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Di-lysine motif-like sequences formed by deleting the C-terminal domain of aquaporin-4 prevent its trafficking to the plasma membrane

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Abstract

Aquaporin-4 is a transmembrane water channel protein, the C-terminal domain of which is facing the cytosol. In the process of investigating the role of the C-terminal domain of aquaporin-4 with regard to intracellular trafficking, we observed that a derivative of aquaporin-4, in which the C-terminal 53 amino acids had been removed (Δ271-323), was localized to intracellular compartments, including the endoplasmic reticulum, but was not expressed on the plasma membranes. This was determined by immunofluorescence staining and labeling of the cells with monoclonal antibody specifically recognizing the extracellular domain of aquaporin-4, followed by confocal microscopy and flow cytometry. Deletion of additional amino acids in the C-terminal domain of aquaporin-4 led to its redistribution to the plasma membrane. This suggests that the effect of the 53-amino acid deletion on the subcellular localization of aquaporin-4 could be attributed to the formation of a signal at the C terminus that retained aquaporin-4 in intracellular compartments, rather than the loss of a signal required for plasma membrane targeting. Substitution of the lysine at position 268 with alanine could rescue the Δ271-323-associated retention in the cytosol, suggesting that the C-terminal sequence of the mutant served as a signal similar to a di-lysine motif.

KEYWORDS

Aquaporin-4, di-lysine motif, endoplasmic reticulum, subcellular localization, transmembrane protein

1 | INTRODUCTION

Aquaporin-4 (AQP4) is a member of a water channel family, having six transmembrane helices linked by three extracellular and two intracellular loops, with both the amino (N)- and carboxy (C)-terminal domains oriented toward the cytosol (Hasegawa et al., 1994; Jung et al., 1994). AQP4 is the most abundant water channel in the central nervous system (CNS), where it is densely expressed in astrocytic end-feet, and plays a pivotal role in water and ion homeostasis (Nagelhus & Ottersen, 2013; Nielsen et al., 1997). AQP4 exists as two main isoforms, the 323-amino acid M1 and 301-amino acid M23 isoforms, with the latter lacking the N-terminal 22 amino acids of M1 due to an alternative transcriptional start site (Lu et al., 1996) and a leaky scanning mechanism (Rossi et al., 2010).

The localization of AQP4 is critical because it defines its physiological role. AQP4 polarization is evident in the kidney, where it is expressed and distributed to the basolateral membrane of distal tubules, highlighting its role in water reabsorption by collaborating with AQP2 and AQP3 (Terris et al., 1995). Analysis of the AQP4 C terminus suggests that tyrosine-based (277-YMEV-280) and di-leucine-like (288-ETEDLIL-294) motifs (Bonifacino & Traub, 2003) are involved in polarized expression and trafficking of AQP4 (Madrid et al., 2001). In addition, it has been reported that perivascular localization of AQP4 in the brain is stabilized by the C-terminal postsynaptic density 95/disk large/zonula occludens-1 (PDZ)-binding motif (321-SSV-323) and requires the subcellular scaffolding protein α-syntrophin (Neely et al., 2001).

Interestingly, subcellular- or super-aquaporins, a subfamily of aquaporins (Ishibashi, 2006; Ishibashi et al., 2014), which is only found in multicellular organisms, is expressed subcellularly, such as in the endoplasmic reticulum (ER). Numerous members of this family have a di-lysine ER retention motif (K(X)KXX) at the C terminus (Teasdale & Jackson, 1996). In mammals, two subcellular-aquaporin isoforms do exist, AQP11 and AQP12 (Morishita et al., 2004). AQP11 is mainly expressed in the testis, kidney, and liver, whereas AQP12 is selectively expressed in acinar cells of the pancreas (Itoh et al., 2005; Morishita et al., 2005). Analysis of the C termini of mouse AQP11 and AQP12 revealed that they contain amino acid sequences similar to di-lysine motifs (-WLHNNQMTNKKE and -SPGSVDAKMHKGE, respectively; Ishibashi, 2006), although these sequences do not completely satisfy the di-lysine motif. In addition, it has been demonstrated that AQP6 is another mammalian aquaporin that is not localized to the plasma membrane, although it does not have a di-lysine motif at its C terminus (Yasui, Hazama, et al., 1999; Yasui et al., 1999).

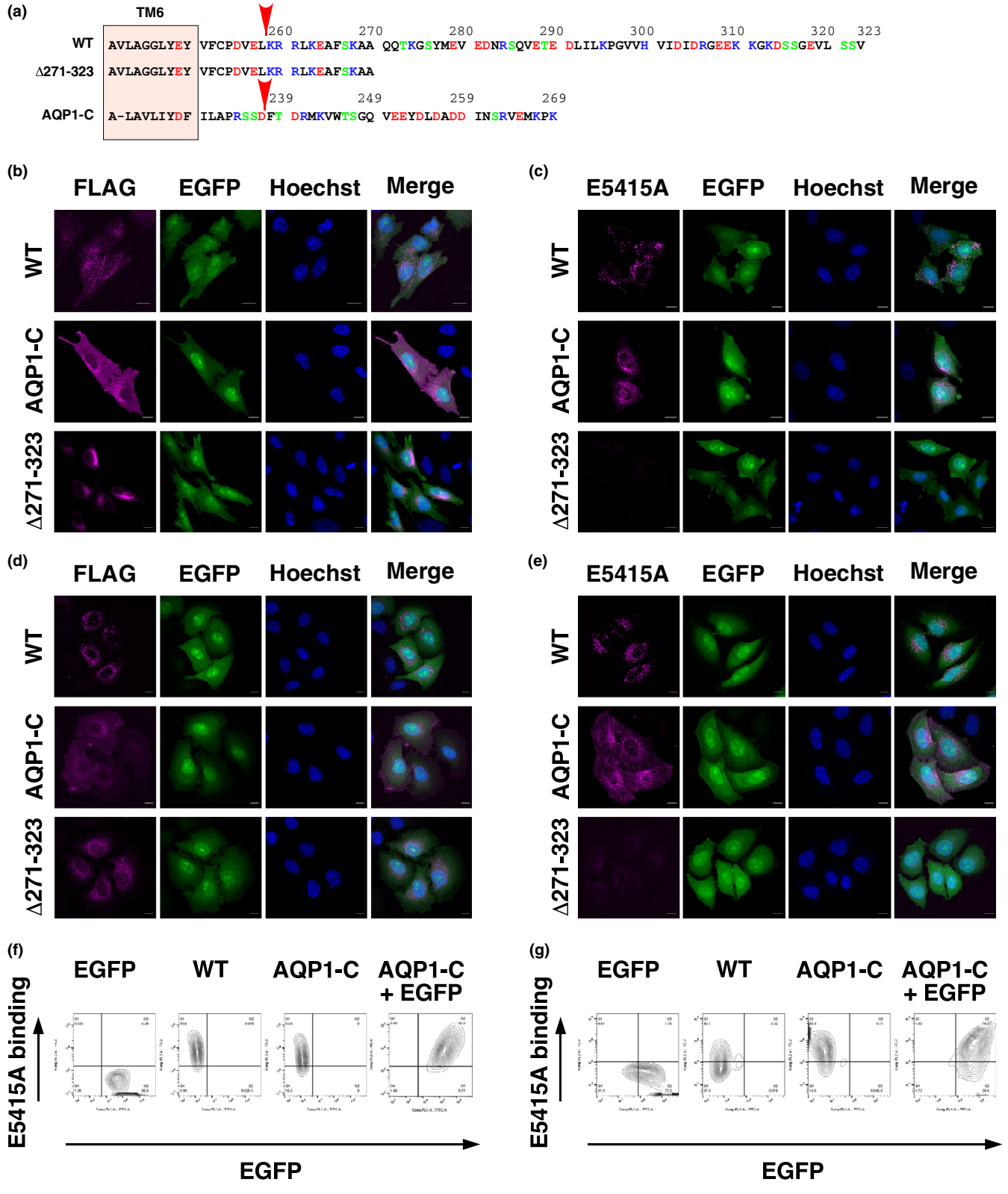
In this study, we demonstrated that deletion of the C-terminal 53 amino acids (Q^{271}-V^{323}) of AQP4 resulted in an abrogation of surface expression, and redistribution to intracellular compartments, including the ER, probably due to the formation of a di-lysine-like C-terminal signal that retained the protein in intracellular compartments.

