Available online at www.sciencedirect.com

SCIENCE DIRECT

Biochimica et Biophysica Acta 1763 (2006) 337–344

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

http://www.elsevier.com/locate/bba

Protein kinase A-regulated membrane trafficking of a green fluorescent protein-aquaporin 5 chimera in MDCK cells ☆

Chisato Kosugi-Tanaka, Xuefei Li, Chenjuan Yao, Tetsuya Akamatsu, Norio Kanamori, Kazuo Hosoi *

Department of Molecular Oral Physiology, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima-shi, Tokushima 770-8504, Japan

Received 20 August 2005; received in revised form 28 January 2006; accepted 6 February 2006
Available online 10 March 2006

Abstract

The green fluorescent protein (GFP) of the jellyfish, *Aeqorea victoria*, was used as an autofluorescent tag to track the trafficking of aquaporin 5 (AQP5), an exocrine gland-type water channel. Two groups of chimeric proteins were constructed; one in which GFP was fused to the amino-terminus of AQP5 (GFP–AQP5) and the other, in which it was fused to the carboxyl terminus of it (AQP5–GFP). In each group, 2 chimeras were produced, a wild-type AQP5 with its normal sequence and a mutant AQP5 having a mutated amino acid at 259, i.e., GFP–AQP5–T259A and AQP5–GFP–T259A. They were used to transfect Madin–Darby canine kidney (MDCK) cells. The GFP–AQP5 chimera was localized in the intracellular vesicles, which trafficked to the plasma membrane in response to N^6, 2′-*O*-dibutyryladenosine 3′, 5′-cyclic monophosphate (dbcAMP). Membrane trafficking was inhibited by N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquimolinesulfonamide (H-89) but not by palmitoyl-DL-carnitine chloride (PCC). In contrast, the AQP5–GFP chimera expressed in MDCK cells was localized constitutively on the plasma membrane. The cellular localization of the latter chimera was not affected by stimulation with dbcAMP in the presence or absence of H-89 or PCC. Replacement of Thr-259 with Ala-259 did not affect the dbcAMP-induced translocation of the chimeric protein, suggesting that phosphorylation of Thr-259 was not necessary for AQP5 trafficking under the present experimental conditions. Thus, the GFP–AQP5 chimera will be a useful tool to study AQP5 trafficking in vitro, whereas the constitutive membrane localization of the AQP5–GFP chimera suggests the importance of the carboxyl terminus of the AQP5 protein for its sorting, whether it is translocated to intracellular vesicles or to the plasma membrane.

Keywords: Aquaporin 5; Trafficking; GFP-tagged AQP5

Abbreviations: AQP5, aquaporin 5; dbcAMP,N^6, 2′-*O*-dibutyryladenosine 3′, 5′-cyclic monophosphate; DMEM/F-12, Dulbecco's modified Eagle's medium F-12 HAM; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; GFP, green fluorescent protein; GLUT, glucose transporter; HRP, horseradish peroxidase; H-89, N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfoamide; MDCK, Madin–Darby canine kidney; PBS, phosphate-buffered saline; PCC, palmitoyl-DL-carnitine chloride; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; PMA, phorbol 12-myristate 13-acetate; PMSF, phenyl methylsulfonyl fluoride; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis

☆ *Nucleotide numbering:* The first nucleotide, adenine at the translation initiation site (ATG) was denoted as nucleotide number one in this paper. *Designation of plasmid constructs:* Plasmid constructs established in the present study are designated as follows: pEGFP-C2 vector ligated to *rAQP5* cDNA, GFP–AQP5; pEGFP-N2 vector ligated to *rAQP5* cDNA, AQP5–GFP; pEGFP-C2 vector ligated to *rAQP5* cDNA with mutation at nt 775, GFP–AQP5–T259A; pEGFP-N2 vector ligated to *rAQP5* cDNA with mutation at nt 775, AQP5–GFP–T259A.

* Corresponding author. Tel.: +81 88 633 7323; fax: +81 88 633 7324.
E-mail address: hosoi@dent.tokushima-u.ac.jp (K. Hosoi).

0167-4889/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2006.02.005

1. Introduction

Aquaporin (AQP) water channels are essential for mediating rapid osmotic water transport across the cell membrane. There are 13 known mammalian AQPs. Among them, AQP5 was first identified in the salivary gland and subsequently in other exocrine glands; and it was found to be localized consistently at the apical membranes of acinar cells in the submandibular and parotid glands of rats [1–4]. The vesicular trafficking is one of mechanisms that regulate AQP functions. AQP5 is translocated from intracellular to the plasma membrane in response to the elevation of intracellular Ca^{2+}, where involvement of the cytoskeleton is suggested [5,6]. In a murine lung epithelial cell line (MLE-12), a cAMP analog, 8-(4-chlorophenylthio)-cAMP (cpt-cAMP), was recently found to induce AQP5 expression both transcriptionally and post-transcriptionally [7]. AQP2 is the water channel, which has been studied more regarding the event after second messenger-induced phosphorylation. It is reported that activation of protein kinase A (PKA) by cAMP leads to phosphorylation of AQP2 at Ser-256, the amino acid residue located at the cytoplasmic domain of carboxyl terminus. By the use of LLC-PK_1 cells (a pig kidney line typical of normal kidney tubular epithelium) transfected with S256A-mutant AQP2, it was shown that phosphorylation of the Ser-256 residue by PKA is required for vasopressin-induced translocation of AQP2 from vesicles toward the plasma membrane [8,9]. van Balkom et al. [10] constructed altered *AQP2* genes, each of which produced a product with a mutation at either putative casein kinase II-target motifs (Ser-148, Ser-229, Thr-244), a protein kinase C (PKC)-target motif (Ser-231), or a PKA-target motif (Ser-256); they were then expressed in Madin–Darby canine kidney (MDCK) cells. All of these mutant proteins, except that of Ser-256 mutant trafficked from intracellular vesicles to the apical membrane via a forskolin-sensitive mechanism, similarly as wild-type AQP2 did [10].

