Mutations in sixth transmembrane domain of AQP2 inhibit its translocation induced by vasopression

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Mutations in sixth transmembrane domain of AQP2 inhibit its translocation induced by vasopression. Am. J. Physiol. Renal Physiol. 278: F395–F405, 2000.—Vasopressin-induced phosphorylation of serine 256 of the aquaporin-2 (AQP2) water channel triggers translocation of the protein from cystolic reservoir vesicles to the apical membrane of collecting duct principal cells. Dileucine motifs are located in the sixth transmembrane domain (6TM) of AQP2 and are known as the signal sequence for internalization, sorting from the trans-Golgi network to endosomes/lysosomes, and basolateral sorting. In this study, involvement of 6TM in vasopressin-induced translocation of the protein was investigated. A series of mutations in 6TM of AQP2 was introduced to rat cDNA and expressed in LLC-PK1 cells. Immunofluorescence microscopy indicated that the mutant AQP2 proteins were retained in the cytoplasm after vasopressin stimulation, which actually promoted the plasma membrane expression of wild-type protein. Immunoelectron microscopy showed that the mutant AQP2 proteins reached the endosomes but did not reach the plasma membrane. These results demonstrate that 6TM has essential domains for vasopressin-induced translocation from endosomes to the plasma membrane.

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THE AQUAPORINS (AQPs) belong to a family of intrinsic membrane proteins that form channels and facilitate selective water transport across the cell membrane (12). AQP2 is the vasopressin-regulated water channel that is expressed exclusively in principal cells of kidney collecting duct (5, 6, 15). Vasopressin regulates the water permeability of the collecting duct through the translocation of AQP2 water channels from intracellular vesicles to the apical membrane (13, 15, 18). The apical membrane expression of AQP2 is dependent on a rise in intracellular 3',5'-cyclic monophosphate and activation of protein kinase A. This intracellular process is activated by the binding of vasopressin to the vasopressin V2 receptor subtype existing on the basolateral membrane of the principal cells. Recent studies have shown that phosphorylation of the serine residue at position 256 in the cytoplasmic COOH terminus of AQP2 protein is necessary for its exocytotic membrane insertion (4, 9).

The shuttle hypothesis predicts that, without vasopressin, AQP2 protein stays on intracellular vesicles just underneath the plasma membrane. In addition, AQP2 protein relocates to the plasma membrane on vasopressin stimulation and then is internalized via clathrin-coated pits to the reservoir vesicles for recycling to the plasma membrane (1, 10, 13–15, 20).

Many recycling membrane proteins have a unique site in their sequence that interacts with cellular components for internalization (11, 17, 21). In many membrane proteins a dileucine motif in the cytoplasmic tail is reported to be essential for internalization from the cell surface, sorting from the trans-Golgi network to endosomes/lysosomes, and basolateral sorting (8, 16, 17). Dileucine-related signals have been described for T-cell receptors (CD3ε, CD3ζ, CD4), cation-dependent and -independent mannose 6-phosphate receptors (CD-M6PR and CI-M6PR, respectively), interferon-γ receptor (IFNγR), lysosomal integral membrane protein (LIMP) II, glucose transporter 4, IgG Fc receptor (FcRII-B2), and the major histocompatibility complex class II invariant chain. Internalization of some of these proteins is proved to proceed via clathrin-coated pits. This event is thought to be caused by the interaction of the dileucine motif in its sequence with adaptor protein complex.

AQP2 is also a recycling membrane protein, and it is supposed that AQP2 protein has a site interacting with some cellular component. In the sixth transmembrane domain (6TM), there is one dileucine motif in human AQP2 (L217 and L218) (19) and two dileucine motifs in rat AQP2 (L217 and L218, L222 and L223) (6). Although existing in the transmembrane domain, it is possible that the sequence possesses an essential property for sorting. For example, the fourth transmembrane domain of Hₐ-K⁺-ATPase is reported to be essential for its apical localization, and it is suggested that the role of the domain in sorting may be realized through an association with a particular γ-subunit (2, 3). Thus, in the study presented here, we investigated...
involvement of 6TM of AQP2 protein in its translocation induced by vasopressin.

