AQP2(NT)] and the second in which it was fused to the carboxyl-terminus [AQP2-GFP(CT)]. The GFP-AQP2(NT) chimera trafficked in a regulated pathway from intracellular vesicles to the basolateral plasma membrane in response to vasopressin or forskolin stimulation of cells. In contrast, the AQP2-GFP(CT) chimera expressed in LLC-PK_1 cells was localized constitutively on both apical and basolateral plasma membranes. The cellular location of this chimera was not modified by vasopressin or forskolin. Thus, while the GFP-AQP2(NT) chimera will be useful to study AQP2 trafficking in vitro, the abnormal, constitutive membrane localization of the AQP2-GFP(CT) chimera suggests that one or more trafficking signals exist on the carboxyl-terminus of the AQP2 protein.

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Introduction

Since the identification of aquaporin 1 (AQP1, formerly known as CHIP28) as a water channel protein (Agre et al. 1993), it has become clear that AQP1 is but one member of a much larger family of proteins that are present in diverse kidney epithelial cell membranes as well as in a multitude of other epithelial and non-epithelial cell types (Agre et al. 1995; Fushimi and Marumo 1995; Van Os et al. 1994; Verkman et al. 1995). The aquaporins serve to regulate renal water reabsorption and body fluid homeostasis, but they also represent a family of functionally important proteins that are of great cell biological interest because of their distinct intracellular targeting and trafficking characteristics. While AQP1 is constitutively expressed on the plasma membrane of several native epithelial cells (Nielsen et al. 1993a,b; Sabolic et al. 1992), aquaporin 2 (AQP2) is located in principal cells of the renal collecting duct (Sasaki et al. 1994) where it is expressed on the cell surface only after vasopressin stimulation (DiGiovanni et al. 1994; Fushimi et al. 1993; Sabolic et al. 1995).

Aquaporins have common structural features, including six transmembrane-spanning domains and two motifs comprised of the three amino acids asparagine-proline-alanine, which are highly conserved among family members (Agre et al. 1995). The cytoplasmic portions of the molecules show more variability and may contain specific sorting sequences, by analogy with many other membrane proteins (Agre et al. 1995; Brown and Stow 1996). We and others have recently shown that protein kinase A-induced phosphorylation of the serine 256 residue in the AQP2 cytoplasmic carboxyl-terminus is necessary for the regulated membrane expression of this protein (Katsura et al. 1997; Kuwahara et al. 1995). However, other regulatory motifs undoubtedly remain to be discovered in the aquaporins, by analogy with other membrane proteins that also enter a regulated membrane expression pathway, such as the insulin-regulatable glucose transporter, GLUT4 (James and Piper 1994; Pascoe et al. 1996).

An increasing number of studies have utilized the green fluorescent protein (GFP) of the jellyfish, *Aequorea victoria*, as a molecular tag to monitor dynamic processes in cells (Carey et al. 1996; De Giorgi et al. 1996; Dobson et al. 1996; Presley et al. 1997; Rizzuto et al. 1996; Scales et al. 1997). The intracellular distribution of exogenously expressed GFP-protein chimeras can be directly monitored without the need for chemical fixation and antibody staining. Such chimeric proteins can ultimately be used to follow intracellular trafficking pathways in real time, but a major caveat is that the AQP2-GFP chimeras may not be handled by the intracellular sorting machinery in the same way as the native protein in any given cell type. For example, GLUT4 has been tagged with GFP (Dobson et al. 1996). While both the amino-terminal and carboxyl-terminal constructs translocated to the plasma membrane in response to insulin, only the carboxyl-terminal GLUT4-GFP construct was re-internalized upon insulin removal. The amino-terminal GFP-GLUT4 construct remained on the plasma membrane.

The present study was designed to assess the utility of AQP2-GFP chimeras by comparing their intracellular trafficking with that of the AQP2 which we have previously shown to enter a vasopressin-stimulated, regulated exocytotic pathway in LLC-PK_1 cells in culture (Katsura et al. 1995, 1996, 1997). For this purpose, two chimeras were constructed, one in which GFP was fused to the cytoplasmic amino-terminus of AQP2, designated GFP-AQP2(NT), and the second in which GFP was fused to the cytoplasmic carboxyl-terminus of AQP2, designated AQP2-GFP(CT). These chimeras were transfected into LLC-PK_1 cells for analysis of their trafficking pathways. The GFP-AQP2(NT) chimera entered a vasopressin-regulated pathway of plasma membrane insertion in LLC-PK_1 cells and retained water channel function, similarly to the AQP2 protein alone. In contrast, the AQP2-GFP(CT) chimera was constitutively expressed on the plasma membrane of LLC-PK_1 cells without the need for vasopressin stimulation, and water channel function was impaired.

Materials and methods

Materials

Unless otherwise stated, all chemicals were purchased from Sigma, (St. Louis, Mo.) and all cell culture reagents were from Gibco BRL (Grand Island, N.Y.).

Rat tissue fixation and immunofluorescence

Sprague-Dawley rats were anesthetized, perfused, and fixed as previously described (Sabolic et al. 1995). Kidney cryosections were incubated with a newly developed antiserum to AQP2 [diluted 1:400 in phosphate-buffered saline (PBS)]. This antibody was raised against the second extracellular loop of AQP2 (C-GDLAVNALHNNATA). Its specificity was shown by western blotting, immunostaining of rat kidney inner stripe of the outer medulla, and peptide inhibition using 1 mg of the immunizing peptide per 1 ml of whole anti-serum. The sections were incubated for 1 h in anti-AQP2 antibody for 1 h, followed by 1-h incubation with Cy3-conjugated goat anti-rabbit IgG. The coverslips were mounted in Vectashield (Vector Laboratories, Burlingame, Calif.) diluted 1:1 in 0.3 M TRIS-Cl, pH 8.9. They were examined by indirect immunofluorescence and photography using a Nikon FXA photomicroscope.

Immunoblotting

Cells were grown on 100-mm tissue culture dishes to subconfluency then rinsed twice with PBS (10 mM sodium phosphate buffer, pH 7.4, containing 0.9% NaCl). Cells were then scraped off the dish with 1 ml lysis buffer per dish [50 mM TRIS, pH 8, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 0.2 mM PMSF, Complete protease inhibitor (Boehringer Mannheim, Indianapolis, Ind.)]. Cellular proteins (20 mg) were incubated at room temperature for 30 min in reducing Laemmli buffer in the case of cells transfected with AQP2 alone, and up to 80°C for 5 min for cells transfected with AQP2-GFP constructs. Protein samples were subjected to SDS-PAGE, then transferred to polyvinylidene difluoride membrane (Millipore, Bedford, Mass.) by Trans-blot SD semi-dry transfer (Bio-Rad, Melville, N.Y.). The transferred proteins were detected by either a polyclonal AQP2 antibody to the cytoplasmic C-terminal 15 amino acids (Immunodynamics, La Jolla Calif.) or an anti-GFP polyclonal antibody (Clontech, Palo Alto, Calif.). Primary antibodies were followed by horseradish peroxidase-conjugated goat anti-rabbit IgG, and detected by Renaissance Western Blot Chemiluminescence Reagent (New England Nuclear, Boston, Mass.).