The green fluorescent protein (GFP) has recently become available for studying the behavior/localization of particular proteins in the living system. The intracellular distribution of exogenously expressed GFP-protein chimeras can be directly tracked 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 GFP–AQP5 chimeras may not be handled by the intracellular sorting machinery in the same way as the native protein in cells of any given type. For example, although both of the glucose transporter-4 (GLUT4) connected to GFP at its amino-terminus and the same protein with GFP at its carboxyl terminus are translocated to the plasma membrane in response to insulin, only the GLUT4 tagged with GFP at its carboxy terminus is re-internalized upon insulin removal [11]. The GLUT4 tagged with GFP at the amino terminus remains on the plasma membrane [11]. In the study of AQP2 trafficking, the GFP–AQP2 chimera (GFP fused to the amino-terminus of AQP2) expressed in LLC-PK_1 cells was found to traffick in a regulated pathway from intracellular vesicles toward the basolateral plasma membrane in response to vasopressin or forskolin stimulation. In contrast, the AQP2–GFP chimera (GFP fused to the carboxyl terminus of AQP2) was localized constitutively on both apical and basolateral plasma membranes. The cellular localization of this chimera was not modified by vasopressin or forskolin [12].

The present study was aimed at assessing the cellular regulation of AQP5 trafficking. For this purpose, we constructed two types of chimeras, one in which GFP was fused to the amino-terminus of AQP5, designated as GFP–AQP5, and the second in which GFP was fused to the carboxy-terminus of AQP5, designated as AQP5–GFP. MDCK cells were transfected with these chimeras for analysis of their trafficking pathways. Furthermore, to determine the role of the putative phosphorylation site at the C-terminal domain in the regulation of AQP5 trafficking, a PKA/protein kinase G (PKG) target site (Thr-259) was altered to Ala so that this residue would not be phosphorylated when the mutated protein was expressed in MDCK cells.

2. Materials and methods

2.1. Reagents

ISOGEN was purchased from Nippon Gene (Tokyo, Japan). RNase H Minus, M-MLV reverse transcriptase and pGEM-T Easy vector were from Promega (Madison, WI). QIA quick Gel Extraction kit was from Qiagen (Tokyo, Japan). Two types of vectors, pEGFP-C2 and pEGFP-N2, and anti-GFP antibody (BD Living Colors™, A.v. Peptide Antibody) were obtained from BD Biosciences Clontech (Palo Alto, CA). Dulbecco's modified Eagle's medium F-12 HAM (DMEM/F-12), N^6, 2′-*O*-dibutyryladenosine 3′, 5′-cyclic monophosphate (dbcAMP), palmitoyl-DL-carnitine (PCC), G418, and phorbol 12-myristate 13-acetate (PMA) were procured from Sigma-Aldrich (Tokyo, Japan). Lipofectamine 2000 Reagent was from Invitrogen (Tokyo, Japan); and N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfoamide (H-89), from Seikagaku Corporation (Tokyo, Japan). Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG and enhanced chemiluminescence (ECL) Western Blotting Detection System were procured from Amersham Biosciences (Piscataway, NJ). Alexa Fluor™ 594-conjugated goat anti-rabbit IgG (H+L) antibody was from Molecular Probes (Eugene, OR). Glass-bottomed microwell dishes came from MatTek Corporation (Ashland, MA).

2.2. PCR-cloning of AQP5 cDNA and construction of GFP-fusion genes

Total RNA was prepared from rat submandibular glands by using ISOGEN and treated with DNaseI at 37 °C for 1 h. cDNA was synthesized from 2 μg of total RNA with random hexamers and M-MLV reverse transcriptase, RNase H Minus, at 37 °C for 1 h. The cDNA of rat *AQP5*

Table 1
Sequence of PCR primer for amplification of the *AQP5* gene

Designation	Primer sequences
rAQP5K-5 (sense)	5′-CTCGAGGCCCCCAAGGCACCATGAA-3′
rAQP5K-4 (antisense)	5′-GAATTCGTGTGCCGTCAGCTCGATGG-3′
rAQP5K-11 (antisense)	5′-GAATTCGTGTGCCGTCAGCTCGATGGCC-3′
rAQP5K-6 (sense)	5′-CTCGAGCACCATGAAAAAGGAGGTGT-3′
rAQP5K-8 (antisense)	5′-GAATTCAGTGTGCCGTCAGCTCGATGG-3′
rAQP5K-12 (antisense)	5′-GAATTCTCAGTGTGCCGTCAGCTCGATGGCC-3′

Underline, restriction enzyme recognition sequence; Double underline, mutational sequence.

(GeneBank accession number U16245), including a Kozak sequence just upstream of the translation initiation codon (ATG), was then amplified by PCR using the primer pairs listed in Table 1. Namely, for construction of GFP–AQP5, 2 primer sets were designed, i.e., rAQP5K-6 (sense) and rAQP5K-8 (antisense) for the wild-type construct, and rAQP5K-6 (sense) and rAQP5K-12 (antisense) for the T259A mutant construct. The PCR products produced by using these primers were sub-cloned in the pGEM-T Easy vector, and then ligated to pEGFP-C2 as described below. Similarly, the primer set rAQP5K-5 (sense) and rAQP5K-4 (antisense) was designed for construction of wild-type AQP5–GFP; and the set rAQP5K-5 (sense) and rAQP5K-11 (antisense), for the T259A mutant-type AQP5–GFP. The PCR products produced by using these primers were sub-cloned, and ligated to pEGFP-N2 (see below). The PCR conditions were set as follows: denaturation at 96 °C for 30 s, annealing at 61 °C for 30 s, and extension at 72 °C for 80 s. These steps were repeated for 35 cycles following an initial denaturation at 96 °C for 10 min. The resulting PCR product was cloned in the pGEM-T Easy vector according to the manufacturer's protocol.

To prepare the GFP–AQP5 or the AQP5–GFP fusion chimeras, we double digested the pGEM-T Easy vector containing the *AQP5* cDNA with *Xho*I/*Eco*RI sites at its 5′ and 3′ ends with *Xho*I and *Eco*R*I* restriction enzymes to excise the insert from the vector. The insert DNA (*AQP5* cDNA) was separated from the vector DNA by agarose gel electrophoresis and purified by using the QIA quick Gel Extraction kit. The DNA was ligated to vector pEGFP-C2 or vector pEGFP-N2 at the *Xho*I/*Eco*RI sites, as indicated above.