2 | RESULTS AND DISCUSSION

2.1 | Deletion of the C-terminal 53 amino acids causes redistribution of AQP4 to intracellular compartments

In the process of investigating the role of the cytosolic C-terminal domain of AQP4 in its subcellular localization, trafficking, and degradation, we generated deletion mutants and observed that deletion of the C-terminal 53 amino acids (Figure 1a, Δ271-323) caused aberrant subcellular localization of AQP4 in two cell lines, that is, CHO-K1 and HeLa cells. While WT AQP4 was found to localize to both the plasma membrane and some intracellular compartments, AQP4 Δ271-323 was exclusively found in intracellular compartments (Figure 1b,d). Unexpectedly, WT AQP4 was mostly detected in intracellular vesicular structures in HeLa cells (Figure 1d, WT, magenta). However, replacing the cytosolic C-terminal domain of AQP4 (K^{259}-V^{323}, Figure 1a, WT) with that of AQP1 (F^{238}-K^{269}, Figure 1a, AQP1-C) led to increased cell-surface AQP4 (Figure 1d, AQP1-C, magenta). This was confirmed by flow cytometry, which revealed a higher level of cell-surface expression of AQP4 by labeling with a monoclonal antibody E5415A, which recognizes the extracellular region of mouse AQP4 (Huang et al., 2016), in cells expressing AQP1-C compared with those expressing the WT protein (Figure 1g). Despite the prevalent intracellular localization of WT AQP4 in HeLa cells, extracellularly added fluorescently labeled E5415A was incorporated into intracellular compartments (Figures 1e and 2b), indicating that at least some WT AQP4 reached the plasma membrane and underwent antibody-induced endocytosis in HeLa cells, as observed in those expressing AQP1-C (Figure 1e). In CHO-K1 cells, both WT and AQP1-C were efficiently

FIGURE 1 Deletion of the C-terminal 53 amino acids causes localization of aquaporin-4 in intracellular compartments. (a) The primary sequence of the C-terminal cytoplasmic region of mouse AQP4 compared with that of mouse AQP1. A part of the 6th transmembrane domain (TM6) is indicated by the box. Amino acids possessing acidic, basic, and hydroxyl groups are indicated in blue, red, and green, respectively. Cutting sites for exchanging the C-terminal domains of AQP4 and AQP1 are indicated by red arrow heads. (b-e) Localization of wild-type (WT) AQP4, as well as AQP4 with the C-terminal domain of AQP1 (AQP1-C), and AQP4 lacking the C-terminal 53 amino acids (Δ271-323). CHO-K1 (b and c) or HeLa (d and e) cells were transfected with a plasmid containing cDNAs encoding either mouse AQP4 or its derivative and EGFP linked by an IRES. Fixed cells (b and d) were stained with anti-FLAG antibody (magenta), while live cells (c and e) were treated with Alexa Fluor-555-labeled monoclonal anti-mouse AQP4 extracellular domain antibody (E5415A, magenta) for 24 hr. In both cases, only transfected cells (green) were specifically labeled with each antibody (magenta). Nuclei were stained with Hoechst 33342 (blue). Scale bars, 10 μm. (f and g) Flow cytometry of CHO-K1 (f) and HeLa (g) cells transfected with a plasmid containing cDNAs encoding EGFP (EGFP), WT AQP4 (WT), AQP4 containing the C-terminal domain of AQP1 (AQP1-C), or both AQP1-C and EGFP linked by an IRES (AQP1-C + EGFP). Binding of E5415A was detected with PE-labeled anti-mouse IgG. The *X* and *Y* axes represent intensities of fluorescence of EGFP and PE, respectively



expressed on the cell surface (Figure 1b,f) and underwent antibody-induced endocytosis (Figures 1c and 2b, c). In contrast, extracellularly added fluorescently labeled E5415A was detected neither on the cell surface nor in intracellular compartments in both CHO-K1 and HeLa cells expressing AQP4 Δ271-323 (Figure 1c,e), indicating that it could not reach the plasma membrane. These results were confirmed by flow cytometry, in which sparse cell-surface labeling was observed in cells expressing AQP4 Δ271-323 (Figure 2c, Δ271-323).

(a)

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                                        260       270
Δ274-323  AVLAGGLYEY VFCPDVELKR RLKEAFSKAA QQT
Δ272-323  AVLAGGLYEY VFCPDVELKR RLKEAFSKAA Q
Δ271-323  AVLAGGLYEY VFCPDVELKR RLKEAFSKAA
Δ270-323  AVLAGGLYEY VFCPDVELKR RLKEAFSKA
Δ268-323  AVLAGGLYEY VFCPDVELKR RLKEAFS
Δ265-323  AVLAGGLYEY VFCPDVELKR RLKE
Δ259-323  AVLAGGLYEY VFCPDVEL
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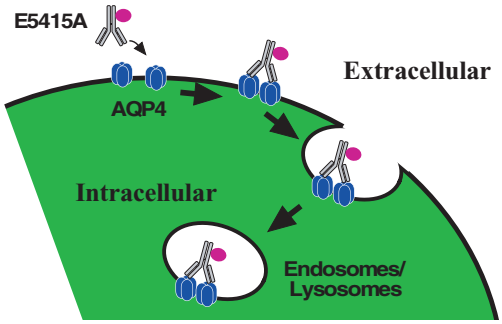
(b)



(c)

