AKAP220 colocalizes with AQP2 in the inner medullary collecting ducts

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During dehydration, protein kinase A phosphorylates aquaporin 2 (AQP2) at serine 256 and this is essential for apical membrane sorting of AQP2 in the collecting ducts. A-kinase anchoring proteins (AKAPs) bind protein kinase A and protein phosphatases conferring substrate specificity to these enzymes and localize them to the appropriate intracellular compartment. We found that AKAP220 bound to AQP2 in a yeast two-hybrid screen. Further, it was highly localized to the papilla compared to other regions of the kidney. Using double immunofluorescence and immunoelectron microscopy we found that AKAP220 co-localized with AQP2 in the cytosol of the inner medullary collecting ducts. Forskolin-mediated phosphorylation of AQP2, transiently expressed in COS cells, was increased by AKAP220 co-expression. Our results suggest that AKAP220 may be involved in the phosphorylation of AQP2 by recruiting protein kinase A.

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Aquaporin 2 (AQP2) is a water channel in the collecting ducts of the kidney, and it is important in urine concentration. In dehydrated animals, arginine vasopressin is released from the pituitary gland and binds to the vasopressin type 2 receptor on the basolateral plasma membrane of the collecting ducts,[1] stimulates adenylate cyclase, and increases the cyclic AMP concentration, thus activating cyclic AMP-dependent protein kinase A (PKA). AQP2 is then phosphorylated at serine 256 by PKA,[2,3] and this phosphorylation is a switch that translocates AQP2 from the cytosol to the apical membrane. Once AQP2 is inserted in the apical membrane, water permeability of the collecting ducts increases, thus enabling water reabsorption.[4–6] Although the overall scheme of the urine concentration mechanism is identified as described above, the detailed mechanisms of AQP2 trafficking by AQP2 phosphorylation remain largely unknown. To gain insight into this mechanism, the identification of molecules surrounding AQP2 is an important step. Previously, we isolated several AQP2-binding proteins by coimmunoprecipitation with anti-AQP2 antibody and mass spectrometry.[7] However, this method only allowed us to identify the proteins tightly bound to AQP2 even in the presence of detergents. Although the yeast two-hybrid assay is a powerful tool to identify proteins showing subtle binding ability within cells, this strategy in our hands has not successfully identified proteins bound to AQP2. Accordingly, to identify novel AQP2-binding proteins, we adopted a new yeast two-hybrid assay that uses split-ubiquitin technology.[8] In contrast to the conventional yeast two-hybrid assay, we could use whole AQP2 as bait. By using the new yeast two-hybrid assay, we identified A-kinase anchoring protein 220 (AKAP220) as an AQP2-binding protein.

RESULTS

Isolation of AKAP220 by the yeast two-hybrid system

After two-hybrid screening under a stringent condition, a clone encoding part of AKAP220 (residues 958–1306) was isolated. To confirm the result of library screening, we again transformed yeast cells with whole AQP2 and the isolated AKAP220 clone alone or in combination (Figure 1a). Only the yeast clone transformed with both plasmids could grow on the selection plate, confirming the binding of both