AQP2-GFP chimeric constructs

To prepare the GFP-AQP2(NT) fusion chimera, a pcDNAI/neo vector containing the AQP2 cDNA with a c-myc epitope tag at its 3′ end (Katsura et al. 1995) was digested with *Sal*I and *Bgl*II restriction endonucleases to cut out the AQP2 cDNA insert. The insert was purified away from the vector by agarose gel electrophoresis using Wizard PCR Preps DNA Purification System (Promega, Madison, Wis.), then ligated into the *Sal*I/*Bam*HI sites of vector pEGFP-C1 (Clontech). To construct the AQP2-GFP(CT) chimeric protein, the AQP2-c-myc insert was digested with *Eco*RI/*Bgl*II, gel purified, and ligated into the *Eco*RI/*Bam*HI sites of vector pEGFP-N1 (Clontech).

Cell culture

The polarized renal epithelial cell line LLC-PK_1 was stably transfected with AQP2-GFP chimeric constructs using the DOTAP transfection reagent (Boehringer Mannheim) according to the manufacturer's recommendations. All cells were grown at 37°C in 5% CO_2. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum and 2 mM L-glutamine. Transfected cells were selected and maintained with 1 mg/ml G418 (Geneticin), and single clones were isolated using cloning rings. Cells were routinely determined to be mycoplasma negative.

LLC-PK_1 cell immunofluorescence

Cells grown on glass coverslips (Bellco, Vineland, N.J.) were incubated in serum-free medium for 2 h prior to the experiment, then treated with 10 nM lysine vasopressin or 10 μM forskolin (Calbiochem, San Diego, Calif.). In the recycling experiment, cells were treated with 10 μg/ml cycloheximide for 30 min prior to vasopressin or forskolin stimulation to inhibit protein synthesis. Subsequent stimulation and washout steps also included cycloheximide. Cells were fixed for 20 min with 4% paraformaldehyde containing 5% sucrose in PBS and mounted in Vectashield:TRIS-Cl, pH 8.9 (1:1). They were examined for GFP expression by indirect immunofluorescence and photography using a Nikon FXA photomicroscope, or confocal laser scanning microscopy (Bio-Rad Microscope Division; MRC/600). Nikon and confocal generated computer images

were analyzed using IP Lab Spectrum imaging software (version 3.0.1; Signal Analytics). Some cells were processed and fixed as above, and then permeabilized with 0.1% Triton X-100 for 4 min, followed by 5-min blocking in 1% BSA in PBS, 1 h at room temperature with antibodies against AQP2 or GFP. In this case, Cy3-conjugated goat anti-rabbit IgG was used to allow simultaneous visualization of the GFP and the red Cy3 fluorescence. Other antibodies used for double staining were an anti-c-myc monoclonal antibody (Oncogene Science, Cambridge, Mass.), an anti-rough endoplasmic reticulum (RER) polyclonal antibody (Meyer et al. 1982), and an anti-β-COP monoclonal antibody (clone M3A5; Sigma) to visualize Golgi-associated vesicles.

Construction of AQP2-GFP chimeras for cRNA transcription

For the amino-terminal GFP-AQP2(NT) fusion protein, GFP was amplified by PCR using a plasmid containing GFP (Clontech) as template with primers, 5′-GAAGGATCCATACATATGGCTAGC-3′ and 5′-TTATTTGTAGAATTCATCCATGCC-3′ with engineered *Bam*HI (5′) and *Eco*RI (3′) sites. The stop codon of GFP was removed and fused in-frame to the rat AQP2 start codon. Rat AQP2 was amplified by RT-PCR using rat kidney cDNA as template and primers 5′-GGAATTCATGTGGGAACTCAGATCC-3′ and 5′-GCTCTAGAGGCCTTGCTGCCGCGAGGCAGG-3′ encoding *Eco*RI (5′) and *Xba*I (3′) sites. The cDNA encoding the chimeric construct was subcloned into pSP64 T (Zhang et al. 1993) for RNA transcription. The carboxyl-terminal AQP2-GFP fusion protein was amplified by PCR using the AQP2-GFP(CT) fusion construct (see AQP2-GFP chimeric constructs above) as template with primers 5′-GAAGGATCCATGTGGGAACTCAGATCC-3′ and 5′-GCTCTAGATTACTTGTACAGCTCGTC-3′ encoding *Bam*HI (5′) and *Xba*I (3′) sites. The PCR product encoding the fusion construct was subcloned in pSP64T at *Bam*HI and *Xba*I sites.

Oocyte water permeability measurements

Complementary RNA was transcribed in vitro using SP6 RNA polymerase and 4 μg of plasmid DNA at 37°C for 2 h in the presence of diguanosine triphosphate (Pharmacia Piscataway, NJ), for capping. cRNA was purified after DNAse digestion by phenol-chloroform extraction and ethanol precipitation. Stage V and stage VI oocytes from *Xenopus laevis* were isolated, defolliculated, and microinjected with 50 nl of water or cRNA suspensions (10 ng/ml). Oocytes were incubated at 18°C for 24 h and osmotic water permeability (Pf) was measured from the time course of oocyte swelling at 10°C in response to a fivefold dilution of the extracellular Barth's buffer with distilled water.