In this study, a series of mutations in 6TM were introduced to rat cDNA, and the mutant and wild-type AQP2 proteins were expressed in LLC-PK1 cells, an epithelial cell line derived from pig kidney. Intracellular localization of expressed AQP2 protein was analyzed by immunofluorescence confocal microscopy and immunoelectron microscopy.

**MATERIALS AND METHODS**

**Cell Culture and Transient Gene Expression**

For recombination of 6TM, the amino acid sequence of 6TM of AQP2 (WVYWVGPGV1AGGLGSLLYDFLLFPR) was replaced with the corresponding part of bovine major intrinsic protein (bMIP; WVFWIGPLVGAIIGSLLYNLFS). MIP is a membrane protein of lens fiber cells and has relatively low water permeability compared with AQP2 protein (7, 22).

To examine the role of the two dileucine motifs in 6TM of rat AQP2, both L217A/L218 and L222A/L223 were replaced with dialanine by PCR-based, site-directed mutagenesis. The nucleotide sequences of these mutants were verified by a fluorescence sequencer (model 373A, Applied Biosystems). PCR fragment codings for open reading frames of wild type and mutants were subcloned into the Hind III and Xba I site of mammalian cell expression vector pcDNA3 (Invitrogen, Carlsbad, CA).

LLC-PK1 cells were used as an expression system in the present experiment. Transfection was performed by electroporation by using Gene Pulser (Bio-Rad, Melville, NY). Subconfluent cells were detached from dishes by trypsin treatment. Approximately $1 \times 10^7$ cells suspended in 600 ml of PBS were electroporated at 340 V with 960 µF, using 30 µg of the appropriate plasmid DNA. Cells were used 24 h after the transfection.

Abbreviations for mutants were as follows: 6TM-MIP, recombinant of 6TM in AQP2 with bMIP; L217A/L218A, double mutation of L217A and L218A; and L222A/L223A, double mutation of L222A and L223A.

**Immunoblotting of AQP2 and Mutants**

Cells grown on plastic dishes (30 mm in diameter) were treated with 150 µl of 1× reporter lysis buffer (Promega, Madison, WI) for 15 min. The cell lysate was centrifuged for 1 min at 15,000 g at 4°C to remove incompletely lysed fragments. Ten microliters of the supernatant were denatured in SDS sample buffer [1.5% SDS, 30 mM Tris·HCl, pH 6.8, 2.5% β-mercaptoethanol, and 5% (vol/vol) glycerol] at 80°C for 10 min, resolved in 10–20% gradient SDS-PAGE for 1 h with a 40 mA current, and electrotransferred to an enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham, Arlington Heights, IL) by using Fastblot semidry blotting apparatus (Biometra, Goettingen, Germany). After the membrane was blocked with Superblock (Promega) for 1 h at 23°C and washed once with TBS-T (20 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.4), it was incubated with affinity-purified antibody against a synthetic peptide, corresponding to 15 COOH-terminal amino acid residues of rat AQP2 (1 µg IgG/ml), washed three times with TBS-T, incubated with a 1:200 dilution of biotin-labeled anti-rabbit IgG antibody (Vector, Burlingame, CA), washed, and incubated again with a 1:500 dilution of ABC mixture (Vector). The blot was visualized by ECL by using an ECL minicamera (Amersham).

![Immunoblotting analysis of LLC-PK1 cells transfected with wild type and mutants of aquaporin-2 (AQP2). Lane 1: pcDNA3 vector. Lane 2: wild type. Lane 3: recombinant of 6th transmembrane domain (6TM) in AQP2 with bovine major intrinsic protein (bMIP; 6TM-MIP). Lane 4: double mutation of L217A and L218A (L217A/L218A). Lane 5: double mutation of L222A and L223A (L222A/L223A). Left: molecular markers (kDa).](image-url)

Fig. 1. Immunoblotting analysis of LLC-PK1 cells transfected with wild type and mutants of aquaporin-2 (AQP2).