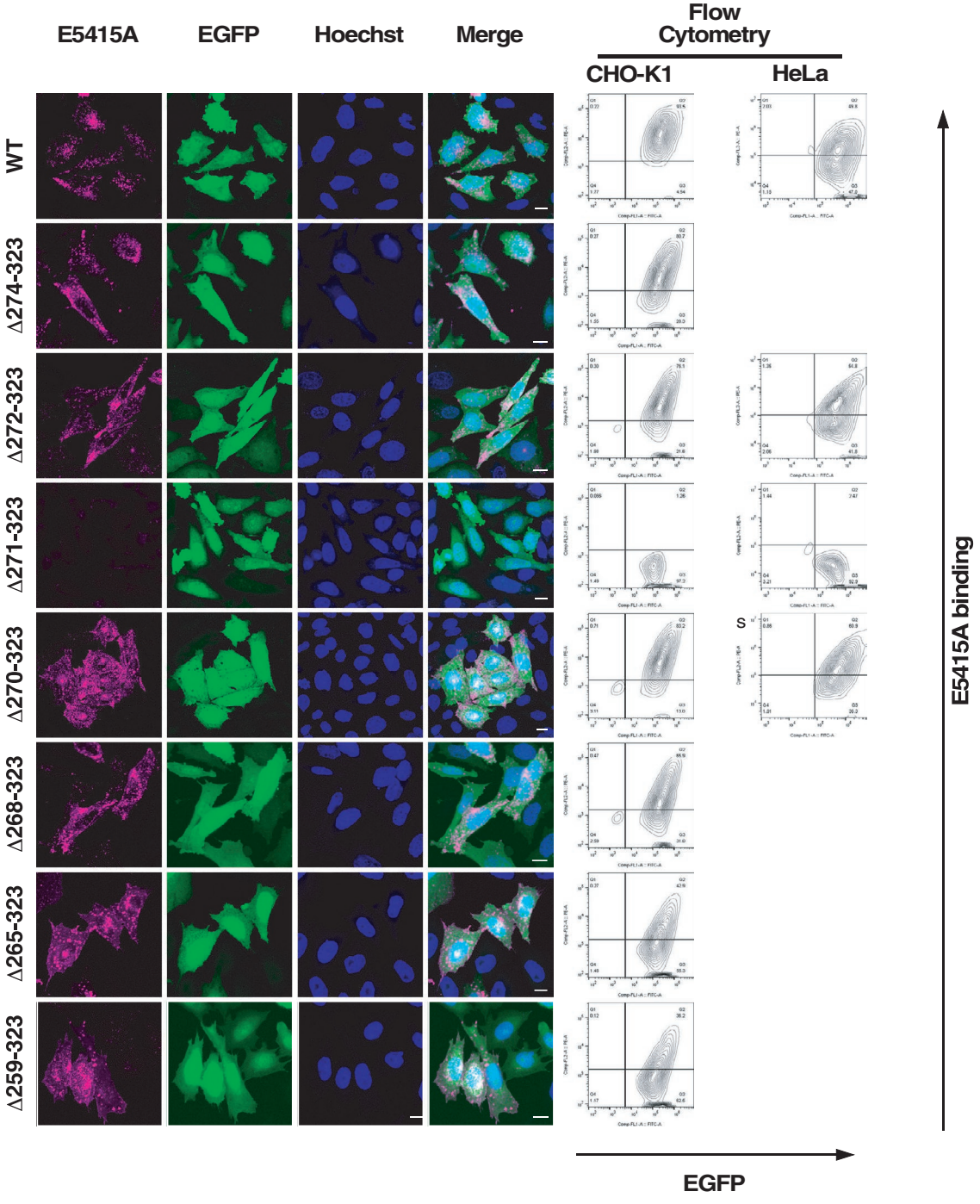


FIGURE 2 Formation of a specific sequence at the C terminus results in a putative motif causing retention of aquaporin-4 in intracellular compartments. (a) Sequential deletion of sequences at the AQP4 C terminus was used in this study. Red, blue, and green letters represent characteristics of the amino acid, as described in Figure 1. (b) Schematic illustration of antibody (E5415A)-induced endocytosis of AQP4. (c) Detection of AQP4 and its derivatives on the cell surface of live cells transfected with each plasmid (EGFP) by confocal microscopic imaging of CHO-K1 cells treated with Alexa Fluor-555-labeled E5415A (magenta), as well as by flow cytometry of CHO-K1 and HeLa cells labeled with E5415A followed by PE-labeled anti-mouse IgG. Scale bars, 10 μm

2.2 | Formation of a specific sequence at the C terminus functions as a putative motif causing retention of AQP4 in intracellular compartments

Next, we examined whether the characteristics of AQP4 Δ271-323 resulted from the loss of a signal in the C-terminal domain within the deleted region, which was necessary for transportation of AQP4 to the plasma membrane. To examine this possibility, we generated additional deletion mutants of AQP4 lacking 50-65 amino acids (Figure 2a) and analyzed their localization with fluorescently labeled E5415A. As a whole, all mutants as well as AQP4 Δ271-323 were expressed with levels higher than WT AQP4 as determined by Western blotting (Figure 3a). Based on our observations, all newly generated mutants, including those characterized by deletion of large stretches (Δ259-323, Δ265-323, Δ268-323, and Δ270-323), were transported to the plasma membrane in CHO-K1 cells, as determined by incorporation of extracellularly added fluorescently labeled E5415A (Figure 2c). It is unlikely that the lack of incorporation of fluorescently labeled E5415A into intracellular compartments in cells expressing AQP4 Δ271-323 was due to a conformational change of its extracellular loops, which may cause a reduction in the binding affinity of E5415A to the corresponding epitope. This conclusion was based on the observation that E5415A was bound to AQP4 in all other mutants, including AQP4 Δ270-323 and Δ272-323, the cytosolic domains of which are only one amino acid shorter and longer, respectively, than that of Δ271-323 (Figure 2a,c). Similar results were obtained by flow cytometry using CHO-K1 and HeLa cells (Figure 2c). Thus, we concluded that the effect of deleting the C-terminal 53 amino acids of AQP4 on subcellular localization could be attributed to the formation of a signal at the C terminus that retained AQP4 in intracellular compartments, rather than a signal required for transportation of AQP4 to the plasma membrane being lost.

2.3 | AQP4 Δ271-323 partially colocalizes with an ER marker

It is well known that many ER-resident proteins have a signal for retention in the ER at their C terminus (Teasdale & Jackson, 1996). In the case of type 1 transmembrane proteins from yeast, plant, and mammalian cells, ER localization can be attributed to the presence of di-lysine motifs at the C terminus (Benghezal et al., 2000; Jackson et al., 1990; Nilsson et al., 1989), which are directly recognized by coat protein complex I (COPI) for retrograde transport from the *cis*-Golgi network to the ER (Jackson et al., 2012). Although the C-terminal five amino acid sequence of AQP4 Δ271-323 (266-FSKAA-270) does not satisfy the classical di-lysine motif (KXKXX or KKXX), it might function as an ER localization signal. Afterward, we examined whether AQP4 Δ271-323 was localized in the ER. Confocal images revealed that AQP4 Δ271-323 (Figure 4, magenta) partially colocalized with an ER marker (Figure 4, green), suggesting that there was a population of AQP4 Δ271-323 residing in the ER. Since AQP4 Δ271-323 did not fully colocalize with the ER marker, it may have also been contained in intracellular compartments other than the ER. However, we cannot exclude that AQP4 Δ271-323 and the ER marker resided in different subcompartments of the ER. The ER marker used in this experiment is a fusion construct of the ER signal sequence of calreticulin (KDEL) and EGFP. KDEL is an ER retention motif for soluble proteins that binds to KDEL receptors, which also interact with COPI (Lewis & Pelham, 1990). According to these properties, AQP4 Δ271-323 is a transmembrane protein that is inserted into ER membranes, and, unlike the ER marker, is not present in the ER lumen, which may have contributed to the observed partial colocalization.