Results

Characterization of the AQP2 external loop antibody

For undetermined reasons, and as we have previously reported (Katsura et al. 1995), the anti-C-terminal AQP2 antiserum used in our previous studies on rat (Sabolic et al. 1995) and mouse kidneys (Breton et al. 1995) failed to stain AQP2 expressed in cultured cells, although it gave positive results by western blotting. Thus, a c-myc tag had previously been added to the carboxyl-terminus of AQP2 for detection purposes (Katsura et al. 1995). Because this AQP2-c-myc construct was previously characterized in LLC-PK_1 cells, we used the same construct in the current study to maintain consistency. However, a new AQP2 antibody was raised in rabbits against a synthetic peptide (C-GDLAVNALHNNATA) from the sec-

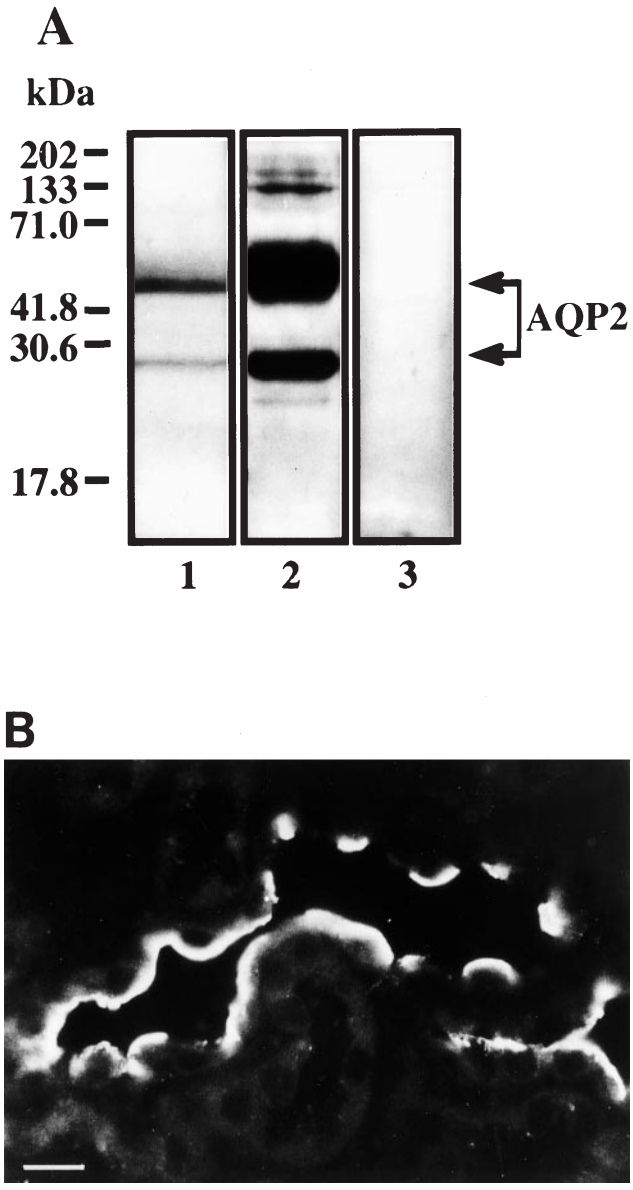


Fig. 1A, B Characterization of a new antibody to the second external loop of aquaporin 2 (*AQP2*). **A** Western blot of rat kidney papilla. The new external domain antibody identified a sharp band at the expected 28 kDa and a broader band at a higher molecular weight corresponding to the glycosylated form of the protein (*lane 1*). When the same dilutions of immune sera were used, the intensity of staining by this new antibody was somewhat weaker by western blotting than with a previous AQP2 antibody directed against the cytoplasmic carboxyl-terminal portion of AQP2 (*lane 2*). Preimmune serum confirmed the absence of this antibody prior to immunization (*lane 3*). Molecular mass markers are shown at the *left* (kDa). **B** Immunofluorescence of rat kidney inner stripe of the outer medulla. The AQP2 external domain antibody gave a strong staining of papillary principal cells by immunofluorescence while the intercalated cells were not stained, demonstrating its specificity and usefulness for immunocytochemsitry. *Bar* 20 μm

ond external loop of the AQP2 protein for future studies, such as cell surface biotinylation. We found that this new external epitope antibody labeled AQP2 in LLC-PK_1 cultured cells, in addition to tissue sections. Thus, it was used here to colocalize with the GFP from the AQP2-GFP chimeras (see below). Because this antibody has not been previously described, we briefly report its characterization here.

Western blot of rat kidney papilla using the new antibody identified a distinct band at 28 kDa and a broader band at a higher relative molecular mass, corresponding to the glycosylated form of the protein (Fig. 1A). When the same dilutions of immune sera were used, the intensity of staining by this new antibody (Fig. 1A, lane 1) was weaker by western blotting than with a previous antibody directed against the cytoplasmic carboxyl-terminal portion of AQP2 (VELHSPQSLPRGSKA) (Fig. 1A, lane 2). Preimmune serum demonstrated the absence of the antibody prior to immunization (Fig. 1A, lane 3). Specificity of the antibody was confirmed by peptide inhibition on AQP2-transfected LLC-PK_1 cells (data not shown).

In tissue sections, the AQP2 external loop antibody gave a strong staining of papillary principal cells by immunofluorescence (Fig. 1B). The intercalated cells remained unlabeled, thus demonstrating the specificity and usefulness of this new antibody for immunocytochemistry.

Expression of AQP2-GFP chimeric proteins

The GFP gene, enhanced by human codon optimization and chromophore mutations, was used to construct two AQP2 chimeric proteins. GFP was fused to the amino-terminus of AQP2, designated GFP-AQP2(NT), or to the carboxyl-terminus of AQP2, designated AQP2-GFP(CT).

Polarized, porcine LLC-PK_1 renal epithelial cells were stably transfected with either GFP-AQP2(NT) or AQP2-GFP(CT) chimeras. Expression of the full-length fusion proteins in LLC-PK_1 stable transfectants was confirmed by western blot using an anti-GFP polyclonal antibody (Fig. 2A) as well as with the AQP2 external loop antibody (data not shown). The vector-transfected control cells exhibited a 27-kDa GFP band when blotted with the GFP antibody, as well as a fainter band at approximately 30 kDa (Fig. 2A, lane 1). In the amino- and carboxyl-terminal AQP2 fusion protein-transfected cells (Fig. 2A, lanes 2 and 3, respectively), a band at 56 kDa was detected, as expected for the chimeric protein (27 kDa GFP+29 kDa AQP2). A higher mass band of approximately 65 kDa was also seen. The mobility of GFP in the SDS-polyacrylamide gel was found to be heat modifiable, with the ratio of the amounts of protein in the lower and higher bands depending on the denaturation temperatures used prior to loading the gel (Fig. 2B). By denaturing the GFP alone at a temperature of 50°C for 5 min, the majority of the GFP was in the higher mass band of approximately 30 kDa (Fig. 2B, lane 1). After 5 min at 60°C, a double band at 27 kDa and approximately 30 kDa was seen (Fig. 2B, lane 2).

