ORIGINAL

Trafficking of GFP-AQP5 chimeric proteins conferred with unphosphorylated amino acids at their PKA - target motif (^{152}SRRTS) in MDCK-II cells

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Abstract : Three constructs having mutated PKA-target motif at ^{152}SRRTS of AQP5, an exocrine type water channel, were prepared and fused to C-terminus of green fluorescence protein cDNA to examine the effects of blocking of phosphorylation at ^{152}SRRTS (a consensus PKA-target motif of AQP5) on translocation or trafficking of the chimeric proteins expressed in the Madin-Darby canine kidney-II (MDCK-II) cells. H-89 treatment increased translocation of wild-type GFP-AQP5 to the apical membrane. All 3 mutant molecules translocated 1.5 to 2 times more than the control wild-type GFP-AQP5. Colchicine but not cytochalasin B inhibited the translocation of wild-type GFP-AQP5. Present results suggest dephosphorylation of this consensus sequence increase GFP-AQP5 translocation, and that microtubules but not microfilaments are involved in this event. J. Med. Invest. 56 : 55-63, February, 2009

Keywords **:** *aquaporin-5, PKA-consensus sequence, trafficking*

INTRODUCTION

Aquaporins (AQPs) are the water channel proteins that have been identified in virtually all living organisms, and they are generally responsible for rapid water movement across the plasma membrane in almost all cells (1, 2). Among the 13 mammalian aquaporins identified so far, *i.e.*, AQP0-AQP12 (2), AQP5 is reported to be expressed in the cell membrane of multiple secretory glands, including the lacrimal, salivary, and airway submucosal glands, as well as in type 1 alveolar cells (3, 4), sweat glands (5), corneal epithelium (6), and duodenal Brunner's gland (7). The essential role of AQP5 has been shown in the study of AQP5 gene knockout mice, which demonstrated significantly increased lethality of the embryo and problems related with water homeostasis (8, 9). In addition, defective cellular trafficking was noted in the lacrimal gland and salivary gland biopsies from patients with Sjögren's syndrome but not in patients with non-Sjögren's dry eye and dry mouth (10, 11), although there are some opposite reports (12).

In a murine lung epithelial cell line (MLE-12), a cAMP analog, 8-(4-chlorophenylthio)-cAMP (cpt-cAMP), was found to induce the AQP5 mRNA and protein expression (13). It is also reported that such stimulations induce translocation of AQP5 from the intracellular storage sites to the apical membrane (14-17) ; *i.e.*, AQP5, majority of which is distributed in the cytoplasm, can be translocated to the plasma membrane in response to cpt-cAMP in MLE-12 cells (13). AQP5 was also targeted to the cell membrane after incubation with cpt-cAMP in Madin-Darby

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canine kidney cells (MDCK) cells (14). In contrast, Sidhaye, *et al.* reported a biphasic effect of cpt-cAMP on AQP5 translocation in MLE-12 cells ; they showed that short-term exposure to this second messenger reduces membrane expression of AQP5, which is subsequently recovered by long-term exposure (18). However, these contradictive results may be mainly due to apparent difference in the basal level of AQP5 expression in the cell membrane ; *i.e.*, the membrane expression of AQP5 was far greater in the later study (18). On the contrary, Woo, *et al.* found that AQP5 membrane targeting may not be regulated solely by PKA phosphorylation and that it can be regulated by more than one mechanism besides that by cAMP dependent phosphorylation (19).

The present study was aimed to determine the role of PKA phosphorylation site at the ^{152}SRRTS in the regulation of AQP5 trafficking. For this purpose we prepared 3 GFP-AQP5 constructs containing mutated PKA target site where one or more of Ser and Thr are replaced with Ala and Val, respectively so that phosphorylation of this residue is restricted or completely prohibited when they were expressed in the host cells. Here, we showed that AQP5 can be expressed on the plasma membrane of MDCK-II cells even phosphorylation of PKA-target sequence is restricted or prohibited, suggesting the existence of PKA-independent trafficking.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM) and cytochalasin B were obtained from Sigma-Aldrich (St.Louis, MO). Lipofectamine 2000, Opti-MEM I, and Alexa Fluor 594-conjugated streptavidin were purchased from Invitrogen (Carlsbad, CA). Sulfosuccinimidyl 6-(biotinamido) hexanoate (sulfo-NHS-LC-biotin) was obtained from Pierce Biotechnology (Rockford, IL). Restriction enzymes, Xho I and EcoR I, came from Takara Bio. Inc (Shiga, Japan). Ligation-Convenience Kit was from Nippon Genetech Co., Ltd (Tokyo, Japan). pEGFP-C2 vector (Cat. No. 6083-1) was obtained from Clontech (Palo Alto, CA). pGEM-T Easy vector was bought from Promega (Madison, WI). Transwell polycarbonate filter culture chambers (Cat. No. 3413) were obtained from Corning Costar (Lowell, MA). Micro slide glass with 70 μm-thickness frame (Cat. No. S 2193A7), and micro cover glasses were products of Matsunami Glass (Osaka, Japan). H-89 came from Seikagaku Corporation (Tokyo, Japan). Colchicine was obtained from Wako Pure Chemical Industries (Osaka, Japan). The QuickChange Site-Directed Mutagenesis Kit was obtained from Stratagene (La Jolla, CA).