2.4 | K^{268} and the alkyl group of the C-terminal amino acid in AQP4 Δ271-323 are essential for localization in intracellular compartments

If the characteristics of AQP4 Δ271-323 are determined by a specific sequence formed at the C terminus, like a di-lysine ER retention motif, disruption of this sequence would result in the release of AQP4 Δ271-323 from intracellular compartments. Therefore, we evaluated whether such a sequence existed at the C terminus of AQP4 Δ271-323 by generating substitution mutants (Figure 5a). Levels of expression of all derivatives of AQP4 Δ271-323 in CHO-K1 cells are comparable to each other and were higher than that of WT (Figure 3b). Substitution of A for K^{268} allowed Δ271-323 to be transported to the plasma membrane (Figure 5b, K268A). Interestingly, substitution of G for A^{270} at the C terminus also promoted cell-surface localization of the mutant (Figure 5b,

A270G). In contrast, substitution of the C-terminal L for A^{270} did not restore cell-surface expression of AQP4 Δ271-323 (Figure 5b, A270L), indicating that an alkyl group at the last amino acid was required for maintaining intracellular localization of AQP4 Δ271-323. Since the A270G and K268A mutants of Δ271-323 reached the cell surface, we measured water permeability of these mutants using a modified calcein self-quenching assay. As shown in Figure 6, the two mutants to a small degree conducted water through the plasma membrane, compared with cells transfected with empty vector (EGFP) and naïve cells. However, the two mutants water permeability was lower than that of WT. This result suggests that in C-terminally truncated mutants, water permeability was reduced. In the case of cell-based assays, the level of expression and subcellular localization of aquaporin molecules should be considered. In our experiment, levels of A270G and K268A mutants of Δ271-323 were higher than that of WT (Figure 3b) while their cell-surface localization was lower than that of WT (Figure 5b, 80% and 85% in EGFP-positive cells for A270G and K268A Δ271-323, respectively, versus 95% for WT). Ho et al. (2009) demonstrated that trypsinized human AQP4 lacking C-terminal 64 amino acids (Δ260-323) inserted into proteoliposomes conducted water at a rate comparable to that of full-length human AQP4. Considering this observation, it is unlikely that a large deletion of C-terminal domain severely affects water permeability of the protein.

There is a cluster of basic amino acids (259-KRRLK-263) located near the 6th transmembrane domain, five amino acids upstream of K^{268} (Figure 5a). This sequence resembles arginine-based ER localization signals, which were also suggested to interact with COPI (Michelsen et al., 2005). To examine whether this cluster of basic amino acids plays a role in the localization of AQP4 Δ271-323 in intracellular compartments by functioning as an arginine-based ER localization motif, we substituted the R^{261} residue for A. Interestingly, AQP4 R261A-Δ271-323 reached the plasma membrane only in cells highly expressing the mutant, as determined by confocal microscopy and flow cytometry (Figure 5b, R261A). This suggests that the cluster of basic amino acids was not essential for intracellular localization of the protein but may influence the efficiency of the C-terminal di-lysine motif-like signal.

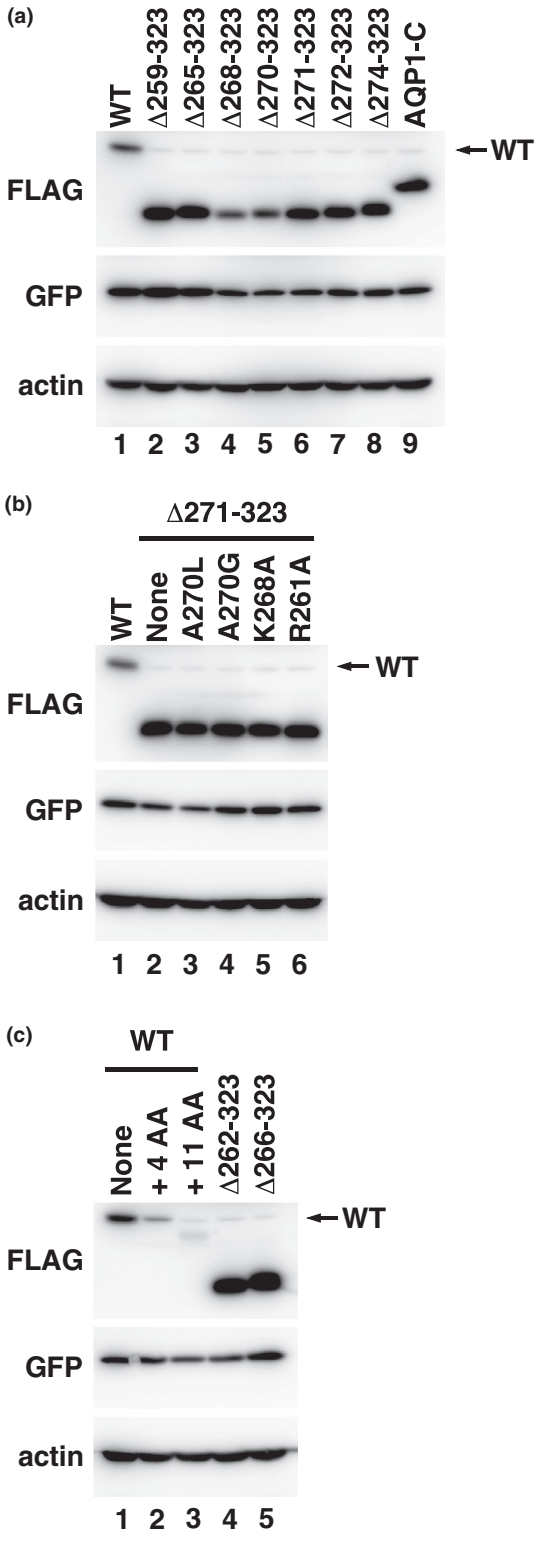


FIGURE 3 Expression of AQP4 and its derivatives in CHO-K1 cells. Expression of AQP4 and its derivatives was determined by Western blotting and detected with an antibody against the FLAG tag added to the N terminus of each protein. All cDNAs encoding each AQP4 or its derivative were connected with that encoding EGFP via IRES, which represents transfection efficiency in each transfected cell. (a) Expression of WT (lane 1) or C-terminally truncated mutants (lanes 2–8) as well as a chimeric mutant that has C-terminal domain of AQP1 (lane 9). (b) Expression of substitution mutants of Δ271-323 (lanes 3–6) comparing with that of WT (lane 1) and AQP4 Δ271-323 (lane 2). (c) Expression of WT (lane 1), WT with a 4-amino acid (+ 4 AA, lane 2) or 11-amino acid (+ 11 AA, lane 3) putative di-lysine like sequence added to its C terminus, or C-terminally truncated mutant of AQP4 (lanes 4 and 5). Unexpectedly, WT AQP4 possessing a sequence (RRLKEAFSKAA) at its C terminus (+ 11 AA) migrated faster than WT AQP4. It is still unclear why this happened. We also experienced the faster migration of full-length AQP4 in which some negatively charged amino acids were replaced by alanine (unpublished observation)