![Time course of intracellular translocation of AQP-like immunoreactivity (AQP2-LIR) in wild-type AQP2-transfected cells during vasopressin (VP) treatment (10 nM). VP−: without VP treatment; AQP2-LIR located in cytoplasm. 5 min: In cells treated with VP for 5 min, AQP2-LIR was located mainly in cytoplasm, whereas fluorescence was faint on plasma membrane. 10 min and 15 min: In cells treated with VP for 10 and 15 min, AQP2-LIR was located both in cytoplasm and on plasma membrane. 20 min and 30 min: In cells treated with VP for 20 and 30 min, respectively, AQP2-LIR was located mainly in cytoplasm. Bar = 10 µm.](image-url)

Fig. 2. Time course of intracellular translocation of AQP-like immunoreactivity (AQP2-LIR) in wild-type AQP2-transfected cells during vasopressin (VP) treatment (10 nM). VP−: without VP treatment; AQP2-LIR located in cytoplasm. 5 min: In cells treated with VP for 5 min, AQP2-LIR was located mainly in cytoplasm, whereas fluorescence was faint on plasma membrane. 10 min and 15 min: In cells treated with VP for 10 and 15 min, AQP2-LIR was located both in cytoplasm and on plasma membrane. 20 min and 30 min: In cells treated with VP for 20 and 30 min, respectively, AQP2-LIR was located mainly in cytoplasm. Bar = 10 µm.
Immunofluorescence Microscopy

The transfected LLC-PK1 cells were grown on a poly-D-lysine-coated coverslip equipped at the center of the bottom of a dish (Glass Bottom Culture Dish, MatTec, Ashland, MA) for 24 h and fixed by incubation with 4% paraformaldehyde in 0.1 M PBS for 30 min at 23°C. Nonspecific immunoreactivity was blocked by preincubation with Superblock (Promega) for 1 h. The specimens were incubated with an affinity-purified rabbit anti-AQP2 antibody (3 µg IgG/ml) for 2 h at 23°C. After being rinsed with PBS, the cells were incubated for 1 h with FITC-conjugated affinity-purified goat anti-rabbit IgG (Sigma Chemical, 1:100 dilution), rinsed again, and filled with a mounting medium (Vectashield, Vector). The samples were examined with a laser scanning confocal microscope (LSM 410, Carl Zeiss). A control preparation, without the primary antibody incubation, revealed no FITC labeling.

The immunoreactivity distributions were quantitatively analyzed on digital confocal images with the public domain National Institutes of Health Image program (developed at NIH). A rectangular area along the radius with a 10-pixel width between the nuclear membrane and the plasma membrane was arbitrarily selected and divided into 10 blocks in a radial direction. Mean brightness of the pixels in each block was reckoned as an immunofluorescence intensity of the area. The intracellular gradient pattern of the AQP2-like immunoreactivity (AQP2-LIR) in the radial direction of the cells was compared among wild-type and mutant AQP2-transfected cells. Each of 10 values of wild-type or mutant AQP2-transfected cells was analyzed with Student’s t-test to clarify the significance of the difference in the intracellular localization pattern among them.