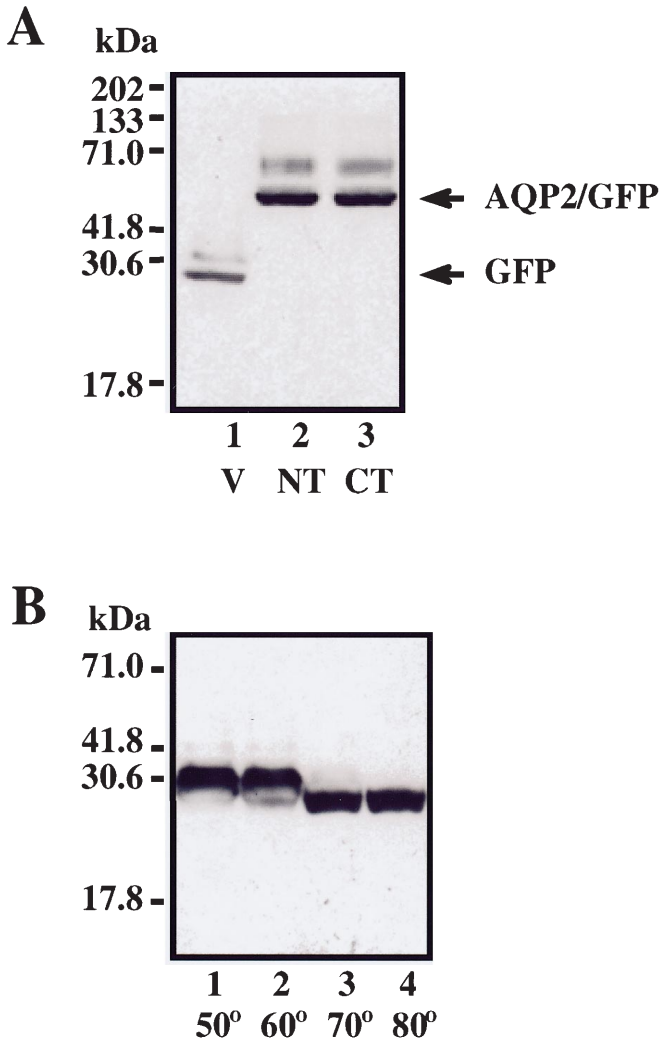


Fig. 2 **A** Western blot using an anti-green fluorescent protein (*GFP*) polyclonal antibody to confirm expression of the full-length fusion proteins in LLC-PK_1 stable transfectants. The GFP vector-transfected control cells exhibited a 27-kDa protein and a faint 30-kDa protein (*lane 1*; *V*). A strong band at 56 kDa was detected in the *N*-terminal GFP-AQP2(NT) fusion protein-transfected cells (*lane 2*; *NT*) and the C-terminal AQP2-GFP(CT) fusion protein-transfected cells (*lane 3*; *CT*), as well as a faint band at approximately 65 kDa. **B** The effect on SDS-PAGE migration of GFP under denaturation temperatures ranging from 50°C to 80°C is shown. Molecular mass markers are shown at the *left* (kDa)

Denaturation at 70°C for 5 min resulted in only a small fraction of the GFP remaining in the 30-kDa form (Fig. 2B, lane 3). Finally, denaturation at 80°C for 5 min was sufficient to cause the GFP to migrate completely at 27 kDa (Fig. 2B, lane 4). The samples shown in Fig. 2A were heated at 65°C for 5 min, thus, a small proportion of the GFP and chimeras migrated at a higher molecular weight in addition to those expected.

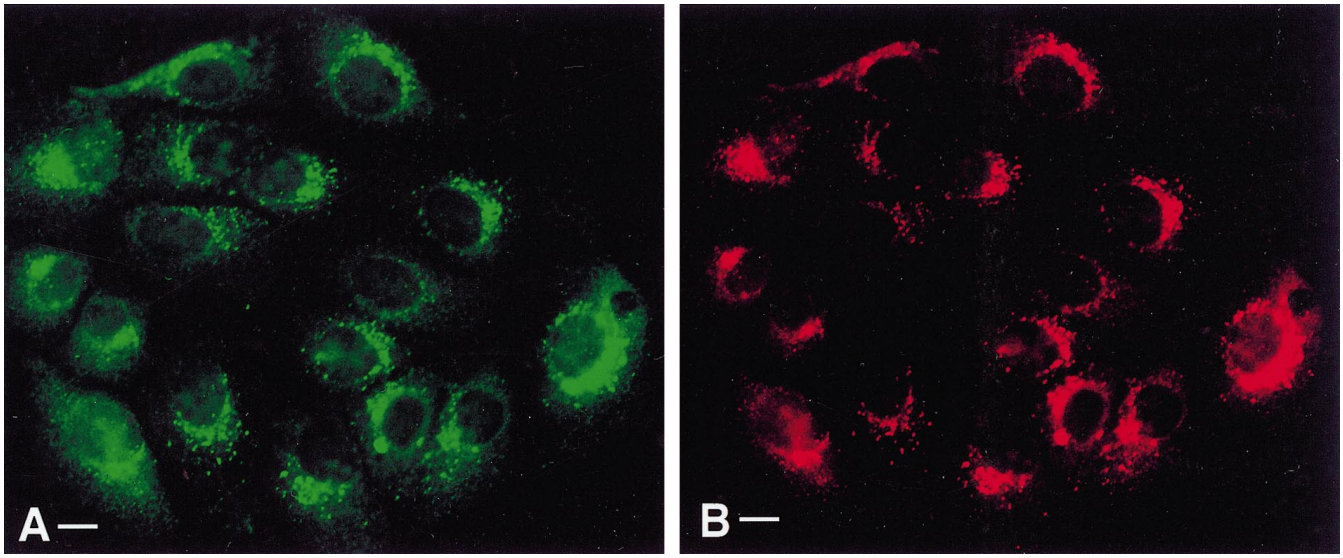
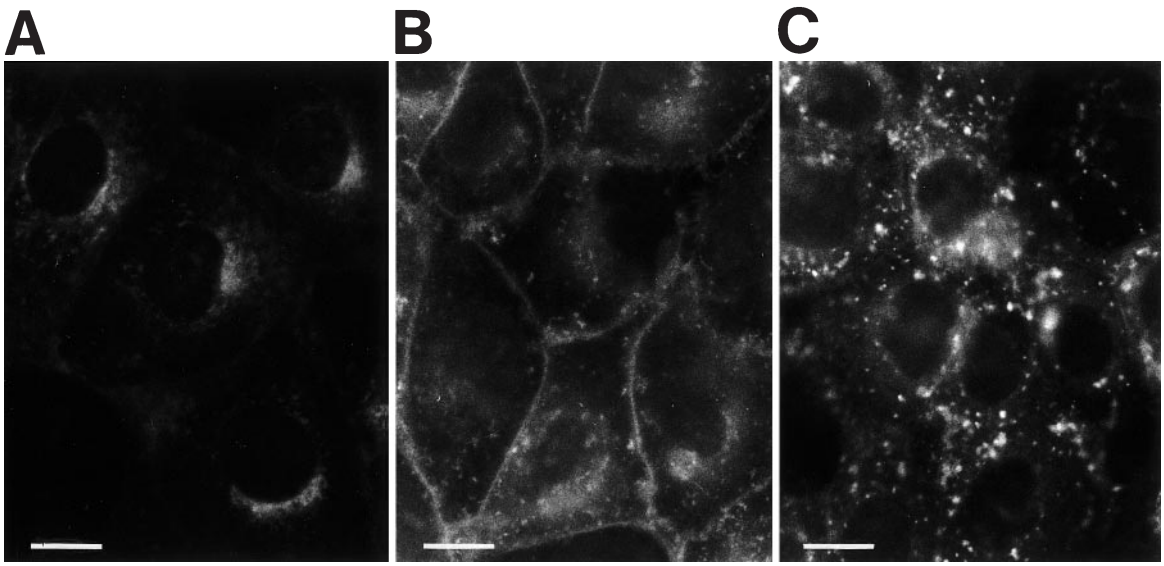