2.3. Cell culture and stable transfection

MDCK cells were cultured in DMEM/F-12 containing 5% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The cells were cultured at 37 °C under a humidified 5% CO_2 atmosphere. By use of the Lipofectamine 2000 Reagent, 10 μg each of wild-type and T259A-mutant of the GFP–AQP5 and AQP5–GFP expression constructs, and the GFP vector without an insert, were used to transfect MDCK cells that had been grown on 9 cm-dishes to a subconfluent density. After 48 h of incubation, the transfected cells were selected upon cultivation for 2 weeks in medium containing 1 mg/ml of G418. The resultant clones were isolated, expanded to large cultures, and subjected to further analysis.

2.4. Antibodies

AQP5 antibody used in the present study was raised by injecting a rabbit with a mixture of Freund's complete adjuvant containing *M. Butyricum* and the carboxyl-terminal peptide of rat AQP5 (NH_2-CDHREERKKTIELL-TAH-COOH) conjugated to keyhole limpet hemocyanin. The specificity of this antibody was confirmed by Western blotting conducted as reported previously [13].

2.5. Western blotting

Transfected MDCK cells grown on 9-cm plastic dishes were treated with 900 μl of lysis buffer (10 mM Tris–HCl, pH 8.0, containing 1% Triton-X-100, 50 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenyl methylsulfonyl fluoride (PMSF), and 1 μg/ml aprotinin) at 4 °C for 5 min. The resulting cell lysates were homogenized by the use of a glass/Teflon homogenizer, and centrifuged at 800×*g* for 10 min at 4 °C to remove the nucleus and cell debris. The supernatant obtained was centrifuged at 105,000×*g* for 1 h at 4 °C, after which the pellet was collected and re-suspended in the homogenization buffer. A small portion of the supernatant was used for protein measurement. Ten micrograms of the total membrane protein was separated by 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose filter. After having been blocked with phosphate buffered saline (PBS) containing 1% or 5% skim milk (for anti-AQP5 and anti-GFP antibodies, respectively), the filter was incubated at 4 °C overnight with either anti-AQP5 antiserum diluted at 1:3000 or anti-GFP antibody diluted at 1:200. To verify the specificity of positive bands, we performed a control experiment with the same concentration of antibody that had been treated with 40 μg/ml of the immunizing peptide. The filter was then washed and incubated with HRP-conjugated goat anti-rabbit IgG diluted at 1:3000 for 1 h at room temperature. The filter was finally subjected to an ECL Western Blotting Detection System and exposed to Fuji RX X-ray film.

2.6. Fluorescence microscopy

For observation of GFP fluorescence, non-fixed cells cultured at a density of near-confluence on glass-bottomed microwell dishes were imaged by means of a fluorescence microscope (Model IX71, Olympus, Japan). For staining of AQP5 by Alexa Fluor™ 594, the cells fixed with paraformaldehyde were treated with 0.1% Triton X-100 in PBS for 15 min at room temperature to expose intracellular antigenic sites. The cells were then washed 3 times with PBS, blocked with 0.2% gelatin in PBS for 1 h, and incubated with 500-times diluted rabbit anti-AQP5 antiserum for 1 h at room temperature. The cells were next washed with the blocking solution and

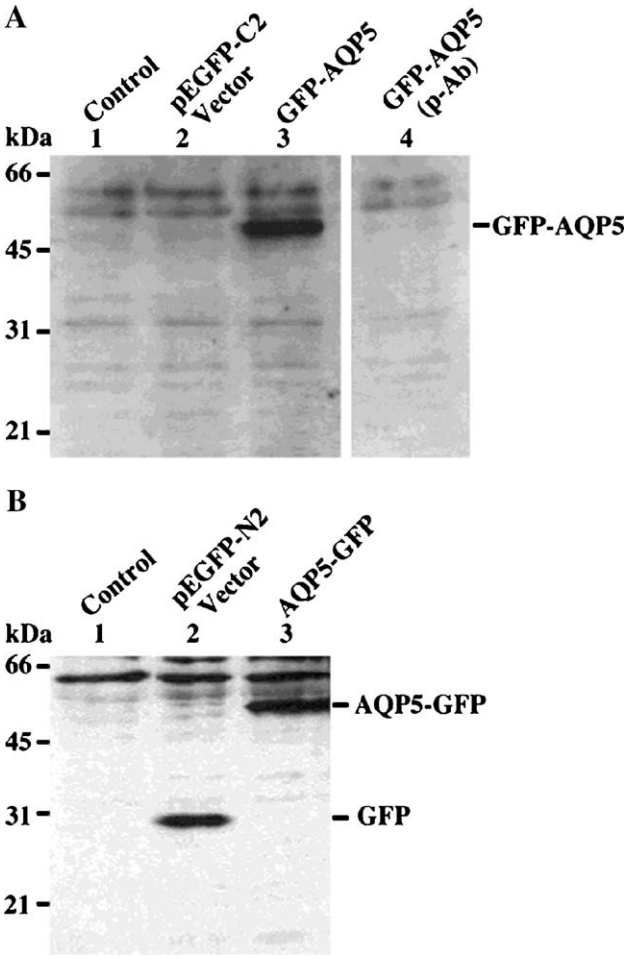

Fig. 1. Immunoblot analysis of the fusion proteins of GFP and AQP5 expressed in MDCK stable transfectants. Aliquots of 10 μg protein of the total membrane fraction from normal and transfected MDCK cells were separated by SDS-PAGE (10% polyacrylamide gel) and probed with the antibodies indicated below. (A) Blots probed with anti-AQP5 antibody (lanes 1–3) and with anti-AQP5 antibody pre-absorbed with antigen peptide (p-Ab; lane 4). Lane 1, control cells (cells without transfection); lane 2, cells transfected with pEGFP-C2 vector; lanes 3 and 4, cells transfected with GFP–AQP5. (B) Blots probed with anti GFP antibody. Lane 1, control cells (cells without transfection); lane 2, cells transfected with pEGFP-N2 vector; lane 3, cells transfected with AQP5–GFP. The molecular weight of standard markers in kDa is indicated on the left.

reacted with 200-times diluted Alexa Fluor™ 594-conjugated goat anti-rabbit IgG (H+L) for 1 h at room temperature. GFP and Alexa Fluor™ 594 fluorescence were observed by using U-MNIBA2 and U-MWIG2 filters (Olympus, Tokyo Japan), respectively.