Immunoelectron Microscopy

Immunoenzyme technique with diaminobenzidine (DAB) reaction. The bottom of a plastic culture dish (30 mm in diameter) was covered with Araldite (Ciba-Geigy) at ~2-mm thickness, and the culture dish was filled with DMEM containing 10% FCS for 24 h at 37°C. The transfected cells were grown on the dishes for 24 h at 37°C, then incubated with DMEM containing 10 nM lysin-vasopressin for 10 min. The cells were fixed with 4% paraformaldehyde in 0.1 M PBS for 30 min at 23°C. Nonspecific binding of the antibody was
blocked by preincubation with 3% BSA for 1 h. The specimens were incubated with the anti-AQP2 antibody (3 µg IgG/ml) for 2 h at 23°C, and washed with PBS. Subsequently, they were incubated for 1 h with a biotin-labeled anti-rabbit IgG antibody (1:100 dilution), washed again, and incubated with an ABC mixture (1:500 dilution). The cells were washed in Tris-buffered saline (TBS) and incubated with 0.5 µg/ml DAB in TBS containing 0.03% H₂O₂ for 10 min. After the DAB reaction was stopped with cold PBS, the cells were postfixed with 2.5% glutaraldehyde for 20 min, washed again with PBS, and osmicated in 2% OsO₄ for 1 h. The cells were washed with distilled water, dehydrated with 70, 80, 90, and 100% ethanol, and embedded in Araldite. Ultrathin sections were cut out with an ultramicrotome (Ultracut OmU4, Leica, Vienna, Austria). The sections without counterstaining were observed by using a transmission electron microscope (H-600, Hitachi, Hitachinaka, Japan).

Double immunogold labeling technique. After the transfected cells were incubated with vasopressin, the cells were fixed with a mixture of 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M PBS for 30 min and postfixed with 0.5% OsO₄ in the same buffer for 10 min. The cells were dehydrated with ethanol and embedded in Epon (TAAB, Berkshire, UK). Ultrathin sections were cut out and etched with 7% H₂O₂ for 10 min. After being washed, the sections were incubated with 3% BSA in PBS overnight at 4°C, followed by incubation with rabbit anti-AQP2 antibody (1 µg IgG/ml) and mouse anti-Grp78 antibody [endoplasmic reticulum (ER) marker, 5 µg IgG/ml, StressGen, Victoria, Canada] for 2 h at 23°C. After being washed with PBS, the sections were incubated with a mixture of 15 nm gold-labeled goat anti-rabbit IgG (1:300 dilution, British BioCell International, Cardiff, UK) and 10 nm gold-labeled anti-mouse IgG (1:300 dilution) for 1 h at 23°C. Sections were stained with uranyl acetate and lead citrate and observed with a transmission electron microscope (H-7100, Hitachi).

RESULTS

Wild-type and mutant AQP2 proteins were expressed in LLC-PK₁ cells by electroporation, and transient expression of the AQP2 proteins was examined by immunoblotting (Fig. 1). Immunoreactivity of AQP2

Fig. 4. Immunofluorescence localization of wild-type and mutant AQP2 proteins with or without VP stimulation. A-D: orthogonal sectioning images are shown at the top (profile of vertical plane sectioned at position indicated by arrowhead) and on the right of each panel (profile of vertical plane sectioned at arrow). A: AQP2-LIR in cells transfected with wild-type AQP2 and incubated with 10 nM VP appeared on both apical and basolateral membranes. B: intracellular localization of AQP2-LIR in wild-type AQP-transfected cells without VP stimulation. C: after VP stimulation, AQP2-LIR remained in cytoplasm of cells transfected with L217A/L218A. D: AQP2-LIR in cells transfected with L217A/L218A without VP stimulation was located in cytoplasm. Mutant AQP2 proteins 6TM-MIP and L222A/L223A were also located in cytoplasm without VP stimulation (data not shown). E and F: AQP2-LIR in cells transfected with mutants 6TM-MIP (E) and L222A/L223A (F) and incubated with VP remained in cytoplasm. Bar = 10 µm.
protein was not detected in LLC-PK1 cells transfected with pcDNA3 vector. In the lane loaded with wild-type AQP2 protein, a 29-kDa band was detected, corresponding to the size of native AQP2 protein. The size of the bands in the lanes loaded with mutant AQP2 proteins was from 29 to 30 kDa. No difference in the band density between wild type and mutants was detected, indicating similar expression efficiency.