Fig. 3A, B Confocal laser microscopy confirmation of GFP-AQP2(NT) location using an AQP2 external loop antibody. **A** The inherent green fluorescence of GFP-AQP2(NT) under baseline conditions (without vasopressin) was located in intracellular vesicles in a perinuclear pattern. **B** The AQP2 external loop antibody followed by Cy3-conjugated goat anti-rabbit, used to label the same set of cells as shown in **A**, exhibited an identical localization to the inherent fluorescence of the GFP-AQP2(NT) chimera. *Bar* 10 μm

Inherent fluorescence of GFP confirmed to be GFP-AQP2(NT)

Using immunofluorescence and confocal microscopy, the GFP-AQP2(NT) fusion protein was located primarily on intracellular vesicles, as seen by the inherent fluorescence of GFP (Fig. 3A). The GFP-AQP2(NT) protein was also localized using the AQP2 external loop antibody followed by Cy3-conjugated goat anti-rabbit IgG (Fig. 3B). A similar pattern of localization was obtained using a c-myc antibody against the tag on AQP2 (data not shown). The location of AQP2 labeled by the external loop antibody overlapped completely with the GFP fluorescence, confirming that the location of the inherent GFP fluorescence was due to the GFP-AQP2(NT) fusion protein, and not due to cleaved GFP protein in the cell alone. Therefore, the inherent fluorescence of the GFP-AQP2(NT) chimera was suitable and appropriate for monitoring trafficking of AQP2 in this cell culture system.

Fig. 4A–C Nikon immunofluorescence microscopy of GFP-AQP2(NT) cells with the plane of focus at an intermediate level through the cells. The regulated recycling of GFP-AQP2(NT) is demonstrated in the continued presence of cycloheximide. **A** Under baseline conditions (without vasopressin), AQP2 exhibited a predominantly intracellular, perinuclear localization. **B** Vasopressin treatment for 30 min caused translocation of intracellular GFP-AQP2(NT) to the basolateral membrane. **C** Withdrawal of vasopressin in the continued presence of cycloheximide allowed re-internalization of GFP-AQP2(NT) after 1 h. *Bar* 20 μm



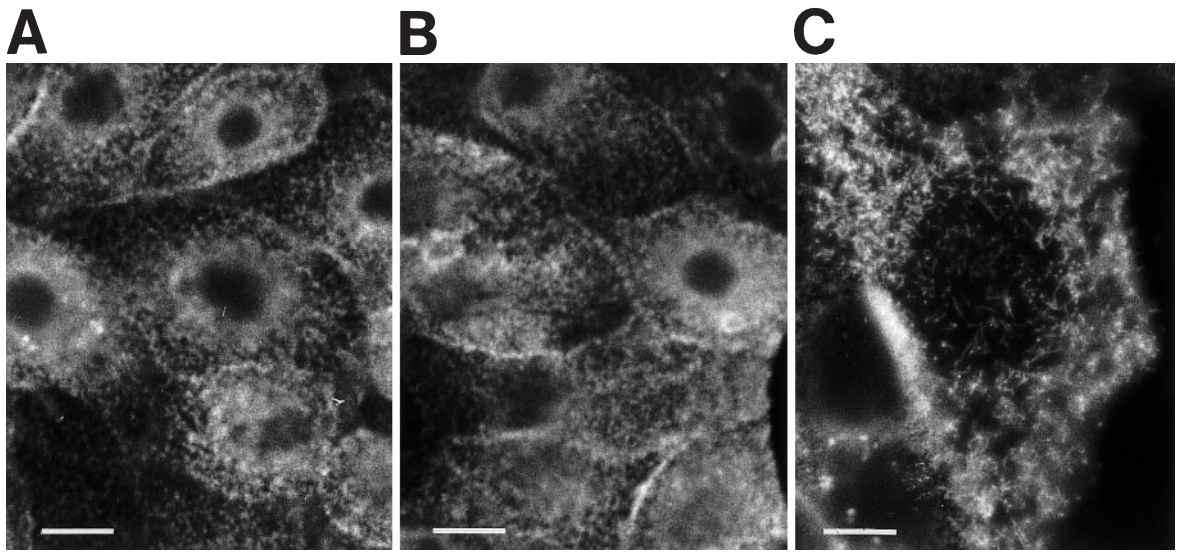


Fig. 5A–C Nikon immunofluorescence microscopy of AQP2-GFP(CT) cells with the plane of focus at the apical surface of the cells. **A** AQP2-GFP(CT) is constitutively located on the apical (and basolateral, not shown) membranes even in the absence of vasopressin. **B** Stimulation by vasopressin resulted in little increase in membrane localization of AQP2-GFP(CT). **C** A higher magnification view of the apical surface of a cell demonstrates the microvillar location of AQP2-GFP(CT) in the unstimulated, baseline state. *Bars* **A**, **B** 20 μm, **C** 5 μm