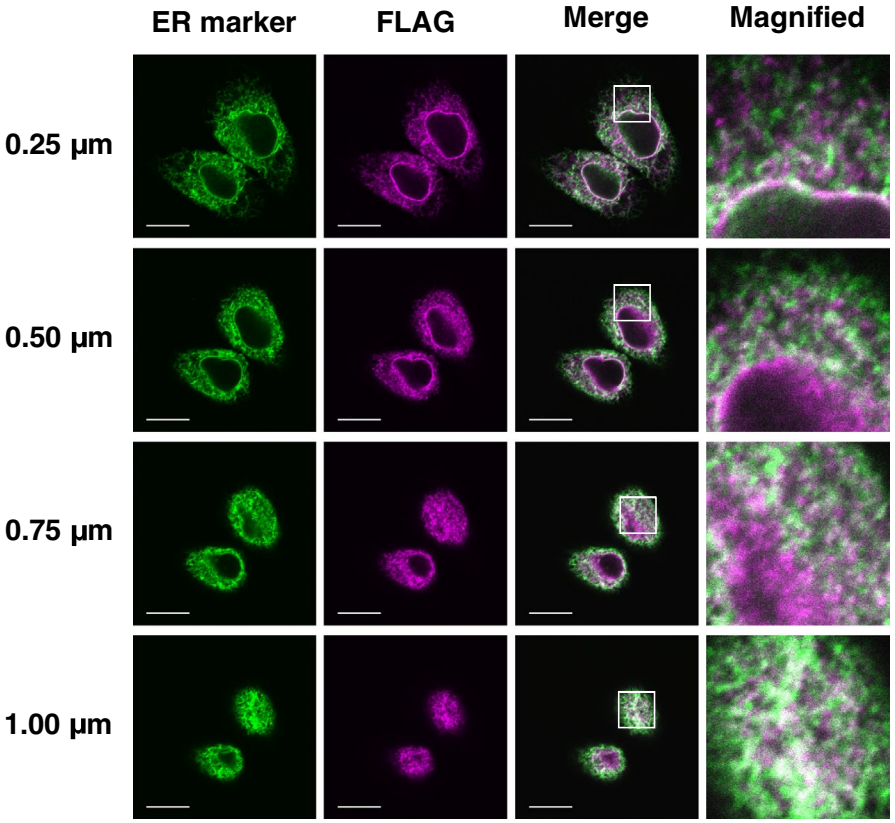


FIGURE 4 Aquaporin-4 Δ271-323 partially colocalizes with an ER-specific marker. HeLa cells transfected with the pEBMulti-Puro vector containing a cDNA encoding AQP4 Δ271-323 were treated with a baculovirus vector containing an ER marker (green). Cells were fixed with 4% paraformaldehyde and stained with anti-FLAG antibody (magenta). Z-stack images were taken at 0.25 μm intervals, and the distance from the bottom of the cells is indicated. The areas magnified in the rightmost panels are indicated with a white box in each merged image. Scale bars, 10 μm

2.5 | Universality of the di-lysine motif-like sequence

To examine the universality of the di-lysine motif-like sequence of AQP4 Δ271-323, we added the last four (267-SKAA-270) or eleven (260-RRLKEAFSKAA-270) amino acids of the mutant to the C terminus of WT AQP4 (Figure 7a, WT-SKAA and WT-RRLKEAFSKAA). Addition of the sequences to WT AQP4 partially prevented the protein from cell-surface expression in CHO-K1 cells as determined by flow cytometry; 98% of EGFP-positive cells expressed cell-surface WT AQP4, whereas only 32% and 69% of EGFP-positive cells expressed cell-surface WT-SKAA and WT-RRLKEAFSKAA mutants, respectively (Figure 7b). One possible explanation for the insufficient effect of the addition of the di-lysine motif-like sequence to the C terminus of WT AQP4 on its subcellular localization is that the distance from the transmembrane domain is critical for the sequence to function as a signal that retains AQP4 in intracellular compartments. This observation is another difference between this signal and the di-lysine motif; a di-lysine motif of 3-hydroxy-3-methylglutaryl coenzyme A reductase functions at a position located more than 500 amino acids from the transmembrane domain (Luskey & Stevens, 1985). On the other hand, in our case, we observed that addition of the shorter sequence, SKAA was more effective compared with the longer sequence, RRLKEAFSKAA, supporting the idea that the distance from the transmembrane domain is critical for the signal and suggests that the last four amino acids are sufficient if the sequence is located at an appropriate position. It should be noted that levels of expression of the two mutants (Figure 3c lanes 2 and 3) were lower than that of WT (Figure 3c, lane 1) as determined by Western blotting, which may contribute to reduced cell-surface expression of the mutants in CHO-K1 cells (Figure 7b).

There are two other sequences that form a C-terminal sequence similar to the last four amino acids of Δ271-323 (SKAA) upstream of this sequence (258-LKRR-261 and 262-LKEA-265) when the C-terminal domain is deleted just after the sequences (Figure 7a, Δ262-323 and Δ266-323, respectively). We transfected these constructs into CHO-K1 cells and examined the level of cell-surface expression of these mutants by flow cytometry. Levels of expression of these mutants in CHO-K1 cells (Figure 3c, lanes 4 and 5) were almost the same and much higher than that of WT (Figure 3c, lane 1). As shown in Figure 7b, similar to cells transfected with the Δ271-323 mutant, only 1% of EGFP-positive cells expressed detectable cell-surface Δ266-323 mutant AQP4, indicating that the C-terminal LKEA also functions as a di-lysine motif-like intracellular retention signal. Approximately 24% of EGFP-positive cells expressed detectable cell-surface Δ262-323 mutant AQP4 (Figure 7), indicating that the C-terminal LKRR also functions as an intracellular retention signal; however, the effect was limited. It is unclear why the C-terminal LKRR