Fig. 5. Quantitative analysis of intracellular localization of AQP2-LIR in cells transfected with wild-type and mutant AQP2 with or without VP stimulation. A: relative immunofluorescence intensities of AQP2-LIR with (+) and without (−) VP were plotted against relative distance from nuclear membrane to plasma membrane (nos. 1–10; as described in Fig. 3A). B and C: relative AQP2-LIR near nuclear membrane (B) and near plasma membrane (C) in cells transfected with wild-type and mutant AQP2. Immunofluorescence intensities in block 3 (B) and block 9 (C) in Fig. 5A are summarized. Values are means ± SE of 20 cells. *Significantly different compared with corresponding block of wild-type transfected cells without VP incubation, P < 0.01.

Fig. 6. Intracellular localization of wild-type and mutants of AQP2 with VP stimulation examined with immunoelectron microscopy (immunoenzyme technique). A: wild-type AQP2-transfected cells. Diaminobenzidine (DAB) precipitation was detected on plasma membrane (arrows), in cytoplasm near plasma membrane, and on endoplasmic reticulum (ER). B: L217A/L218A-transfected cells. DAB precipitation was detected on ER and in cytoplasm near ER (arrows) but could not be detected on plasma membrane. Bar = 0.5 µm.
Time Course of Intracellular Localization of Wild-Type and Mutant AQP2 Proteins

The time course of intracellular translocation of AQP2 protein during vasopressin treatment in LLC-PK1 cells transfected with wild-type AQP2 was analyzed by immunofluorescence confocal microscopy (Fig. 2). Without vasopressin stimulation, AQP2-LIR for wild-type AQP2 protein was located in the cytoplasm. After 10- or 15-min incubation with 10 nM vasopressin, the AQP2-LIR was found in both the plasma membrane and the cytoplasm. After 20- or 30-min vasopressin incubation, the AQP2-LIR again appeared mainly in the cytoplasm. The intensity of intracellular immunofluorescence was plotted against relative distance from the nuclear membrane to the plasma membrane (Fig. 3). In wild-type AQP2-transfected cells treated with vasopressin for 10 or 15 min, fluorescence intensity of the AQP2-LIR near the plasma membrane (nos. 9 and 10 in Fig. 3A) was significantly higher than that in the cells without vasopressin treatment. When wild-type AQP2-transfected cells were treated with vasopressin for 5, 20, or 30 min, there was no significant difference in the intensity of the AQP2-LIR compared with that in the cells without vasopressin treatment. These results indicate that wild-type AQP2 protein expression on the plasma membrane reaches maximum at 10- or 15-min treatment with vasopressin, and, conversely, that the expression level on the plasma membrane decreased by vasopressin treatment longer than 20 min. The fluorescence intensity of the AQP2-LIR near the nuclear membrane (nos. 3 and 4 in Fig. 3A) in wild-type AQP2-transfected cells significantly decreased if cells were treated with vasopressin for 10 or 15 min. Figure 3, B and C (no. 3 and no. 9, respectively), shows the time course of relative immunofluorescence intensity near the nuclear membrane and that near the plasma membrane, respectively.

Mutations in 6TM Inhibit Vasopressin-Induced Translocation of AQP2 Protein

The effect of vasopressin stimulation on the intracellular localization of the AQP2-LIR in wild-type or mutant AQP2-transfected cells was examined by immunofluorophotometry (Fig. 4). Without vasopressin stimulation, both mutant AQP2-transfected cells and wild-type AQP2-transfected cells showed similar cytoplasmic staining. After vasopressin stimulation (10 nM for 10 min), wild-type AQP2 protein relocated to the plasma membrane, both the apical and the basolateral sides. In the cells transfected with each mutant of 6TM-MIP, L217A/L218A, and L222A/L223A, intracellular localization of the AQP2-LIR did not change after vasopressin stimulation; i.e., the immunoreactivity remained in the cytoplasm, and negligible fluorescence was detected on the plasma membrane. The intracellular localization of the AQP2-LIR in wild-type and each mutant AQP2 protein in the transfectants did not differ significantly from that in wild-type-transfected cells. In wild-type AQP2-transfected cells with vasopressin stimulation, the AQP2-LIR near the plasma membrane (nos. 9 and 10 in Fig. 5A) was significantly higher, and the AQP2-LIR near the nuclear membrane (nos. 3 and 4 in Fig. 5A) was significantly lower than that without vasopressin stimulation (see also Fig. 3). On the other hand, in the cells transfected with each mutant (6TM-MIP, L217A/L218A, or L222A/L223A), vasopressin stimulation did not change the intracellular localization of the AQP2-LIR.