GFP-AQP2(NT) enters a regulated membrane insertion pathway in LLC-PK_1 cells

For GFP to be used as an effective tag, it was imperative that it not interfere with the normal sorting or trafficking characteristics of AQP2 expressed in LLC-PK_1 cells. To determine whether the GFP-AQP2(NT) fusion protein retained the ability of AQP2 to recycle between intracellular vesicles and the plasma membrane in LLC-PK_1 cells, the cells were first incubated for 30 min in serum-free medium containing 10 mg/ml cycloheximide to inhibit protein synthesis. This allowed the same population of GFP-AQP2(NT) proteins to be monitored over a 2-h period, as we have previously described in LLC-PK_1 cells (Katsura et al. 1996). All subsequent treatments were also carried out in the continued presence of cycloheximide. Cycloheximide treatment for 1–3 h had little effect on the baseline distribution of GFP-AQP2(NT) compared to its intracellular localization in untreated cells (data not shown). Under non-stimulated conditions, GFP-AQP2(NT) localized to intracellular vesicles that were concentrated in the perinuclear region (Fig. 4A), as has been previously shown for AQP2 alone (Katsura et al. 1995). Stimulation of the cells with the antidiuretic hormone, vasopressin, caused a translocation of GFP-AQP2(NT) to the basolateral membrane (Fig. 4B). A similar effect was also observed with forskolin stimulation (data not shown). Subsequent washout and further incubation in vasopressin-free medium for 1 h caused the reappearance of GFP-AQP2(NT)-containing vesicles in the cytoplasm (Fig. 4C). Thus, both regulated exocytosis and endocytosis of GFP-AQP2(NT) was observed, as has been demonstrated for AQP2 alone (Katsura et al. 1995). The only difference between AQP2 and the GFP-AQP2(NT) protein was the time difference required for the translocation of GFP-AQP2(NT). While AQP2 alone was seen to translocate to the plasma membrane in 10 min following vasopressin stimulation, the GFP-AQP2(NT) fusion protein required 30 min before a similar level of plasma membrane expression was observed. Similarly, following washout of the hormone, re-internalization into intracellular vesicles resulted, although this was more scattered than the baseline distribution after just 1 h post washout.

AQP2-GFP(CT) enters a constitutive membrane insertion pathway in LLC-PK_1 cells

In contrast to the AQP2 amino-terminal fusion with GFP, the carboxyl-terminus fusion protein did not enter a regulated pathway of membrane insertion. The localization of AQP2-GFP(CT) under non-stimulated conditions was predominantly on the apical and basolateral membranes, with only a small amount located intracellularly. The punctate pattern of apical surface localization of AQP2-GFP(CT) is also shown (Fig. 5A). A cross-sectional view of AQP2-GFP(CT) cells demonstrating its basolateral localization by confocal microscopy is shown in Fig. 6C. The plasma membrane AQP2-GFP(CT) location is in contrast to the intracellular vesicular location of GFP-AQP2(NT) (Fig. 4A) and AQP2 (Katsura et al. 1995) under non-stimulated conditions. Vasopressin stimulation of AQP2-GFP(CT) cells caused only a marginal, if any, increase in basolateral membrane localization (Fig. 5B). Thus, the presence of the GFP protein on the carboxyl-terminus of AQP2 caused the chimeric protein to enter a constitutive pathway of membrane insertion. At higher magnification, the apical microvillar location of the AQP2-GFP(CT) chimera is visible (Fig. 5C). The behavior of this fusion protein, therefore, parallels the constitu-

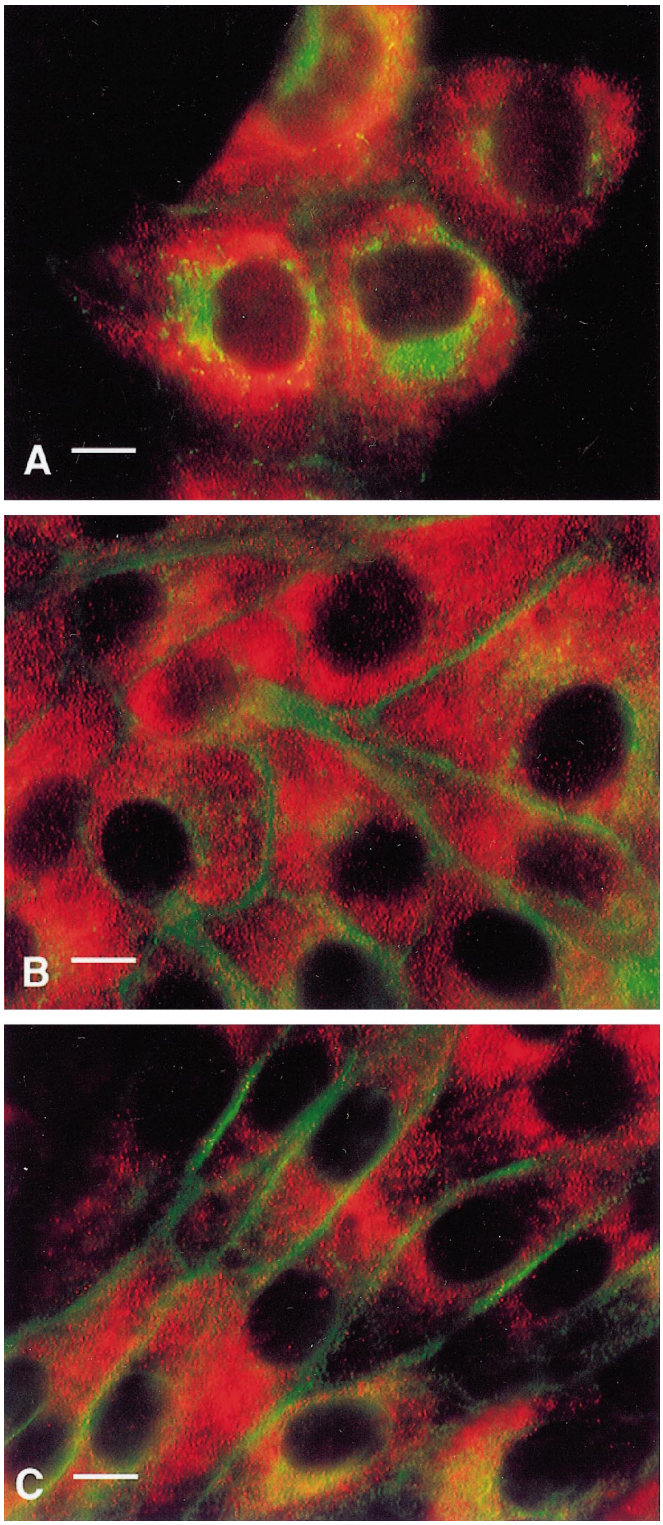


Fig. 6A–C Confocal laser microscopy with the plane of focus at an intermediate section through the cells illustrates rough endoplasmic reticulum (RER) location in relation to the AQP2-FP chimeric proteins. **A** Golgi region localization of GFP-AQP2(NT) in the unstimulated baseline state is distinct from the Cy3-labeled RER. **B** Basolateral membrane translocation of GFP-AQP2(NT) following vasopressin stimulation is distinct from the Cy3-labeled RER. **C** Constitutive basolateral membrane localization of AQP2-GFP(CT) in the unstimulated, baseline state is distinct from the Cy3-labeled RER. *Bar* 10 μm