2.7. Treatments with dbcAMP and H-89 or PCC

Approximately 72 h before dbcAMP treatment, cells were plated on glass-bottomed microwell dishes and cultured for 48 h in the medium described above. The medium was then replaced with DMEM/F12 containing 0.5% fetal bovine serum. After 24 h of serum-starvation, different concentrations of dbcAMP were added to separate cultures, which were then incubated for 18 h. Thirty micromolar H-89 or 10 μM PCC was added to the cultures 6 h before the addition of dbcAMP to inhibit PKA or PKC, respectively.

3. Results

3.1. Expression of GFP–AQP5 and AQP5–GFP chimeric proteins

Two GFP vectors were used to construct two AQP5 chimeric proteins. Normal *AQP5* cDNA was fused to the 3′- or 5′-end of the *GFP* gene in pEGFP-C2 or pEGFP-N2 vectors, and the resulting protein products expressed in transfected cells were designated as GFP–AQP5 and AQP5–GFP, respectively. MDCK cells ware stably transfected with either GFP–AQP5 or AQP5–GFP or with their mutant constructs. Expression of the full-length fusion proteins in stably transfected MDCK cells was confirmed by Western blotting using anti-AQP5 antibody and anti-GFP polyclonal antibody.

In the MDCK cells transfected with GFP–AQP5, the anti-AQP5 antibody detected a 54-kDa band which concurred with the expected size for the chimeric protein (27 kDa GFP+27-kDa AQP5; Fig. 1A, lane 3). When the antibody was preabsorbed with AQP5 C-terminal peptide, this band was completely absent thus indicating that the antiserum detected specifically the chimeric protein (Fig. 1A, lane 4). Similarly, the chimeric protein of 54 kDa was detected with anti-GFP polyclonal antibody in the AQP5–GFP transfected cells (Fig. 1B, lane 3). The anti-AQP5 antibody did not detect AQP–GFP (data not shown) since this antibody was reactive only to C-terminal sequence of AQP5 (see Materials and methods and [13]), and since GFP was connected to this domain in the chimeric protein. The pEGFP-N2-vector transfected cells exhibited a 27-kDa GFP band with anti-GFP antibody (Fig. 1B, lane 2), and no bands were detected in control cells without transfection (Fig. 1A and B, lane 1). These results suggest that the GFP vector and GFP–AQP5 or AQP5–GFP chimeras were successfully expressed in MDCK cells.

3.2. Localization of GFP–AQP5 and AQP5–GFP chimeric proteins in MDCK cells

The fusion proteins of GFP and AQP5 expressed in MDCK cells were detected by using a fluorescent microscopy. The GFP–AQP5 protein was localized primarily on intracellular vesicles, as seen by the inherent fluorescence of GFP (Fig. 2B). By staining with the anti-AQP5 antibody and Alexa Fluor™ 594-conjugated goat anti-rabbit IgG (H+L) antibody (Fig. 2C), the AQP5 label was shown to overlap completely the GFP fluorescence, indicating that the localization as revealed by the inherent GFP fluorescence was due to the GFP–AQP5 fusion protein, not to GFP protein that might be

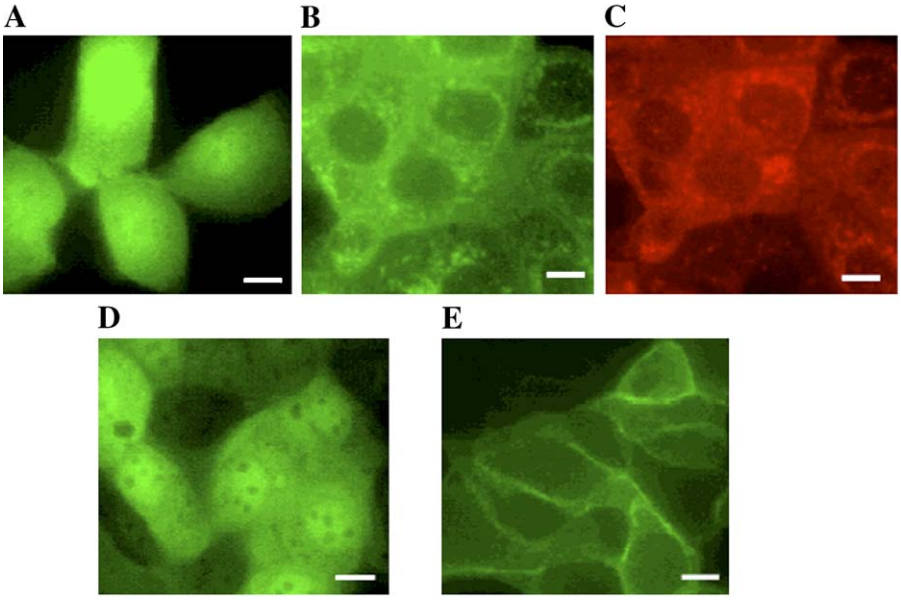

Fig. 2. Cellular localization of fusion proteins of GFP and AQP5 in MDCK stable transfectants. (A) Cells transfected with pEGFP-C2 vector, (B and C) cells transfected with GFP–AQP5; (D) cells transfected with pEGFP-N2 vector, (E), cells transfected with AQP5–GFP. (A, B, D, and E) Inherent green fluorescence of GFP. (C) Red fluorescence of Alexa Fluor™ 594 from the conjugate with goat anti-rabbit IgG, used as a secondary antibody to localize the primary antibody (rabbit anti-AQP5 antibody). This micrograph exhibits fluorescence with a location identical to that of the inherent fluorescence by GFP shown in (B). Scale bars: 30 μm.

generated from the fusion protein by cleavage. On the other hand, MDCK cells transfected with the pEGFP-C2 vector exhibited localization of GFP fluorescence mainly on the cytoplasmic area (Fig. 2A). Therefore, the inherent fluorescence of the GFP–AQP5 chimera was suitable and appropriate for monitoring the trafficking of AQP5 in this cell culture system.