Intracellular Localization of Wild-Type and Mutant AQP2 Proteins by Immunoelectron Microscopy

Intracellular localization of AQP2 protein in the cells transfected with wild-type and mutant AQP2 was also examined by immunoelectron microscopy employing immunoenzyme technique with the DAB reaction (Fig. 6). In the cells expressing wild-type AQP2 protein and stimulated with vasopressin (10 nM for 10 min), DAB precipitation was observed on the plasma membrane, in the cytoplasm near the plasma membrane, and on the ER (Fig. 6A). In the cells transfected with the mutant L217A/L218A, DAB precipitation representing the AQP2-LIR was detected on the ER and in the cytoplasm near the ER, but not on the plasma membrane (Fig. 6B). The immunoreactivity of other mutants, 6TM-MIP and L222A/L223A, was also detected on the ER, but not on the plasma membrane, similar to that of L217A/L218A (data not shown). These observations were consistent with the results obtained with immunofluorophotometric study (Figs. 4 and 5).

In these samples, precise localization of AQP2 protein could not be specified, because of the mild fixation with 4% paraformaldehyde and the diffuse DAB precipitation. To conserve fine structure of the organelle and define precise localization of AQP2 protein, we em...
ployed glutaraldehyde fixation and performed double immunogold labeling with ER marker (Fig. 7).

To verify antigen immunoreactivity in this fixation, immunofluorescence intensity in wild-type AQP2-transfected cells was compared between the cells fixed with 4% paraformaldehyde only and the cells fixed with 4% paraformaldehyde, 1% glutaraldehyde, and 0.5% OsO₄. There was no difference in the
intensity between the two fixation methods (data not shown).

As control, sections were incubated with anti-AQP2 antibody preabsorbed with excess immunizing peptide (250 µg peptide to 40 ng IgG) and anti-Grp78 antibody followed with the respective second antibodies (Fig. 7A). AQP2-LIR (15 nm) was not detected, whereas Grp78-LIR (10 nm) was detected on the ER.

In the cells expressing wild-type AQP2 protein and stimulated with vasopressin, AQP2 label was found on the plasma membrane, endosomes, in the Golgi apparatus, and on the ER (Fig. 7, B–D). In the cells expressing
the mutant L217A/L218A and stimulated with vasopressin, AQP2 label was found on endosomes, in the Golgi apparatus, and on the ER (Fig. 7, E–G). Very little or no gold labeling was found on the plasma membrane (Fig. 7E). In the cells expressing each mutant of L222A/L223A and 6TM-MIP, and stimulated with vasopressin, AQP2 label was found on endosomes (Fig. 7, H and J), in the Golgi apparatus (Fig. 7, I and K), and on the ER (data not shown), but very faintly on the plasma membrane (Fig. 7, H and J), similar to that of L217A/L218A.

Without vasopressin stimulation, AQP2 label was found on endosomes, in the Golgi apparatus, and on the ER, but not on the plasma membrane in cells expressing whichever wild-type or each mutant AQP2 protein (data not shown). There was no difference in the distribution of the AQP2-LIR among wild-type and mutant-expressing cells without vasopressin stimulation and mutant-expressing cells with vasopressin stimulation.

**DISCUSSION**

In the present study, an affinity-purified antibody against a synthetic peptide corresponding to 15 amino acid residues in the COOH terminus of rat AQP2 was used as a primary antibody to detect the expression of AQP2 proteins. All mutations employed in this study were restricted in 6TM but not located in the cytoplasmic terminus where the immunoreactive site for the antibody against AQP2 protein is located. Thus the antibody could equally recognize wild-type and mutant AQP2 proteins.