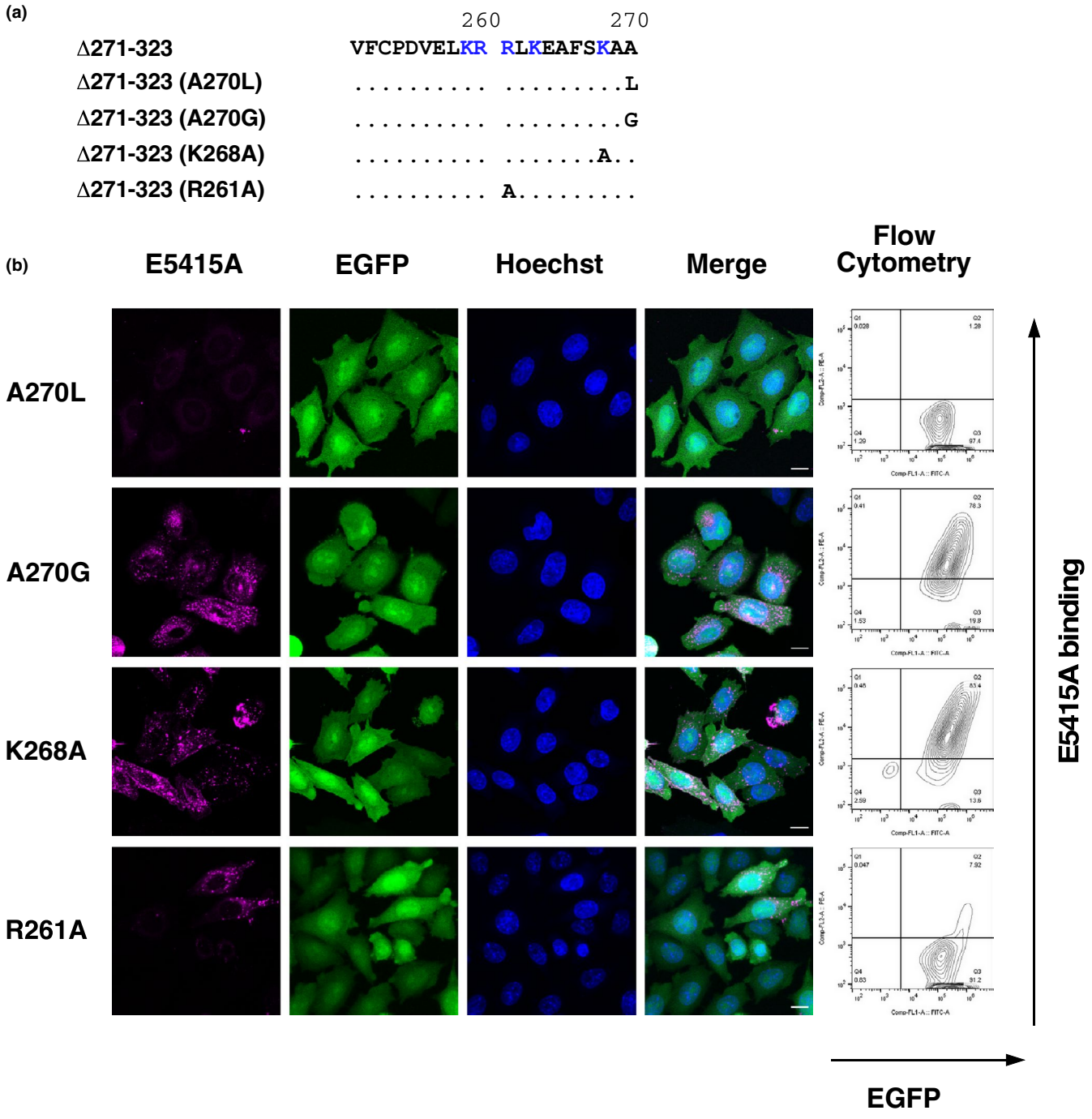


FIGURE 5 Lysine at position 268 and the alkyl group of the C-terminal amino acid in aquaporin-4 Δ271-323 are essential for localization in intracellular compartments. (a) Variants of AQP4 Δ271-323 possessing substitution mutations, which were used in this study. Basic amino acids are indicated in blue. (b) Detection of cell-surface expression of AQP4 Δ271-323 variants in CHO-K1 cells transfected with each plasmid (EGFP) by confocal microscopic imaging of cells treated with Alexa Fluor-555-labeled E5415A (magenta), as well as by flow cytometry of cells labeled with E5415A followed by PE-labeled anti-mouse IgG. Scale bars, 10 μm

located adjacent to the transmembrane domain did not completely retain the mutant in intracellular compartments. We can speculate that a positive charge of the C-terminal amino acid is unsuitable for the motif or the distance from the transmembrane domain of the sequence is too near to function as an intracellular retention signal. Further study is necessary to clarify the mechanism.

2.6 | Comparison with other aquaporins

Members of the subcellular- or super-aquaporin family were found to localize to intracellular compartments, including the ER (Ishibashi, 2006). Interestingly, many of them carry a di-lysine ER retention motif (Ishibashi, 2006). In the case of mammals, AQP11 and AQP12 are

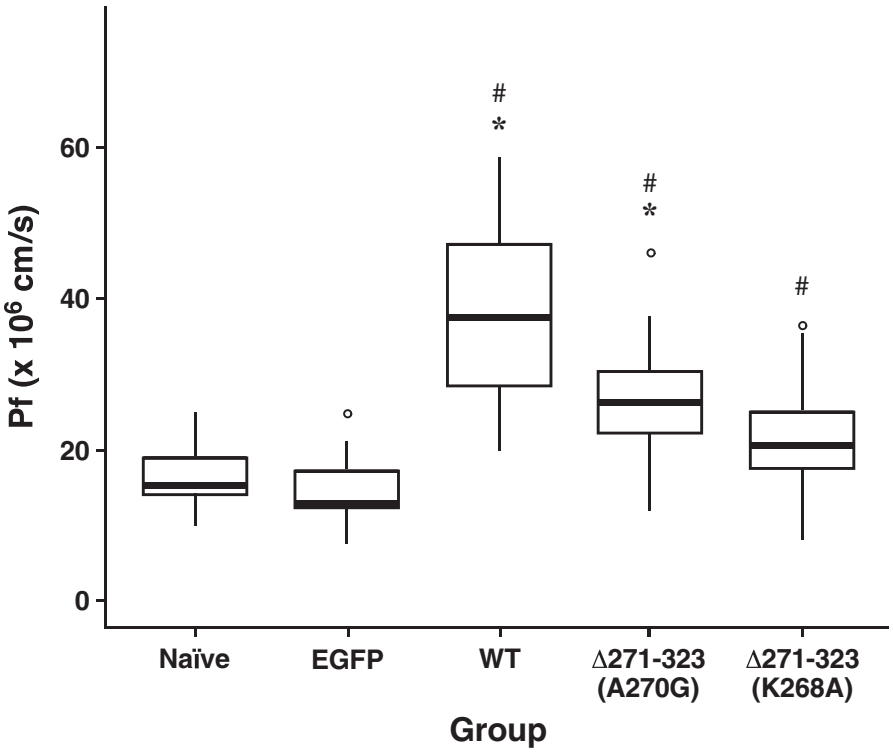


FIGURE 6 Water permeability of AQP4 mutants. Pf refers to the osmotic water permeability coefficient (Miyazaki et al., 2013). Group refers to each transfection of either an EGFP containing plasmid or AQP4 wild-type/mutant AQP4 containing plasmid ($n = 32$). Circles are statistical outliers as determined by RStudio. Naïve CHO-K1 cells are used as a negative control. Thick black bars indicate median Pf values for each group. * and # represent significant ($p < .05$) differences versus naïve and EGFP-expressing cells, respectively as determined by the Tukey's test