PCR cloning and preparation of the pcDNA 3.1/Hygro (+) construct containing wild-type (wt) AQP5 cDNAs

Total RNA was isolated from the submandibular gland (SMG) of rats by using TRI Reagent. A full-length AQP5 cDNA was synthesized by RT-PCR with the SuperScript One-Step RT-PCR System in a thermal cycler (Takara Thermal Cycler MP, Model TP 3000). To a final volume of 25 μl, the following components were mixed on ice : 12.5 μl of 2-times concentrated reaction mixture, 5 pmol of each primer, 0.5 μl of a mixture of reverse transcriptase and Taq DNA polymerase and 1 μg of template RNA. The RT reaction (cDNA synthesis) was carried out at 45°C for 30 min. The reaction mixture was then incubated at 94°C for 2 min to inactivate the enzyme and denature the RNA/cDNA hybrid. The DNA amplification by PCR was next performed for 30 cycles, each cycle consisting of denaturation at 94°C for 15 s, primer annealing at 55°C for 30 s, and extension at 72°C for 1.5 min, followed by 1 cycle of extension at 72°C for 5 min. The primer set used was 5'-AAGCTTCCCC-AAGGCACCATGAAAAA-3' (sense) and 5'-CTCG-AGTCACGAATCTCTGAGGTCTG-3' (anti-sense), which had Hind III and Xho I restriction sites (underlined) at 5' and 3' end, respectively (20). The AQP5 cDNA (1073 bp) obtained by RT-PCR were cloned into the pGEM-T Easy vector, from which the insert was next cut by Hind III and Xho I restriction enzymes and subcloned into a multiple cloning site of the pcDNA 3.1/Hygro (+) vector. The resultant plasmid was termed as wild-type AQP5-Hygro (AQP5(wt)-Hygro). This construct was used for preparation of AQP5 genes with mutated PKA-consensus sequence.

Preparation of AQP5-Hygro having mutated PKA-target motif at deduced amino acids 152-156 (^{152}SRRTS) by site-directed mutagenesis

The AQP5-Hygro cDNAs having mutated PKA-target motif at nucleotides 454-468 (5'-CCTCCAC-CGAC**TCTCGCCGAACCAGC**CCTGTGGGCTC-3'), which corresponds to the amino acid sequence ^{152}SRRTS, were prepared by using a QuickChange site-directed mutagenesis kit. First, AQP5(wt)-Hygro plasmid described above was used as a template,

and artificial mutations were introduced. AQP5's with mutated nucleotide sequences were next subcloned into pEGFP-C2. The following is the experimental design employed : 3 primers, PK-1, PK-3, and PK-5 were synthesized ; the sequence of PK-1 was 5'-CCTCCACCGAC**GCT**CGCCGAACC**GCC**CCTGTGGGCTC-3', which had 3 mutated nucleotides (indicated by the underline), resulting in ^{152}ARRTA. The sequence of PK-5 was 5'-CCACCGACTCTCGCCGA**GTC**AGCCCTGTGG-3', which had 2 mutated nucleotides, resulting in ^{152}SRRVS. Lastly, the sequence of PK-3 was 5'-CCACCGAC**GCT**CGCCGA**GTCGCC**CCTGTGG-3', which had 5 mutated nucleotides, resulting in ^{152}ARRVA. PK-2, PK-4, and PK-6 were complementary DNAs of PK-1, PK-3, and PK-5, respectively. The AQP5-Hygro plasmids having these mutated sequences were designated as AQP5(S152A/S156A)-Hygro, AQP5(T155V)-Hygro, and AQP5(S152A/T155V/S156A)-Hygro, respectively. In this nomenclature, S152A, T155V, and S156A denote that serine (S) at position 152, threonine (T) at position 155, and serine (S) at position 156 were replaced with alanine (A), valine (V), and alanine (A), respectively. Plasmid, AQP5(S152A/S156A)-Hygro was prepared directly by using AQP5(wt)-Hygro as a template and the set of primers, PK-1 and PK-2. Similarly, AQP5(T155V)-Hygro was synthesized by using the same plasmid and the primer set, PK-5 and PK-6. Plasmid, AQP5(S152A/T155V/S156A)-Hygro was prepared from AQP5(S152A/S156A)-Hygro used as a template and the set of primers, PK-3 and PK-4. The brief experimental procedure was as follows. To a final volume of 50 μl, the following components were mixed : 5 μl of 10 x reaction buffer, each set of primers (125 ng/3 μl), 1 μl of dNTP mixture, 50 ng of the template plasmid, 1 μl of PfuTurbo DNA polymerase, and distilled water. The PCR was performed for 18 cycles, each cycle consisting of denaturation at 95°C for 30 s, primers annealing at 55°C for 1 min, and extension at 68°C for 13 min. The PCR products were examined by electrophoresis on 1% agarose gel to ensure sufficient amplification. The PCR product was then treated with Dpn I endonuclease at 37°C for 1 h to digest methylated, parental DNA templates, and purified by electrophoresis on 1% agarose gel. The plasmids with mutated AQP5 sequences were next used for transformation and introduced into *E.coli* JM109 ; and the sequences of cloned plasmids were then verified by DNA sequencing. The AQP5-Hygros having a mutated PKA-target motif were subcloned into pEGFP-C2 as described below.