In contrast to the AQP5 fusion protein with GFP at its amino-terminus, the carboxyl terminus fusion protein was predominantly localized on plasma membranes under non-stimulated condition, with only a small amount detected on the intracellular structures (Fig. 2E). MDCK cells transfected with the pEGFP-N2 vector exhibited localization of GFP fluorescence mainly on the cytoplasmic area (Fig. 2D). Therefore, AQP5 fusion proteins with GFP at its carboxy terminus and amino terminus were concluded to be localized in completely different places; i.e., on the plasma membrane and on intracellular vesicles, respectively (Fig. 2B and E). Thus, the presence of the GFP protein at the carboxyl terminus of AQP5 appears to have caused spontaneous movement of this molecule toward the plasma membrane.

3.3. Effects of dbcAMP on the trafficking of GFP–AQP5 protein in MDCK cells

To determine whether the GFP–AQP5 chimera retains its capacity to traffick in response to second messengers, we treated MDCK cells expressing GFP–AQP5 protein with 100, 300, 500, 700 or 1000 μM dbcAMP for different time. We initially examined the membrane trafficking of GFP–AQP5 for 20 min with 15-s interval and 4 h with 30-min interval. No translocation of the present molecules took place during this time span. The time course study taking longer time was then conducted and translocation was observed around 18 h after which the membrane localization was stable until 24 h. The dose response experiment was also carried out. Compared with the control cells receiving no additive (Fig. 3A), cells treated with 500–1000 μM dbcAMP showed the apparent translocation of GFP–AQP5 from cytoplasmic vesicles to the plasma membrane, and therefore the result with 500 μM dbcAMP is shown in Fig. 3B. Obscure and modest translocation of the chimeric protein was observed at 100–300 μM dbcAMP. To determine whether PKA activity was required for the effects of dbcAMP on GFP–AQP5 translocation, we tested the effect of H-89, a PKA inhibitor; i.e., H-89 (30 μM) was added at 6 h before the addition of 500 μM dbcAMP. Immunofluorescent micrographs of cells treated with dbcAMP in the presence of H-89 showed a strong fluorescence at the cytoplasmic vesicles (Fig. 3D) as compared with cells treated with dbcAMP alone (Fig. 3B), thus suggesting the involvement of PKA in this trafficking. The addition of H-89 alone did not cause any change in the cellular localization of GFP–AQP5 (Fig. 3C). By using a motif search program (GENETYX-MAC), we found a putative PKC target motif at amino acid residues 152–154 (Ser–Arg–Arg) which partially overlaps the PKA/PKG target motif (amino acid residue 153–156; Arg–Arg–Thr–Ser). We therefore examined if activation of PKC would affect the translocation of AQP5. The incubation of the transfected cells with 100 nM PMA, however, did not cause trafficking of AQP5 (data not shown). We also examined the effects of PCC, a PKC inhibitor, to determine whether the inhibition of this kinase would affect GFP–AQP5 translocation by dbcAMP. dbcAMP in the presence of PCC (Fig. 3F) showed a similar effect as that seen in the absence of PCC (Fig. 3B). PCC alone did not have any effect on the translocation of GFP–AQP5 (Fig. 3E).

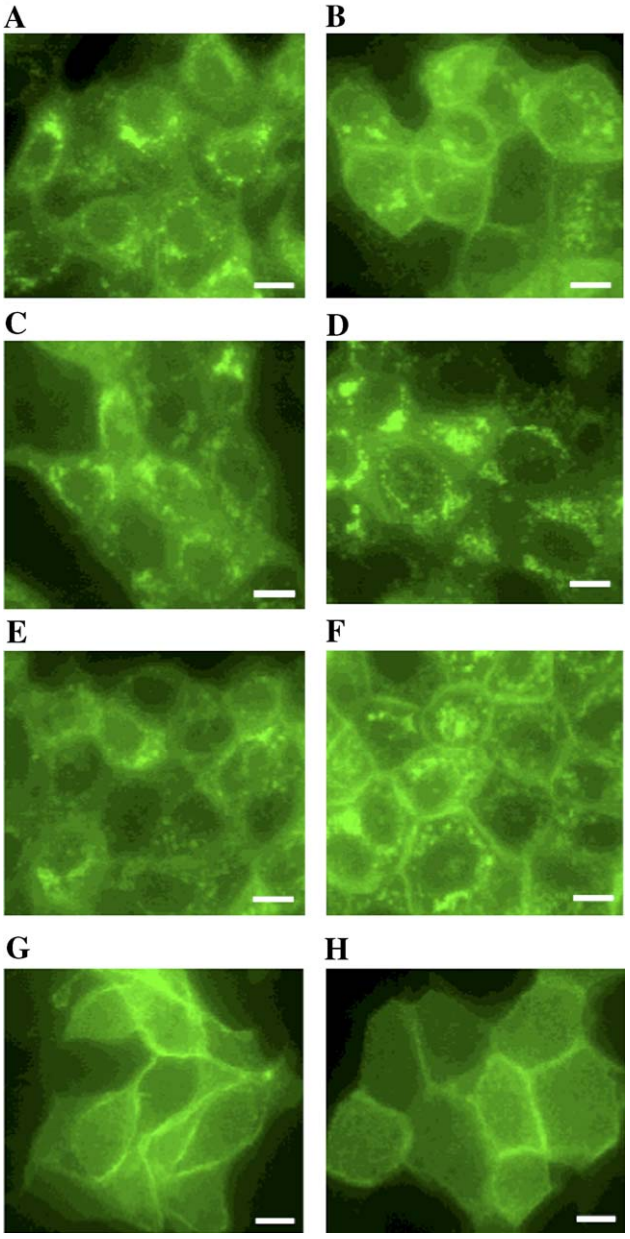

Fig. 3. Effects of dbcAMP, H-89, and PCC on cellular localization of GFP–AQP5 and AQP5–GFP in MDCK stable transfectants. (A–F) MDCK cells transfected with GFP–AQP5; (G and H) MDCK cells transfected with AQP5–GFP. Cells were incubated in the absence (A, B, G, and H) or presence of 30 μM H-89 (C and D) or 20 μM PCC (E and F) for 24 h. B, D, F, and H, 500 μM dbcAMP was added to cell cultures identical to those of A, C, E, and G 6 h after addition of inhibitors, and the cells were cultured for a further 18 h. Scale bars: 30 μm.