categorized as members of this family (Ishibashi, 2006). Although the classical di-lysine motif is absent from the C terminus of AQP11 and AQP12 (-WLHNNQMTNKKE and -SPGSVDAKMHKGE, respectively), there is a lysine residue at the third amino acid from the C terminus (Ishibashi, 2006), like Δ271-323. However, it is important to note that the C-terminal sequence of AQP11 may not function in a similar manner as a di-lysine motif, considering that a C-terminally added V5 tag did not affect its ER localization (Morishita et al., 2005). In addition, the C-terminal sequences of AQP12 are not conserved among species. Thus, it is possible that the mechanisms responsible for localization in intracellular compartments are different between AQP4 Δ271-323 and AQP11/AQP12. Similarly, AQP6 is another mammalian aquaporin exclusively localized in the ER; however, it does not have di-lysine motif or di-lysine motif-like sequence at its C terminus (Yasui, Hazama, et al., 1999; Yasui, Kwon, et al., 1999). Therefore, similar to AQP11 and AQP12, the mechanism responsible for the subcellular localization of AQP6 is still unclear. AQP8 is localized not only in the plasma membrane but also to intracellular compartments, including the inner mitochondrial membrane (Laloux et al., 2018; Takata et al., 2004). Interestingly, AQP8 has a sequence that satisfies XKXX at its C terminus (LKXR) adjacent to the transmembrane domain (Kirscht et al., 2018). However, considering our flow cytometry results for the Δ262-323 mutant, this sequence will not completely function as a intracellular localization signal for AQP8. This is because the position of the LKXR sequence of AQP8 is as close as that of 258-L<u>K</u>RR-261 of the Δ262-323 mutant. Furthermore, similar to the Δ262-323 mutant, the C-terminal amino acid of AQP8 is positively charged (Kirscht et al., 2018).

2.7 | Perspectives

So far, it is unclear whether an enzymatic cleavage after A^{265} as well as after A^{270} occurs as a physiological event, which drastically changes subcellular localization and function of AQP4 in vivo. Considering an important role for AQP11 in intravesicular homeostasis (Morishita et al., 2005), this is an intriguing scenario. Even if this was the case, the AQP4 Δ266-323 and Δ271-323 variants cannot be detected, since commercially available antibodies that are used for Western blotting and/or immunofluorescence staining exclusively recognize the C-terminal domain of AQP4. It has been reported that the amount of AQP4 protein in the central nervous system decreases in the acute phase of traumatic brain injury and ischemic stroke (Frydenlund et al., 2006; Kiening et al., 2002; Monai et al., 2019). This kind of post-translational modification of AQP4 might be involved in the pathogenesis of the aforementioned conditions. To address this issue, an antibody against AQP4 specifically recognizing a portion other than the C-terminal domain is required.

It is unclear whether the di-lysine like C-terminal signals of membrane proteins also function in vivo, including epithelial cells in the kidney, lung, and gastrointestinal tract; skeletal muscle; and astrocytic end-feet in the CNS.

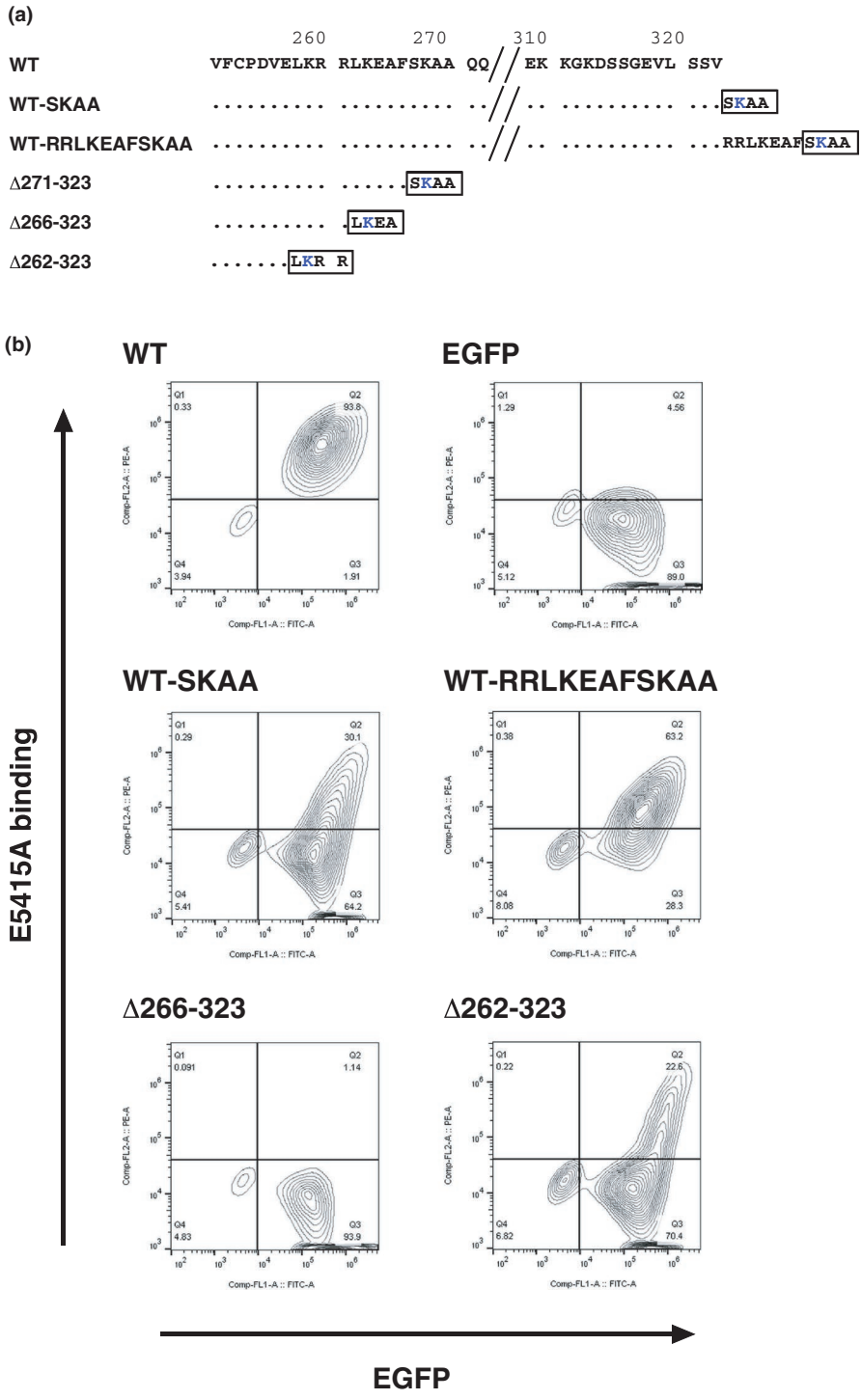


FIGURE 7 Positional effect of the last four amino acid of Δ271-323 mutant on subcellular localization of aquaporin-4. (a) Comparison of the sequences used in the experiments. Four amino acid sequences forming a di-lysine motif-like sequence are indicated with boxes. Lysine residues positioned at the third amino acid from the C terminus is indicated in blue. (b) Detection of AQP4 and its derivatives on the cell surface of live cells transfected with each plasmid by flow cytometry of CHO-K1 cells labeled with E5415A followed by PE-labeled anti-mouse IgG

It is interesting to investigate this issue by generating mice expressing each aforementioned truncated mutant of AQP4 in a future study.