Construction of chimeras, GFP-AQP5(wt) and GFP-AQP5s having mutated PKA-target motif

For transfection experiments, we prepared the constructs of chimeric genes of AQP5 and EGFP following the report which used GFP-AQP2 for study of AQP2 trafficking (21). Complementary DNAs of AQP5's were connected to 3'-end of EGFP DNA in the pEGFP-C2 plasmid because AQP5 connected to N-terminus of EGFP is known to be expressed constitutively at the plasma membrane (14) and is not suitable for the trafficking study. Wild-type (wt) and mutant AQP5 inserts in the Hygro vector were recovered by digestion with Xho I and EcoR I and subcloned into the multiple cloning site of pEGFP-C2 plasmid. The resultant plasmids were termed GFP-AQP5(wt), GFP-AQP5(S152A/S156A), GFP-AQP5(T155V), and GFP-AQP5(S152A/T155V/S156A), respectively. The sequence of AQP5 inserts in all constructs was verified by use of an ABI PRISM 3100-Avant genetic analyzer (Applied Biosystems, Foster City, CA).

Cell culture, transient transfection, cell surface biotinylation, and observation under a confocal laser scanning microscope

MDCK-II cells, kindly provided by Dr. Mikio Furuse (Kobe University), were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate at 37°C in 5% CO_2 ; they were supplemented with fresh medium every third day. For experiments, MDCK-II cells were plated at the cell density of 6×10^4 cells/well on 6.5-mm polycarbonate membranes 48 h prior to transfection. By use of Lipofectamine 2000, the cells were transfected with GFP-AQP5(wt) or GFP-AQP5 having mutated PKA-target motif ; *i.e.*, the cells were covered with a mixture of 0.5 μg of DNA and 2 μl of Lipofectamine 2000 in a final volume of 100 μl of Opti-MEM I medium per well as described by the manufacturer's protocol (Invitrogen). The transfection medium was removed after 5.5 h of incubation and replaced with fresh DMEM containing 10% FBS without antibiotics. After cultivation for 24 h following transfection, in which time point MDCK II cells had been cultured for 3 days in total (22), polycarbonate membranes containing cell monolayers were washed with phosphate-buffered saline (PBS) 3 times, fixed with 3% paraformaldehyde for 20 min, washed, and incubated with 50 mM NH_4Cl for 15 min. For biotinylation,

cells were washed, and blocked with 1% BSA (fraction V) in PBS for 1 h at room tenperature and reacted with cell-impermeable biotinylation reagent (2 mM sulfo-NHS-LC-biotin) in PBS containing 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 7.4 at room temperature for 40 min. After having been washed, cells were incubated with 50 mM NH_4Cl in PBS to quench excess biotinylation reagent. Cells were next reacted with Alexa Fluor 594-conjugate streptavidin (1 : 200), washed, and mounted with Vectashield mounting medium (Vector Laboratories, CA, USA) on slide glasses with frames of 70-μm thickness. They were covered with a coverslip, and sealed with nail polish. Samples were stored in the dark at 4°C until they were examined under a confocal laser scanning microscope (Leica TCN NT, Heidelberg, Germany) with excitations at 488 nm (for FITC) and 568 nm (for Alexa Fluor 594).

The samples were examined by scanning at horizontal directions for 32 times at different vertical planes (0.5 μm intervals). The cells expressing GFP-AQP5 were selected and their transverse images were saved. These data were examined to verify whether AQP5 was completely targeted to apical cell membrane. This can be determined by merging 2 pictures, GFP fluorescence (green) and biotin (red) as the cells expressing AQP5 at cell membrane show the yellow signal. For the sake of convenience, the cell membrane facing to the medium will be referred to as the "apical membrane," and the one facing to the polycarbonate membrane, the "basal membrane." In all transfection experiments, 4-6 wells were prepared and means± S.E. were calculated.