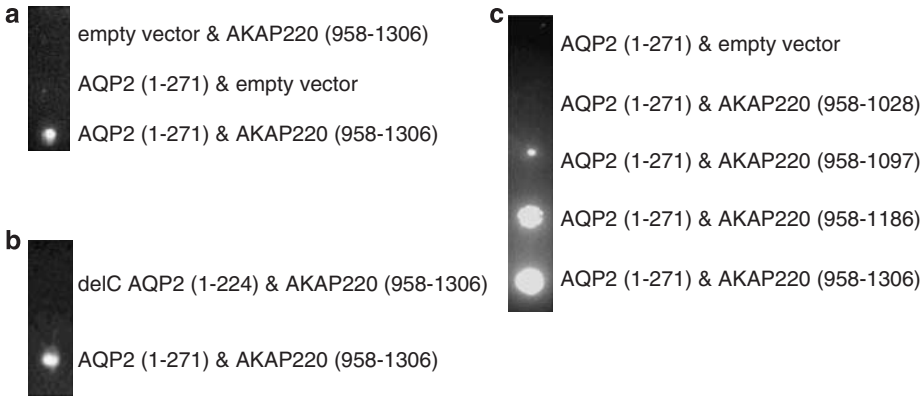

Figure 1 | AKAP220 is an AQP2-binding protein. (**a**) Whole AQP2 (1-271) binds to a partial AKAP220 (958-1306). A yeast colony expressing both AQP2 and AKAP220 could grow on the selection plate. (**b**) AQP2 without the C-terminal cytosolic region (1-224) could not bind to AKAP220. (**c**) Residues from 1098 to 1186 in AKAP220 are involved in the binding to AQP2.

proteins in yeast cells. To further confirm that the binding was not an artifact and to narrow down the binding sites within each protein, we prepared deletion mutants of each clone. As shown in Figure 1b, the deletion of the C-terminal portion of AQP2 abolished the binding, suggesting that the interaction site within AQP2 was located in the cytosolic C terminus. In AKAP220, residues 1098–1186 contained a binding site to AQP2 (Figure 1c). We could not further narrow down the binding sites, as some yeast cells transformed with other mutants did not grow well even on the full medium for an unknown reason.

Immunolocalization of AKAP220 in the inner medullary collecting ducts of rat kidney

To investigate whether AKAP220 could interact with AQP2 *in vivo*, we performed immunoblot, immunofluorescence, and immunoelectron microscopy of AKAP220 in rat kidney. Immunoblot of the cortex, outer medulla, and papilla of rat kidney clearly showed that AKAP220 was most abundant in papilla (Figure 2a). Double immunofluorescence with AQP2 clearly showed that AKAP220 was mainly present in the inner medullary collecting ducts, and under higher magnification AKAP220 was colocalized with AQP2 in the subapical cytoplasmic region (Figure 2b). Whether AKAP220 is also present in the cortical and outer medullary collecting ducts remains to be determined because of the relatively low sensitivity of the antibody in immunofluorescence. To confirm the close localization of AQP2 and AKAP220 *in vivo*, we performed double immunoelectron microscopy (Figure 2c). Although we detected fewer signals for AKAP220 (20-nm gold particles) as compared to the AQP2 signals (10-nm gold particles), probably due to the lower sensitivity of the anti-AKAP220 antibody, almost all AKAP220 was colocalized with AQP2, confirming the direct affinity of AKAP220 to AQP2 *in vivo*.

AKAP220 increased phosphorylation of AQP2

To gain insight into the functional role of AKAP220 in AQP2 phosphorylation, we investigated the phosphorylation status of AQP2 transiently expressed in COS cells with or without AKAP220 coexpression. Before the AQP2 phosphorylation experiments, we confirmed that AKAP220 formed a complex with PKA. As shown in Figure 3a, the catalytic and regulatory subunits of PKA coimmunoprecipitated with AKAP220 as previously reported. Under this condition, AQP2 phosphorylation induced by forskolin was much higher ($P<0.01$, 1.98 ± 0.07-fold increase, mean ± s.d., $n=4$) than in the condition without AKAP220 coexpression (Figure 3b). This phosphorylation of AQP2 measured by autoradiography was confirmed to reflect the phosphorylation of AQP2 at serine 256 (the third lane in the upper panel of Figure 3b). In addition, AKAP220 lacking the binding domain to AQP2 did not elicit the phosphorylation of AQP2 (the fourth lane), confirming that the binding site determined by the yeast two-hybrid assay is important in the AQP2-AKAP220 interaction in mammalian cells.