Incidentally, MDCK cells transfected with AQP5–GFP and cultured under the normal condition (with no added stimulants/inhibitors) expressed the chimeric protein at cell membrane (Fig. 3G), which localization was not affected by dbcAMP (Fig. 3H).

3.4. Effects of T259A on the trafficking of AQP5 protein in MDCK cells

The sequence of the PKA target motif in a C-terminal intracellular domain of AQP2 (253–256) is believed to be important for AQP2 to translocate to the plasma membrane *via* a mechanism in which cAMP is involved [8]. AQP5 has a consensus PKA/PKG-target motif (Arg–Lys–Lys–Thr) in a cytoplasmic loop located similarly as that of AQP2. To determine whether the putative PKA/PKG target motif participated in translocation of AQP5 to the plasma membrane, we mutated Thr-259 in this site to Ala to retain the non-phosphorylated state. MDCK cells were stably transfected with GFP–AQP5–T259A and AQP5–GFP–T259A expression constructs of these mutant proteins. In immunoblot analysis of selected clones, these transfectants expressed 54-kDa mutant proteins, the size of which was exactly the same as that for GFP–AQP5 (Fig. 4A). Fluorescence microscopy of the clones expressing fusion proteins for mutant AQP5 with GFP at the amino- or carboxy-terminus showed localization similar to that of the respective wild-type AQP5 (Fig. 4B and E). Stimulation of the cells with the dbcAMP caused translocation of GFP–AQP5–T259A to the plasma membrane, in similar way as demonstrated for GFP–AQP5 (Fig. 4C). H-89 inhibited the

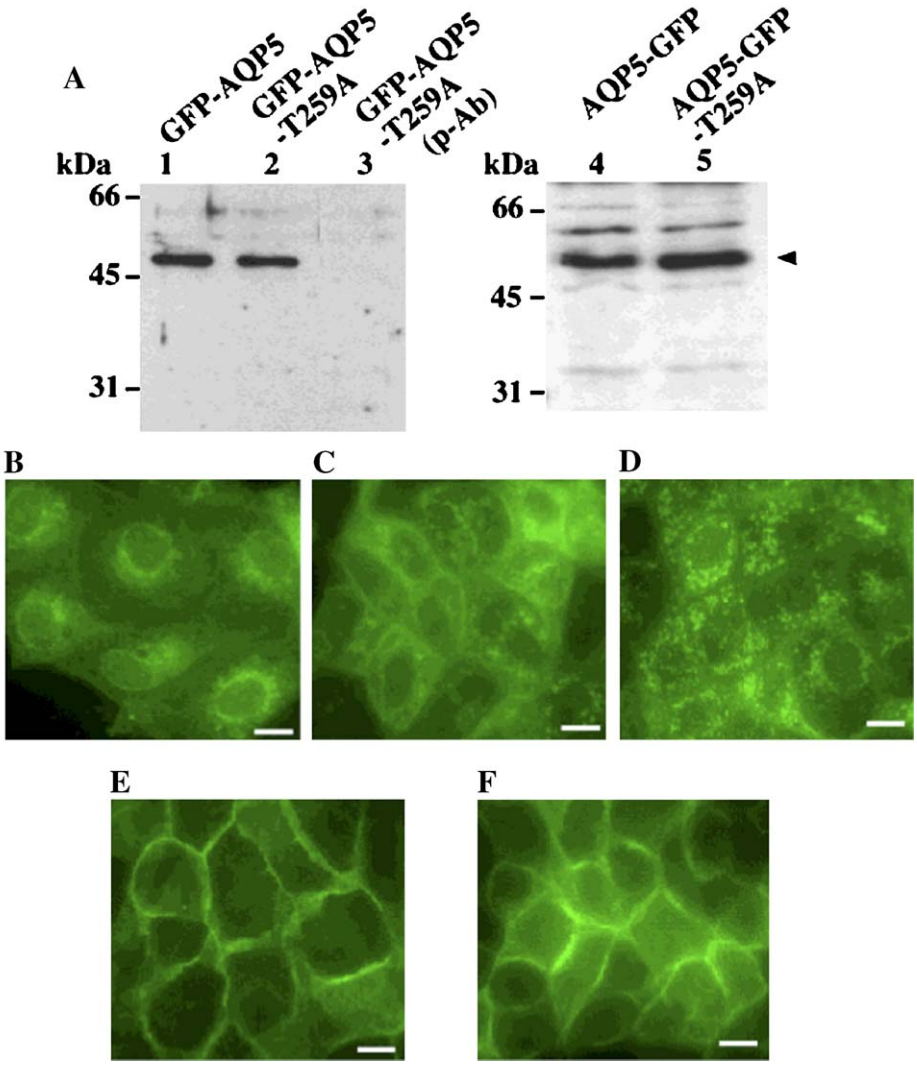

Fig. 4. Western blot analysis and cellular localization of T259A mutant of GFP–AQP5 and AQP5–GFP fusion protein in MDCK cells stable transfectants. (A) Western blotting of wild-type and mutant AQP5 chimeric proteins. Aliquots of 10 μg cell lysates from transfected MDCK cells were separated by SDS-PAGE (10% polyacrylamide gel) and probed with various antibodies. Lanes 1–3, blots probed with anti-AQP5 antibody (lanes 1 and 2) and with anti-AQP5 antibody pre-absorbed with antigen peptide (p-Ab; lane 3). Lane 1, cells transfected with wild-type GFP–AQP5; lanes 2 and 3, cells transfected with a mutant, GFP–AQP5–T259A. Lanes 4 and 5, blots probed with anti GFP antibody. Lane 4, cells transfected with wild-type AQP5–GFP; lane 5, cells transfected with the mutant AQP5–GFP–T259A. (B–F) Fluorescence microscopy of MDCK cells transfected with GFP–AQP5–T259A (B–D) and AQP5–GFP–T259A (E and F). (B and E) Incubation in the absence of additives; (C and F) incubation in the presence of 500 μM dbcAMP for 24 h. (D) Incubated in the presence of 500 μM dbcAMP and 30 μM H-89 (for 24 h and 18 h, respectively). Scale bars: 30 μm.