Treatment with H-89, colchicine, and cytochalasin B

For examination of the effects of inhibitors of PKA, microtubules, and microfilaments, cells cultured on polycarbonate membranes for 48 h were transfected with the chimeric gene, GFP-AQP5(wt) as described above and cultured under a growth-arrest condition (14), *i.e.*, DMEM containing 0.5% FBS, for 18 h. The medium was then replaced with fresh medium containing 0.5% FBS and either 30 μM H-89 (14), 10 μM colchicine or 10 μM cytochalasin B (17) ; and the cells were cultured for another 6 h (14). During 6 h-culture, medium was replaced with fresh ones containing same inhibitors once at 3 h. The cells were then fixed, biotinylated, and examined under a confocal laser scanning microscope as described above.

Statistics

Data are expressed as means±SE. For statistical analysis of results, Mann-Whitney U-test was applied.

RESULTS

AQP5 expression in MDCK-II cells

We examined physiological behavior of AQP5 in MDCK-II cells, since these cells are known to provide a well-defined polarized cell model during cultivation on a polycarbonate membrane, and are widely utilized in studies of epithelial cell polarity and intracellular protein trafficking (23). Thus trafficking and/or translocation of particular proteins, including AQP5 protein (22), toward apical or basolateral membranes can be studied in this model system. MDCK-II cells were transfected with plasmids generating chimeric proteins for GFP-AQP5(wt) or GFP-AQP5 proteins with mutations in PKA consensus sequence, and cytoplasmic or apical-membrane localization of these gene products was analyzed (Figs. 1 and 3). In Fig. 1, typical MDCK-II cells expressing GFP-AQP5(wt) presenting prominent GFP signals and indicating the localization of the AQP5 chimeric protein in them are shown (Fig. 1A). We counted the number of cells that expressed GFP-AQP5(wt) and those showing localization of GFP-AQP5(wt) at apical membrane (Fig. 1B) ; membrane localization of the chimeric protein was confirmed by yellow signal which verified its co-localization with surface-labeled biotin.

At 6 h after transfection, GFP-AQP5(wt) was already expressed but it stayed in the cytosol until 12 h. At 24 h after transfection, 33% of the cells among those expressing GFP-AQP5(wt) showed localization at the apical membrane. This value increased to 73% at 48 h after transfection.

Effects of H-89, colchicine, and cytochalasin B on translocation of GFP-AQP5(wt)

We used several reagents known to stimulate or inhibit cell dynamics in order to understand and confirm the machinery involved in the translocation of GFP-AQP5(wt) in the MDCK-II cells.

Thus, at first, we determined whether PKA activity was required for GFP-AQP5(wt) translocation. An experiment to examine the possible involvement of microfilaments or microtubules was also concomitantly performed. MDCK-II cells, plated

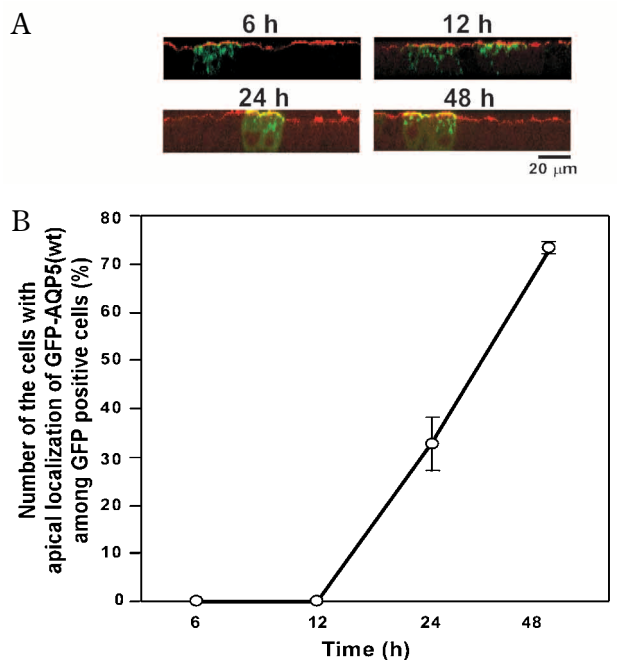

Fig. 1 AQP5 expression in polarized MDCK-II cells. Cultures expressing chimeric proteins were observed under the confocal microscope. A, Vertical pictures (x-z vertical sections ; see MATERIALS AND METHODS section for detail methods) of cells in typical cultures at 6, 12, 24, and 48 h post-transfection, expressing the wt chimeric protein, are shown. The merge technique was used to visualize the co-localization of biotin/Alexa Fluor 594-conjugated streptavidin (red) and GFP signal of GFP-AQP5 (green) in the apical cell membrane which appears as yellow areas in cells. Bar, 20 μm. B, Confocal images shown in "A" were assessed, and the histogram showing the percentages of the cells expressing GFP-AQP5(wt) protein at their apical membrane among GFP-positive cells at different post transfection times is presented. The number of sample wells analyzed was 4 to 6.