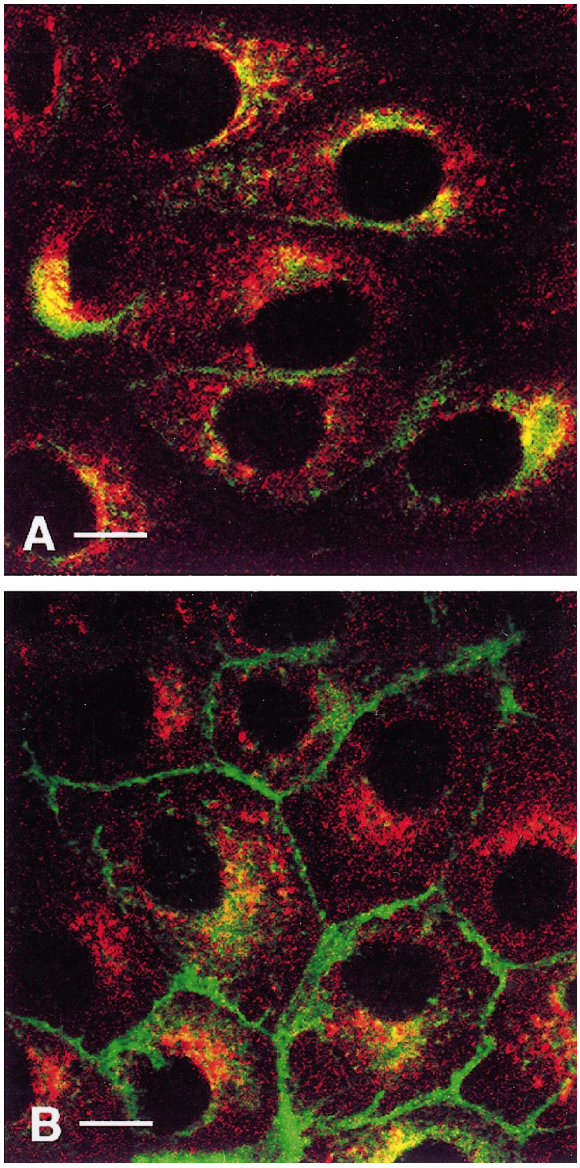


Fig. 7A, B Confocal laser microscopy with the plane of focus at an intermediate section through the cells illustrates β-COP location in relation to the GFP-AQP2(NT) chimeric protein. **A** Golgi region localization of GFP-AQP2(NT) in the unstimulated baseline state overlaps only partially with the Cy3-labeled β-COP. **B** Basolateral membrane translocation of GFP-AQP2(NT) following vasopressin stimulation is distinct from the Cy3-labeled β-COP. Some GFP-AQP2(NT) remains in the Golgi region of a few cells, and only partially overlaps with β-COP. *Bar* 10 μm

tive membrane insertion pathway that we have previously described for another water channel protein, AQP1, in transfected LLC-PK_1 cells (Katsura et al. 1995).

Colocalization of GFP-AQP2(NT) and other cellular markers

A further advantage of using the AQP2-GFP chimeras is for use in colocalization studies of AQP2 with other intracellular proteins without the limitation of using an an-

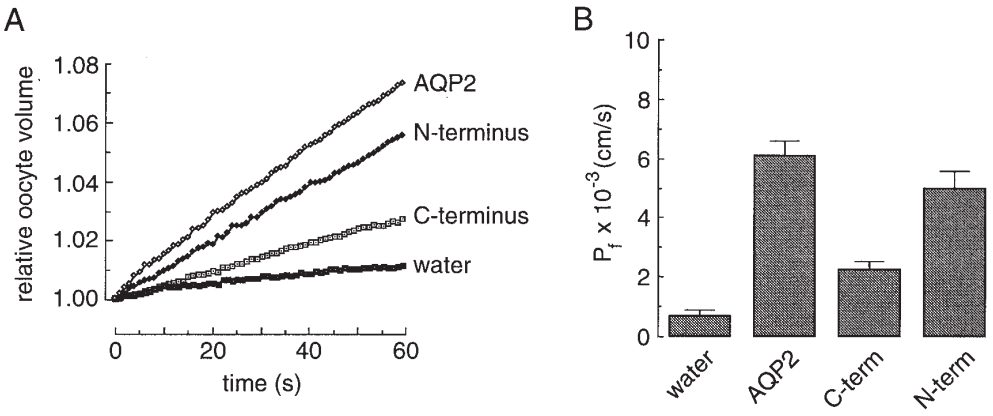


Fig. 8A, B Measurements of water permeability of *Xenopus* oocytes expressing the GFP chimeras. **A** Expression of both constructs significantly increased the rate of oocyte swelling compared to water injected controls. **B** Expression of the GFP-AQP2(NT) construct was similar to that of AQP2, and much more effective in increasing oocyte water permeability than the AQP2-GFP(CT) construct

tibody raised in a particular species to avoid secondary antibody crossreactivity.

Initially, to confirm that proper protein folding of the GFP-AQP2(NT) chimera occurred, resulting in export from the RER, colocalization with an antibody against the RER was performed. A clear delineation between the GFP-AQP2(NT) and the Cy3-labeled RER marker is shown in both the unstimulated (Fig. 6A) and vasopressin-stimulated cells (Fig. 6B). A similar result was obtained for the unstimulated AQP2-GFP(CT) chimera, which, in this confocal image, is seen to be located only on the basolateral plasma membrane, with no apical localization illustrated due to the plane at which the optical section was taken (Fig. 6C). The vasopressin-stimulated AQP2-GFP(CT) chimera appeared identical to that of the unstimulated state (data not shown).

A future goal of using AQP2-GFP-transfected cells is to determine the nature of the intracellular compartment in which the aquaporin resides prior to stimulation. To demonstrate the feasibility of this approach, the distribution of β-COP Golgi vesicle coat protein with respect to GFP-AQP2(NT) was analyzed. β-COP overlapped with GFP-AQP2(NT) to some degree in the Golgi region in the unstimulated state (Fig. 7A, yellow shows overlap of Cy3 and GFP), although there were many vesicles which appeared to contain only one or the other protein. After vasopressin stimulation, GFP-AQP2(NT) was still observed in some cytoplasmic vesicles, but was mostly relocated to the basolateral membrane, whereas β-COP remained mainly in the Golgi region (Fig. 7B).