Since regulatory mechanisms that determine trafficking and localization of membrane proteins by recognizing particular signals within the intracellular domains have not been fully understood, our findings provide a new tool for further investigating a mechanism that regulates subcellular localization of membrane proteins.

3 | EXPERIMENTAL PROCEDURES

3.1 | Plasmid construction

The mouse AQP4 M1 isoform, in which M^{23} was changed to L (Huang et al., 2016) to avoid expression of the M23 isoform by a leaky scanning mechanism (Rossi et al., 2010), had been fused to a FLAG tag at the N terminus and was then

used to construct cDNAs encoding wild-type (WT) AQP4, as well as its C-terminally mutated derivatives. PCR-based mutagenesis was performed to introduce nonsense and/or substitution mutations. All PCR products were inserted into a pGEM-T vector (Promega) for sequencing. The desired cDNAs encoding WT or mutant AQP4 and enhanced green fluorescent protein (EGFP) linked by an internal ribosomal entry site (IRES) were inserted between the SalI and NotI sites of the mammalian expression vector pEBMulti-Puro (FUJIFILM Wako Pure Chemical Corporation).

3.2 | Confocal microscopy

CHO-K1 (RCB0285) and HeLa (RCB0007) cells obtained from RIKEN BRC were grown in Ham's F12 and Dulbecco's modified Eagle's medium (high glucose; FUJIFILM Wako Pure Chemical Corporation), respectively, supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. CHO-K1 and HeLa cells were seeded onto 24-well plates at densities of 1×10^5 and 5×10^4 cells/well, respectively, and transfected with each plasmid using Lipofectamine LTX with Plus Reagents (Thermo Fisher Scientific) according to the manufacturer's instructions. At 48 hr after transfection, the cells were selected with 10 μg/ml of puromycin (InvivoGen) for additional 48 hr. Afterward, the cells were trypsinized and seeded onto 4-well plates containing a 13-mm cover glass (Matsunami Glass Ind. Ltd) coated with poly-D-lysine and cultured for another 48 hr.

For immunofluorescence staining, the cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS. FLAG-tagged AQP4 and its derivatives were stained with monoclonal anti-FLAG antibody (clone M2, 1:200, Sigma-Aldrich Corp.), followed by Alexa Fluor-555-conjugated goat anti-mouse IgG (1:200, Thermo Fisher Scientific).

For visualizing antibody-induced endocytosis of AQP4, 2 μg/ml of Alexa Fluor-555-labeled E5415A, an antibody recognizing the extracellular region of mouse AQP4 (Huang et al., 2016) was added to live cells on a cover glass and incubated for 24 hr at 37°C.

For examining AQP4 Δ271-323 colocalization, HeLa cells were transfected with pEBMulti-Puro containing the cDNA encoding FLAG-tagged AQP4 Δ271-323. Subsequently, the ER was visualized using CellLight™ ER-GFP, BacMam 2.0 (Thermo Fisher Scientific) according to the manufacturer's instructions. Cells were fixed with 4% paraformaldehyde in PBS, washed twice with PBS, permeabilized with 0.1% Triton X-100 in PBS, and blocked with 4% goat serum in PBS for 1 hr at room temperature. AQP4 Δ271-323 was stained using mouse monoclonal anti-DDDDK-tag antibody (clone FLA-1, 1:10,000, MBL Co. Ltd.), followed by Alexa Fluor-555-conjugated goat anti-mouse IgG.

All images were acquired using an LSM 710 confocal laser scanning microscope (Carl Zeiss).

3.3 | Western blotting

Cell lysates were obtained, and the amount of protein in each lysate was measured as described previously (Abe et al., 2008). Thirty microgram of cellular protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 12.5% gel containing 3 mol/L urea, followed by transfer to polyvinylidene difluoride membrane and blocking with 5% skim milk in PBS containing 0.05% Tween 20 (FUJIFILM Wako Pure Chemical Corporation). Signals were visualized with Immobilon Western Chemiluminescent HRP substrate (EMD Millipore Corporation). The antibodies used were anti-β-actin pAb-HRP-DirecT (1:10,000; Medical & Biological Laboratories Co., LTD), anti-DDDDK-tag mAb-HRP-DirectT (1:10,000; Medical & Biological Laboratories Co., LTD), anti-GFP (mFX75, 1:700; FUJIFILM Wako Pure Chemical Corporation), and HRP-conjugated goat anti-mouse IgG (1:5,000, Sigma-Aldrich Corp.).

3.4 | Flow cytometry

CHO-K1 and HeLa cells were seeded onto 60-mm dishes at densities of 5×10^5 and 2×10^5 cells/dish, respectively, and transfected with each plasmid using Lipofectamine LTX with Plus Reagents. At 48 hr after transfection, the cells were selected by adding 10 μg/ml puromycin to the culture media. The cells were then trypsinized and resuspended in 100 μl of 0.1% bovine serum albumin (BSA) in PBS. Next, the cells were incubated with 2 μg/ml of E5415A for 1 hr at 4°C. Afterward, the cells were washed with 0.1% BSA in PBS and subsequently stained with PE-conjugated goat anti-mouse IgG (1:100, SouthernBiotech) for 1 hr at 4°C. Flow cytometry was performed on a BD Accuri C6 Plus flow cytometer (BD Biosciences), and data were analyzed using Flowjo software.

3.5 | Cell water permeability assay

Cell water permeability was conducted via a modified calcein self-quenching assay (Hamann et al., 2002). In brief, AQP4 plasmid transfected CHO-K1 cells were seeded at a density of 9×10^4/well in a black sided, clear bottom 96-well plate one day prior to conducting the permeability assay. On the day of the assay, cells were washed with 100 μl Hanks' balanced salt solution (HBSS), followed by incubation with 20 μl Calcein AM (Thermo Fisher Scientific) in HBSS for 30 min. Postcalcein flooding, cells

were washed once with 100 µl HBSS, and then incubated in 100 µl HBSS. The 96-well plate was then loaded into a SpectraMax i3x (Molecular Devices, LLC.) for high osmotic concentration media injection and fluorescent intensity readings. 25 µl of 800 mosm media was injected into each well (100 µl, 300 mosm) for an increase in osmotic concentration of 100 mosm and intensity was read every 0.067 s for 30 s postinjection at 23–25°C. Raw data were collected using SOFTMAX Pro 7.1 (Molecular Devices, LLC.) and imported into MATLAB 2019b (Mathworks, Inc.) for analysis. Statistical analysis was performed using RSTUDIO version 1.1.463 (RStudio PBC). Data were analyzed using one-way ANOVA followed by the Tukey's test.

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