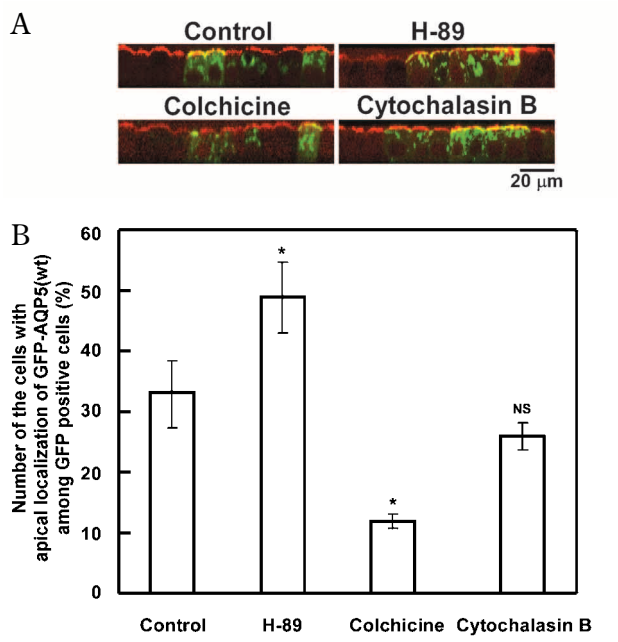

Fig. 2 Effects of H-89, colchicine, and cytochalasin B on the apical translocation of GFP-AQP5(wt). MDCK-II cells were transfected as described in the text. Eighteen hours after transfection, the cells were incubated under growth-arrest conditions (incubation in the medium containing 0.5% FBS) for the next 6 h in the presence or absence of either H-89 (30 μM), colchicine (10 μM) or cytochalasin B (10 μM) and examined under the confocal laser scanning microscope. A, Vertical pictures of GFP-AQP5 positive cells. Bar, 20 μm. B, Histogram showing the percentage of the cells expressing GFP-AQP5(wt) at apical membrane is presented as in Fig. 1. *$p < 0.05$, significantly different from the GFP-AQP5(wt) group. NS, not significantly different from the GFP-AQP5(wt). The number of sample wells analyzed was 4 to 6.

and transiently transfected as described above, were treated with either H-89 (30 μM ; 13, 14), colchicine or cytochalasin B (10 μM, each) at 18 h after transfection ; they were cultured further for 6 h under a growth-arrest condition as described above. In good accordance with our previous finding (17), colchicine, an inhibitor of cytoskeleton assembly, inhibited strongly the translocation of the chimeric proteins ; *i.e.*, the number of cells expressing the GFP-AQP5(wt) at their apical membrane was only 38% of that of the non-treated control cultures (Fig. 2), suggesting that translocation of AQP5-bearing vesicles, required the microtubule system. A microfilament inhibitor, cytochalasin B had no or only little effect on the trafficking of the GFP-AQP5(wt) (81% of control).

On the other hand, percentage of the cells that expressed GFP-AQP5(wt) at their apical membrane among GFP-AQP5(wt) positive cells increased in the presence of the PKA inhibitor H-89, as compared with that for the non-treated cells. The percentage increase in apical translocation by H-89 was 1.5 times of that for non-treated control (Fig. 2).

The above results suggest that the translocation of AQP5-bearing vesicles towards the apical membrane was increased by decreasing the phosphorylation of Ser/Thr of AQP5. From these initial observations, we hypothesized that the phosphorylation itself is probably not crucial for membrane expression of AQP5. The phosphorylation target site blocked by H-89 may be amino acid 152-156 (SRRTS), since this is a PKA-target motif found in AQP5 (3). We, therefore constructed 3 GFP-AQP5 mutants having mutated consensus PKA-target motifs to examine the role of this PKA-target motif in membrane expression of AQP5.

Effects of mutation in PKA-target motif (^{152}SRRTS) on translocation of the AQP5 chimeric proteins

Twenty-four hours after transfection of MDCK-II cells with GFP-AQP5 (S152A/S156A), GFP-AQP5 (T155V) or GFP-AQP5 (S152A/T155V/S156A),

the number of cells expressing each mutant chimeric proteins at cell membrane were 76, 57, and 74%, respectively of the GFP-AQP5 positive cells. These numbers were significantly greater compared to the 33% for the cells expressing GFP-AQP5(wt) at the cell membrane (Fig. 3).