effect of dbcAMP (Fig. 4D). Similar to wild-type AQP5–GFP, AQP5–GFP–T259A was localized in the plasma membrane (Fig. 4E), and dbcAMP did not induce any change in its cellular localization (Fig. 4F).

4. Discussion

In this report, we described the production and initial characterization of two MDCK cell lines, one expressing GFP–AQP5 and the other AQP5–GFP. Our study was conducted to examine the effect of the GFP fusion site of the AQP5 molecule on its intracellular translocation or trafficking. The amino-terminal fusion chimera, GFP–AQP5, was shown to exist at intracellular vesicles under the unstimulated condition and to translocate from these vesicles to the plasma membrane upon stimulation with dbcAMP (Fig. 3A and B). In contrast to GFP–AQP5, the AQP5–GFP fusion protein was localized consistently at the plasma membrane (Fig. 3G and H). Gustafson et al. [12] constructed the amino-terminal and/or carboxy-terminal fusion chimeras of another water channel, AQP2 (GFP–AQP2, and AQP2–GFP, respectively), and transfected LLC-PK_1 cells with them. They reported that the GFP–AQP2 fusion protein was localized on intracellular vesicles whereas the other one, AQP2–GFP, constitutively localized at the apical and basolateral membrane under the unstimulated condition. Their GFP–AQP2 fusion protein was translocated to the plasma membrane upon stimulation of the cells with vasopressin or forskolin. Therefore, the behavior of the present two fusion proteins, AQP5–GFP and GFP–AQP5, is very similar to that of those two AQP2 fusion proteins, supporting the hypothesis that an important targeting motif(s) exist on the carboxy-terminus of AQP5 as well as that of AQP2. In fact, Noda et al. [14] recently reported that PDZ domain of the protein product, signal-induced proliferation-associated gene-1, which is a GAP for Rap1, binds to AQP2 at its carboxy-terminus. Existence of similar mechanism for AQP5 trafficking needs to be determined.

The consensus sequence of the PKA target motif is located in cytoplasmic loop D of AQP5 at amino acid residues 153–156 (Arg–Arg–Thr–Ser, [15]). Based on the results obtained from a motif search program, we found a putative PKC target motif at amino acid residues 152–154 (Ser–Arg–Arg), which overlaps with the PKA target consensus sequence. Further, we found a putative PKA/PKG phosphorylation site at amino acid residues 256–259 (Arg–Lys–Lys–Thr) in the carboxyl terminus cytoplasmic region. Yang et al. [7] examined the effects of cAMP on expression and translocation of AQP5 in MLE-12 cells; they showed that cAMP regulates AQP5 at multiple levels, by increasing synthesis of *AQP5* mRNA and by triggering translocation of AQP5 to the plasma membrane. However, there are not many reports concerning the function of these motifs in the trafficking of AQP5. In this study, we showed that cells treated with 500 μM dbcAMP exhibited trafficking of some vesicles bearing GFP–AQP5 protein toward the plasma membrane although many of these vesicles still remained in the cytoplasm. Furthermore, we examined if PKA, PKC or PKG is involved in dbcAMP-induced trafficking of GFP–AQP5. We showed that the addition of PCC, a PKC specific inhibitor, prior to incubation with dbcAMP induced translocation of AQP5 to the plasma membrane, indicating that PKC was not involved in the dbcAMP-induced trafficking in the present MDCK cells (Fig. 3). Activation of PKC by PMA did not induce trafficking, either (data not shown).

In HSG, a submandibular gland cell line, AQP5 expressed by transfection is translocated to the plasma membrane depending upon increase of cellular calcium ion [6], while it is trafficked to the plasma membrane by PKA-dependent fashion in the duodenal Brunner's gland [15]. Therefore, the cell type AQP5 is expressed may in part affect the protein kinase species which become involved in the phosphorylation of AQP5. In MDCK cells, but not the submandibular gland cells, PKA-anchoring proteins (AKAPs) might be expressed to induce the cAMP-dependent translocation, although the detail mechanism is still unknown.

On the other hand, sodium nitroprusside, L-arginine, and atrial natriuretic peptide as well as permeable cGMP analogs are known to increase cGMP levels and induce AQP2 trafficking in kidney collecting duct cells both in vivo and in vitro. Bouley et al. [16] reported that PKG phosphorylates the carboxyl terminus of AQP2 in vitro and that cGMP-stimulated membrane accumulation of AQP2 does not occur in cells expressing the S256A mutation of AQP2. In this report, to determine whether a putative PKA/PKG target motif (256–259 (Arg–Lys–Lys–Thr)) was involved in the phosphorylation and the translocation of GFP–AQP5, we prepared GFP–AQP5–T259A and AQP5–GFP–T259A expression constructs in which Thr-259 was mutated to Ala to generate the non-phosphorylated state and used these constructs to stably transfect MDCK cells. Stimulation of the cells with dbcAMP caused the translocation of GFP–AQP5–T259A to the plasma membrane, whereas H-89 inhibited such translocation, as has been demonstrated for wild-type GFP–AQP5. GFP–AQP5–T259A having a mutation at a putative PKA/PKG phosphorylation site did not show any effect on the translocation by dbcAMP, suggesting that this putative PKA/PKG target motif (256–259 (Arg–Lys–Lys–Thr)) was not involved in the regulation of GFP–AQP5 in MDCK cells.