It has been reported that in LLC-PK₁ cells constitutively transfected with wild-type AQP2 and treated with vasopressin, the staining of the AQP2-LIR was largely on the basolateral membrane, with little detectable staining on the apical side (10). In the present study, the AQP2-LIR was obviously detected on the plasma membrane on both the apical and the basolateral sides in LLC-PK₁ cells transfected with wild-type AQP2 and treated with vasopressin. To establish functional polarity, LLC-PK₁ cells must grow confluent and form tight junctions. To be confluent the cells must be cultured for >72 h after transfection, but at that time most cells do not express AQP2 protein. It is supposed that the expression level of the protein in these transiently transfected cells was so high that AQP2-expressing cells could not survive for long. In this system, we could not clearly discriminate between apical and basolateral sorting, but we could confirm whether the plasma membrane sorting occurred or not.

Quantitative evaluation was performed on the time course of intracellular localization of AQP2 protein during vasopressin treatment. AQP2 protein expression on the plasma membrane reached maximum at 10 or 15 min of vasopressin treatment. With vasopressin treatment >20 min, the expression level on the plasma membrane decreased to the level of the transfectants without vasopressin treatment. This result suggests that, after 15 min, endocytosis of AQP2 protein was enhanced relatively more than exocytosis even though vasopressin stimulation continued. In the present study, to compare the intracellular localization of wild-type and mutant AQP2 proteins, we selected a 10-min incubation time for vasopressin treatment to minimize the effect of endocytosis.

The AQP2-LIR in confocal immunofluorescence images of the mutant AQP2-transfected cells did not translocate from the cytoplasm to the plasma membrane on vasopressin stimulation, which actually promoted the relocation of the AQP2-LIR in wild-type AQP2-transfected cells. It is concluded that the mutations in 6TM of AQP2 hinder the vasopressin-induced translocation process of the AQP2 protein from an intracellular site to the plasma membrane.

To determine the processing step of AQP2 protein inhibited by introducing the mutations, immunoelectron microscopy was employed. In the experiment using immunoenzyme technique with the mild fixation by 4% paraformaldehyde only and the diffuse DAB labeling, precise localization of AQP2 protein on the ER, endosomes, and/or others could not be specified. The electronmicroscopically fine structure was well preserved in the cells firmly fixed with 4% paraformaldehyde, 1% glutaraldehyde, and 0.5% OsO₄. The precise localization of AQP2 protein could be identified by using double immunogold labeling with the ER marker, anti-Grp78 antibody (Fig. 7). In the cells expressing wild-type AQP2 protein and stimulated with vasopressin, AQP2 label was found not only on the plasma membrane, on endosomes, and in the Golgi apparatus but also on the ER labeled for Grp78. The AQP2 label on the ER may indicate that the protein synthesis continued at the time of tissue fixation because of the high expression level of AQP2 protein.

In the cells expressing each mutant of L217A/L218A, L222A/L223A, and 6TM-MIP and stimulated with vasopressin, AQP2 label was found on endosomes, in the Golgi apparatus, and on the ER, but very faintly on the plasma membrane. It is supposed that the AQP2 label on the ER may reflect newly synthesized protein, similarly to that of wild-type AQP2 protein, and does not indicate its retention in the ER.

From these experiments, it is shown that each mutant protein reached the endosomes but did not reach the plasma membrane on vasopressin stimulation. Translocation of 6TM-MIP mutant protein was inhibited at the same step as the other mutant proteins (L217A/L218A, L222A/L223A). Thus not only the dileucine motifs are essential for the translocation but also the other sequence domain, which may keep the threedimensional arrangement of 6TM, might be important for the translocation.

The results from this study show that 6TM of AQP2 has essential sequences for its translocation. Although it remains unclear how these sequences operate during the translocation, this is the first study demonstrating that 6TM of AQP2 is important for vasopressin-induced translocation from endosomes to the plasma membrane. To understand the mechanism of translocation, it will be also necessary to identify the cellular compo-
ments that recognize the domain and act through the translocation.

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