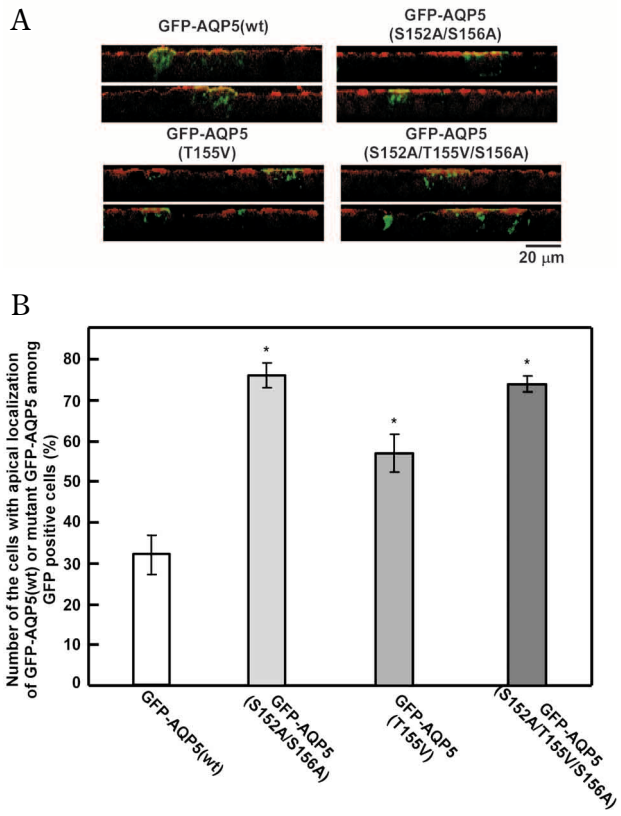

Fig. 3 Effects of mutation of PKA-target motif, ^{152}SRRTS, in GFP-AQP5 on the apical trafficking of the chimeric proteins. GFP-AQP5 plasmids with a mutated PKA-target motif, *i.e.*, GFP-AQP5 (S152A/S156A), GFP-AQP5 (T155V), and GFP-AQP5 (S152A/T155V/S156A), as well as GFP-AQP5(wt), were used to transfect MDCK-II cells, as described in Fig. 1. A, Vertical pictures (x-z vertical section) of cells at 24 h post-transfection, expressing the wt chimeric protein or chimeric protein with mutated PKA-target motif, are shown. The merge technique was used as it is described above. Bar, 20 μm. B, confocal images shown in "A" were assessed, and a histogram showing the percentages of the cells expressing wt AQP5 or AQP5 with a mutated PKA motif at their apical membrane at 24 h post-transfection is presented. *$p < 0.05$, significantly different from the GFP-AQP5 (wt) group. The number of sample wells analyzed was 4 to 6.

DISCUSSION

The salivary glands are innervated by both sympathetic and parasympathetic branches of the autonomic nervous system. The parasympathetic stimulation and activation of M3 muscarinic receptors on the acinar cells produces the largest increase in the salivary flow rate (24). The presence of an exocrine-type water channel, such as AQP5, in acinar cells is essential at least for transcellular water transport as shown by the results of its knockout experiment (8, 9). Trafficking of AQP5 is thought to be involved in the regulation of salivary water secretion *via* this channel protein ; *e.g.*, in the parotid gland, AQP5 was reported to be trafficked from the intracellular vesicles to the apical membrane in response to stimulation by muscarinic receptors *in vitro* (15). In human salivary gland cells (HSG cells) transfected with AQP5 cDNA, the AQP5 protein is trafficked to the plasma membrane following an increase in $[Ca^{2+}]_i$ (17). All of these reports imply that trafficking of AQP5 from intracellular vesicles to the apical membrane is provoked by secretogogue-induced $[Ca^{2+}]_i$ increase leading to an increase in water permeability at the apical membrane. From the aspect of molecular phylogenic comparison, AQP5 is known to be closest to AQP2, suggesting that their physiological and biochemical properties would be close as well (3, 25). AQP5 is trafficked from intracellular vesicles toward the apical membrane in response to a muscarinic agonist (15), which response is similar to the vasopressin-induced trafficking of AQP2 to the plasma membrane (26). A key event that triggers signals for prompting AQP2 trafficking is phosphorylation of the amino acid residue located at the carboxyl terminus, Ser-256, by protein kinase A (27). For instance, van Balkom, *et al.* (28) constructed several *AQP2* genes having 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) ; these constructs were then expressed in MDCK cells. All of these mutant proteins, except Ser-256 mutant, trafficked from the intracellular vesicles to the apical membrane *via* a forskolin-sensitive mechanism, similarly as wild-type AQP2 did, suggesting that phosphorylation of Ser-256 is essential in this cellular event (28).

GFP has been used for studying the behavior and localization of particular proteins in the living system. For example, GFP-AQP2 chimera (GFP fused to the amino-terminus of AQP2) expressed in cultured porcine kidney epithelial cells ($LLC\text{-}PK_1$ cells) was found to traffick in a regulated pathway from the intracellular vesicles toward the basolateral plasma membrane in response to vasopressin or forskolin stimulation (21). Similar to 2 different constructs of GFP with AQP2, GFP-AQP5 was localized primarily in the intracellular vesicles, while AQP5-GFP was predominantly localized on plasma membranes under non-stimulated condition (14).