The AQP-GFP chimeras are functional water channels

To determine whether the AQP2-GFP chimeras retained their capacity to serve as functional transmembrane water channels, the chimeras were expressed in *Xenopus* oocytes and the oocyte water permeabilities were measured as previously described (Zhang et al. 1993). Expression of both constructs significantly increased the rate of oocyte swelling compared to that of water injected controls (Fig. 8), but expression of the GFP-AQP2(NT) construct was much more effective in increasing oocyte water permeability than the carboxyl-terminal AQP2-GFP(CT) construct.

Discussion

The mutationally enhanced GFP of *A. victoria* was fused to the AQP2 protein for the ultimate purpose of non-invasively monitoring the localization and real-time trafficking of AQP2 in live cells, as well as colocalization with other cellular markers. This report describes the production and initial characterization of two AQP2-GFP-transfected LLC-PK_1 cell lines, and highlights an important effect of the AQP-GFP fusion site on intracellular trafficking. The amino-terminal fusion chimera, GFP-AQP2(NT), was shown to translocate to the plasma membrane upon stimulation with vasopressin or forskolin. Furthermore, with the use of cycloheximide to inhibit further protein synthesis, a single cohort of GFP-AQP2(NT) proteins was shown to undergo exocytosis from intracellular vesicles to the membrane upon vasopressin stimulation, and subsequent endocytosis following washout of the hormone. The time course of this GFP-AQP2(NT) recycling was slower than that for the AQP2 protein alone (Katsura et al. 1995), indicating that the presence of the GFP protein did have a slight disruptive effect on trafficking. However, the regulated trafficking of GFP-AQP2(NT) follows the same qualitative pattern as previously demonstrated by Katsura et al. (Katsura et al. 1995, 1996) for AQP2. Furthermore, the water permeability of *Xenopus* oocytes expressing GFP-AQP2(NT) was similar to those expressing AQP2 alone. Thus, fusion of the GFP protein to the amino-terminus of AQP2 did not disrupt proper protein folding or porin functionality, nor did it appear to disturb any putative targeting motifs which may be located in the ten amino acids which, by hydropathy plot, are predicted to be located in the cytoplasm at the amino-terminus of this protein.

In contrast to GFP-AQP2(NT), fusion of GFP to the carboxyl-terminus of AQP2 partially disrupted both the function and trafficking of AQP2. The oocyte permeability of AQP2-GFP(CT) was significantly greater than that of the water-injected controls, however, it was much less than either AQP2- or GFP-AQP2(NT)-expressing oocytes. Furthermore, AQP2-GFP(CT) did not follow the regulated trafficking pathway observed for AQP2 and GFP-AQP2(NT). The AQP2-GFP(CT) fusion protein

was localized constitutively at the apical and basolateral membrane, and very little was observed within cytoplasmic vesicles. This carboxyl-terminus fusion protein of AQP2 appeared to traffic via a constitutive pathway similar to that of AQP1, which has previously been shown to be constitutively located on the basolateral and apical membranes, with a small amount located in the perinuclear Golgi area in LLC-PK_1 cells (Katsura et al. 1995). This trafficking pattern of the AQP2-GFP(CT) chimera provides support for the hypothesis that an important targeting motif(s) exists on the carboxyl-terminus of AQP2 which directs AQP2 to a regulated pathway of endo- and exocytosis in response to hormone stimulation of cAMP. Indeed, we have recently shown that phosphorylation of the serine 256 residue in the AQP2 carboxyl-terminus is required for the vasopressin-stimulated accumulation of AQP2 on the plasma membrane of LLC-PK_1 cells (Katsura et al. 1997). We have not yet determined whether this critical S256 residue can be phosphorylated in either of the AQP2-GFP chimeras.

It was confirmed that both the GFP-AQP2(NT) and AQP2-GFP(CT) chimeras were not misfolded and retained in the endoplasmic reticulum by staining with an RER marker antibody. There was a clear delineation of RER and GFP fluorescence. The GFP-AQP2(NT) chimera was analyzed for colocalization with the β-COP coat protein. Vesicles which bud from Golgi membranes contain several COP proteins (Duden et al. 1991; Rothman and Orci 1992; Serafini et al. 1991). β-COP is located on vesicles which are predominantly involved in transport within the Golgi, and between the Golgi and RER. The precise role of COPI-containing vesicles is somewhat controversial, but recent work has shown that COPI-coated vesicles can be involved in both anterograde and retrograde transport in the Golgi (Orci et al. 1997). Immunofluorescence with an anti-Golgi β-COP antibody showed some overlap with GFP-AQP2(NT), but numerous GFP-containing vesicles were observed that did not have β-COP staining. The converse was also observed, suggesting that β-COP is either not associated with AQP2-containing vesicles or, possibly, only transiently so. The identification and further characterization of GFP-AQP2(NT) containing vesicles in these cells, as well as in kidney collecting duct principal cells will require a detailed study with compartment-specific markers at the electron microscope level (Griffiths et al. 1993), and this is the subject of future work in our laboratory.

Studies on cells expressing the GLUT4 glucose transporter have shown that this protein, whose membrane expression is regulated by insulin, has a complex pattern of vesicular expression. While colocalization with recycling transferrin receptors can be demonstrated for some vesicles, about 60% of the GLUT4-containing structures appear to be a unique class of recycling vesicle (Hanpeter and James 1995). The regulation of GLUT4 vesicles and AQP2 vesicles occurs via different mechanisms, i.e., GLUT4 exocytosis is inhibited by the PI3-kinase inhibitor, wortmannin (Kotani et al. 1995), but that of AQP2 is not (C.E. Gustafson, T. Katsura and D. Brown, unpublished results). Thus, the GFP-AQP2(NT) construct will now facilitate a similar series of studies to identify the AQP2 recycling vesicle population.

In conclusion, these results demonstrate that AQP2-GFP chimeras can be expressed in LLC-PK_1 epithelial cells and can be used to follow trafficking pathways within these cells. The results demonstrate that the position at which the GFP protein is fused to the AQP2 molecule has a major effect on the intracellular pathway into which the chimera is directed, as well as on its water channel functionality. Both of these constructs will be useful to further dissect the intracellular sorting signals that direct different members of the aquaporin family of water channel proteins to different cellular locations in a variety of cell types, both in situ and in cell culture models.

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