Some AKAPs are abundant in the fraction of AQP2-immunopurified vesicles prepared from IMCD cells. Inhibition of forskolin-induced AQP2 translocation with a peptide that prevents PKA–AKAP interaction demonstrated that, besides its enzymatic activity, tethering of PKA to subcellular compartment is essential for AQP2 translocation [17]. The use of the present cells has, of course, a limitation and the experiment with a salivary gland cell line or MLE12 cells would significantly support the present finding.

Based on our observations, the GFP–AQP5 but not AQP5–GFP chimera will be useful to study AQP5 trafficking in vitro; exploring the protein that interacts with carboxyl-terminal sequence of AQP5 is important to understand the mechanism of AQP5 trafficking, and is need to be determined in the future.

Acknowledgements

This work was supported in part by a grant-in-aid for scientific research (15791062) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

[1] S. Raina, G.M. Preston, W.B. Guggino, P. Agre, Molecular cloning and characterization of an aquaporin cDNA from salivary, lacrimal, and respiratory tissues, J. Biol. Chem. 270 (1995) 1908–1912.

[2] S. Nielsen, L.S. King, B.M. Christensen, P. Agre, Aquaporins in complex tissues: II. Subcellular distribution in respiratory and glandular tissue of rat, Am. J. Physiol., Cell Physiol. 273 (1997) C1549–C1561.

[3] L.S. King, S. Nielsen, P. Agre, Aquaporins in complex tissues: I. Developmental patterns in respiratory and glandular tissues of rat, Am. J. Physiol., Cell Physiol. 273 (1997) C1541–C1548.

[4] T. Matsuzaki, T. Suzuki, H. Koyama, S. Tanaka, K. Takata, Aquaporin-5 (AQP5), a water channel protein, in the rat salivary and lacrimal glands: immunolocalization and effect of secretory stimulation, Cell. Tissue Res. 295 (1999) 513–521.

[5] Y. Ishikawa, T. Eguchi, M.T. Skowronski, H. Ishida, Acetylcholine acts on M3 muscarinic receptors and induces the translocation of aquaporin5 water channel via cytosolic Ca^{2+} elevation in rat parotid glands, Biochem. Biophys. Res. Commun. 245 (1998) 835–840.

[6] J. Tada, T. Sawa, N. Yamanaka, M. Shono, T. Akamatsu, K. Tsumura, M.N. Parvin, N. Kanamori, K. Hosoi, Involvement of vesicle–cytoskeleton interaction in AQP5 trafficking in AQP5-gene-transfected HSG cells, Biochem. Biophys. Res. Commun. 266 (1999) 443–447.

[7] F. Yang, J.D. Kawedia, A.G. Menon, Cyclic AMP regulates aquaporin 5 expression at both transcriptional and post-transcriptional levels through a protein kinase a pathway, J. Biol. Chem. 278 (2003) 32173–32180.

[8] K. Fushimi, S. Sasaki, F. Marumo, Phosphorylation of serine 256 is required for cAMP-dependent regulatory exocytosis of the aquaporin-2 water channel, J. Biol. Chem. 272 (1997) 14800–14804.

[9] T. Katsura, C.E. Gustafson, D.A. Ausiello, D. Brown, Protein kinase A phosphorylation is involved in regulated exocytosis of aquaporin-2 in transfected LLC-PK1 cells, Am. J. Physiol., Renal Phsiol. 272 (1997) F817–F822.

[10] B.W.M. van Balkom, P.J.M. Savelkoul, D. Markovich, E. Hofman, S. Nielsen, P. van der Sluijs, P.M.T. Deen, The role of putative phosphorylation sites in the targeting and shuttling of the aquaporin-2 water channel, J. Biol. Chem. 277 (2002) 41473–41479.

[11] S.P. Dobson, C. Livingstone, G.W. Gould, J.M. Tavare, Dynamics of insulin-stimulated translocation of GLUT4 in single living cells visualized using green fluorescent protein, FEBS Lett. 393 (1996) 179–184.

[12] C.E. Gustafson, S. Levine, T. Katsura, M. McLaughlin, M.D. Alexio, B.K. Tamarappoo, A.S. Verkman, D. Brown, Vasopressin regulated trafficking of a green fluorescent protein-aquaporin 2 chimera in LLC-PK_1 cells, Histochem. Cell Biol. 110 (1998) 377–386.

[13] M.N. Parvin, K. Tsumura, T. Akamtsu, N. Kanamori, K. Hosoi, Expression and localization of AQP5 in the stomach and duodenum of the rat, Biochim. Biophys. Acta 1542 (2002) 116–124.

[14] Y. Noda, S. Horikawa, T. Furukawa, K. Hirai, Y. Katayama, T. Asai, M. Kuwahara, K. Katagiri, T. Kinashi, M. Hattori, N. Minato, S. Sasaki, Aquaporin-2 trafficking is regulated bt PDZ-domain containing protein APA-1, FEBS Lett. 568 (2004) 139–145.

[15] M.N. Parvin, S. Kurabuchi, K. Murdiastuti, C. Yao, C. Kosugi-Tanaka, T. Akamatsu, N. Kanamori, K. Hosoi, Subcellular redistribution of AQP5 by vasoactive intestinal polypeptide (VIP) in the Brunner's gland of the rat duodenum, Am. J. Physiol-GI and Liver Physiol. 288 (2005) G1283–G1291.

[16] R. Bouley, S. Breton, T. Sun, M. McLaughlin, N.N. Nsumu, H.Y. Lin, D. A. Ausiello, D. Brown, Nitric oxide and atrial natriuretic factor stimulate cGMP-dependent membrane insertion of aquaporin 2 in renal epithelial cells, J. Clin. Invest. 106 (2000) 1115–1126.

[17] E. Klussmann, K. Maric, B. Wiesner, M. Beyermann, W. Rosenthal, Protein kinase A anchoring protein are required for vasopressin-mediated translocation of aquaporin-2 into cell membranes of renal principal cells, J. Biol. Chem. 274 (1999) 4934–4938.