Although present and previous inhibitor experiments suggested that blocking the PKA-dependent phosphorylation increased the trafficking of AQP molecule toward the apical membrane, this does not necessarily mean that the inhibitor blocked phosphorylation of the PKA-target motif of AQP5 molecules ; it might have blocked proteins to which AQP5 may be associated. The present *in vitro* mutagenesis study for the first time explored that blocking the PKA-target motif of AQP5 molecule increases the trafficking of this molecule toward the apical membrane.

Our study was conducted to examine the effects of the PKA phosphorylation site of the AQP5 molecule on its intracellular translocation or trafficking. The consensus sequence of the PKA-target motif is located in cytoplasmic loop D of AQP5 at amino acid residues 152-156 (Ser-Arg-Arg-Thr-Ser ; 3). Since there are not many reports which describe the function of this motif in the trafficking of AQP5, we investigated the cell physiological properties of rat AQP5 expressed in MDCK-II cells used as a model system. We prepared GFP-AQP5 constructs in which PKA consensus sequence motif was mutated to Ala and/or Val to generate the un-phosphorylated state and these constructs were used to transfect MDCK-II cells.

The amino-terminal fusion chimera, GFP-AQP5 (wt), was shown to be translocated to the apical membrane from the intracellular compartments in polarized MDCK-II cells during cultivation for more than 24 h after transfection. In cells transfected with GFP-AQP5(wt) or mutant GFP-AQP5s, all chimeric proteins were expressed on the plasma membrane ; *i.e.*, even PKA-dependent phosphorylation was blocked, GFP-AQP5 was trafficked toward apical membrane. By the experiment using GFP-AQP5 with replacement of various amino acids at ^{152}SRRTS (S152A/T155V/S156A), we found that blocking the phosphorylation of AQP5 at this PKA-target motif increased its translocation to the apical membrane. Also, the cells expressing the wild-type molecule at their apical membrane increased in number by treatment with H-89 comparing to non-stimulated conditions. This membrane trafficking of GFP-AQP5 (wt) required the involvement of microtubules.

In the present study, we have demonstrated here that GFP-AQP5 chimeric molecules having mutation at their PKA consensus sequence can be expressed at the apical membrane. In contrast with phosphorylation-dependent translocation of AQP2 (28), our data suggest that AQP5 can be trafficked toward the cell membrane irrespective of phosphorylation of PKA-target motif. Thus the present study imply that a mechanism(s) independent of phosphorylation of PKA-target motif (^{152}SRRTS) is involved in translocation of AQP5 in MDCK-II cells.

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REFERENCES

1. Verkman AS, Mitra AK : Structure and function of aquaporin water channels. Am J Physiol Renal Physiol 278 : F13-F28, 2000
2. Verkman AS : More than just water channels : unexpected cellular roles of aquaporins. J Cell Sci 118 : 3225-3232, 2005
3. Raina S, Preston GM, Guggino WB, Agre P : Molecular cloning and characterization of an aquaporin cDNA from salivary, lacrimal, and respiratory tissues. J Biol Chem 270 : 1908-1912, 1995
4. Nielsen S, King LS, Christensen BM, Agre P : Aquaporins in complex tissues. II. Subcellular distribution in respiratory and glandular tissues of rat. Am J Physiol Cell Physiol 273 : C1549-C1561, 1997
5. Nejsum LN, Kwon TH, Jensen UB, Fumagalli O, Frokiaer J, Krane CM, Menon AG, King LS, Agre PC, Nielsen S : Functional requirement of aquaporin-5 in plasma membranes of sweat

glands. Proc Natl Acad Sci 99 : 511-516, 2002
6. Hamann S, Zeuthen T, La Cour M, Nagelhus EA, Ottersen OP, Agre P, Nielsen S : Aquaporins in complex tissues : distribution of aquaporins 1-5 in human and rat eye. Am J Physiol Cell Physiol 274 : C1332-C1345, 1998
7. Parvin MN, Kurabuchi S, Murdiastuti K, Yao C, Kosugi-Tanaka C, Akamatsu T, Kanamori N, Hosoi K : Subcellular redistribution of AQP5 by vasoactive intestinal polypeptide in the Brunner's gland of the rat duodenum. Am J Physiol Gastrointest Liver Physiol 288 : G1283-G1291, 2005
8. Krane CM, Melvin JE, Nguyen HV, Richardson L, Towne JE, Doetschman T, Menon AG : Salivary acinar cells from aquaporin 5-deficient mice have decreased membrane water permeability and altered cell volume regulation. J Biol Chem 276 : 23413-23420, 2001
9. Ma T, Song Y, Gillespie A, Carlson EJ, Epstein CJ, Verkman AS : Defective secretion of saliva in transgenic mice lacking aquaporin-5 water channels. J Biol Chem 274 : 20071-20074, 1999
10. Tsubota K, Hirai S, King LS, Agre P, Ishida N : Defective cellular trafficking of lacrimal gland aquaporin-5 in Sjögren's syndrome. Lancet 357 : 688-689, 2001
11. Steinfeld S, Cogan E, King LS, Agre P, Kiss R, Delporte C : Abnormal distribution of aquaporin-5 water channel protein in salivary glands from Sjögren's syndrome patients. Lab Invest 81 : 143-148, 2001
12. Beroukas D, Hiscock J, Jonsson R, Waterman SA, Gordon TP : Subcellular distribution of aquaporin 5 in salivary glands in primary Sjögren's syndrome. Lancet 358 : 1875-1876, 2001
13. Yang F, Kawedia JD, Menon AG : Cyclic AMP regulates aquaporin 5 expression at both transcriptional and post-transcriptional levels through a protein kinase A pathway. J Biol Chem 278 : 32173-32180, 2003
14. Kosugi-Tanaka C, Li X, Yao C, Akamatsu T, Kanamori N, Hosoi K : Protein kinase A-regulated membrane trafficking of a green fluorescent protein-aquaporin 5 chimera in MDCK cells. Biochim Biophys Acta 1763 : 337-344, 2006
15. Ishikawa Y, Eguchi T, Skowronski MT, Ishida H : 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 : 835-840, 1998
16. Li J, Lee S, Choi SY, Lee SJ, Oh SB, Lee JH, Chung SC, Kim JS, Lee JH, Park K : Effects of pilocarpine on the secretory acinar cells in human submandibular glands. Life Sci 79 : 2441-2447, 2006
17. Tada J, Sawa T, Yamanaka N, Shono M, Akamatsu T, Tsumura K, Parvin MN, Kanamori N, Hosoi K : Involvement of vesicle-cytoskeleton interaction in AQP5 trafficking in AQP5-gene-transfected HSG cells. Biochem Biophys Res Commun 266 : 443-447, 1999
18. Sidhaye V, Hoffert JD, King LS : cAMP has distinct acute and chronic effects on aquaporin-5 in lung epithelial cells. J Biol Chem 280 : 3590-3596, 2005
19. Woo J, Chae YK, Jang SJ, Kim MS, Baek JH, Park JC, Trink B, Ratovitski E, Lee T, Park B, Park M, Kang JH, Soria JC, Lee J, Califano J, Sidransky D, Moon C : Membrane trafficking of AQP5 and cAMP dependent phosphorylation in bronchial epithelium. Biochem Biophys Res Commun 366 : 321-327, 2008
20. Murdiastuti K, Purwanti N, Karabasil MR, Li X, Yao C, Akamatsu T, Kanamori N, Hosoi K : A naturally occurring point mutation in the rat aquaporin 5 gene, influencing its protein production by and secretion of water from salivary glands. Am J Physiol Gastrointest Liver Physiol 291 : G1081-G1088, 2006
21. Gustafson CE, Levine S, Katsura T, McLaughlin M, Alexio MD, Tamarappoo BK, Verkman AS, Brown D : Vasopressin regulated trafficking of a green fluorescent protein-aquaporin 2 chimera in LLC-PK_1 cells. Histochem Cell Biol 110, 377-386, 1998
22. Wellner RB, Baum BJ : Polarized sorting of aquaporins 5 and 8 in stable MDCK-II transfectants. Biochem Biophys Res Commun 285 : 1253-1258, 2001
23. Mostov KE, Verges M, Altschuler Y : Membrane traffic in polarized epithelial cells. Curr Opin Cell Biol 12 : 483-490, 2000
24. Baum BJ : Principles of saliva secretion. Ann NY Acad Sci 694 : 17-23, 1993
25. King LS, Kozono D, Agre P : From structure to disease : the evolving tale of aquaporin biology Nat Rev Mol Cell Biol 5 : 687-698, 2004
26. Katsura T, Gustafson CE, Ausiello DA, Brown D : Protein kinase A phosphorylation is involved in regulated exocytosis of aquaporin-2

in transfected LLC-PK1 cells. Am J Physiol Renal Physiol 272 : F817-F822, 1997
27. Fushimi K, Sasaki S, Marumo F : Phosphorylation of serine 256 is required for cAMP-dependent regulatory exocytosis of the aquaporin-2 water channel. J Biol Chem 272 : 14800-14804, 1997
28. Van Balkom BWM, Savelkoul PJM, Markovich D, Hofman E, Nielsen S, Van der Sluijs P, Deen PMT : The role of putative phosphorylation sites in the targeting and shuttling of the aquaporin-2 water channel. J Biol Chem 277 : 41473-41479, 2002