DISCUSSION

Phosphorylation of AQP2 by PKA is necessary for its apical translocation in the collecting ducts. As this process occurs within a minute,[9,10] molecular mechanisms supporting efficient AQP2 phosphorylation are necessary. As PKA has broad substrate specificity and is present in relatively high concentrations within cells, it is crucial to localize its activity to its substrate. One way to accomplish this localization is by anchoring the regulatory subunit of PKA to AKAPs,[11,12] which target PKA in close proximity to relevant substrates. Although more than 50 AKAPs have been identified by their ability to bind to the regulatory subunits of the PKA holoenzyme, there is no overall sequence similarity among different AKAPs. Previously, several AKAPs were shown to be present in AQP2-bearing vesicles by PKA overlay assays.[13,14] Among them, AKAP18δ was present on the same intracellular vesicles as AQP2.[15] Furthermore, AKAP18δ was translocated to the plasma membrane with AQP2 by vasopressin stimulation, suggesting that AKAP18δ was involved in the AQP2 sorting machinery. However, neither its direct binding ability to AQP2 nor its effect on AQP2

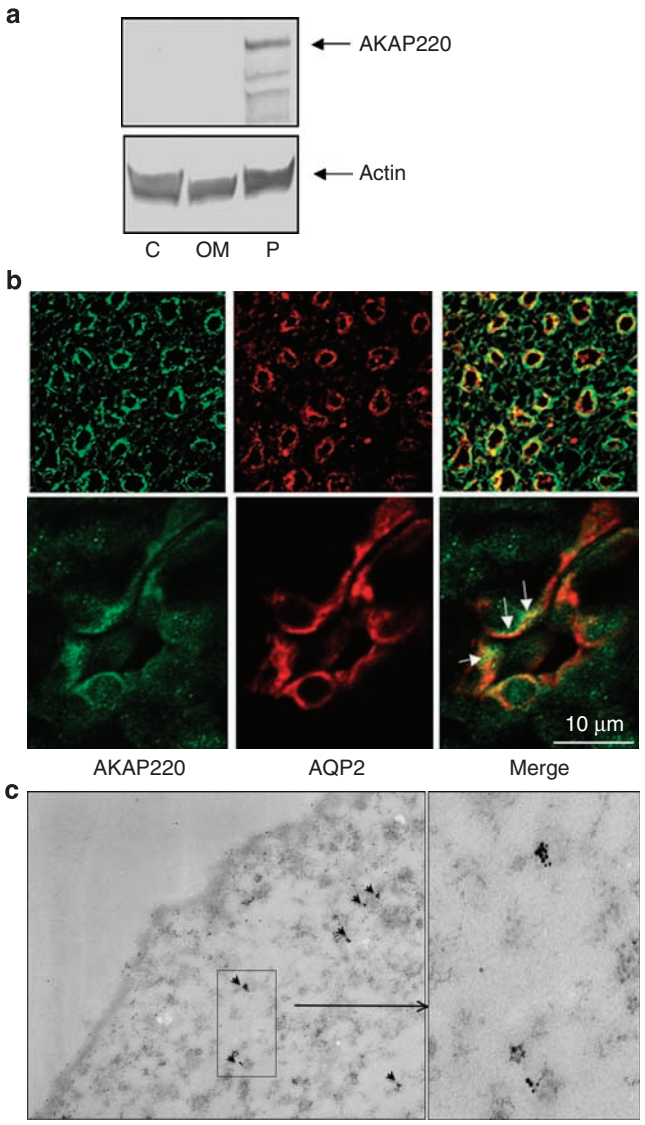

Figure 2 | AKAP220 is present in the papillary collecting ducts of the kidney. (**a**) Total lysates from rat cortex (C), outer medulla (OM), and papilla (P) were separated by SDS–PAGE (27 μg of protein per lane) and immunoblotted with anti-AKAP220 antibody and anti-actin antibody. AKAP220 was detected only in papilla. (**b**) Double immunofluorescence of AKAP220 and AQP2 in the rat kidney. AKAP220 was mainly present in the collecting ducts (upper panels) and colocalized with AQP2 in the subapical cytoplasmic region of the collecting ducts (indicated by arrows). (**c**) Double immunoelectron microscopy of AKAP220 and AQP2 in the rat kidney. Almost all large (20-nm) gold particles (AKAP220) were colocalized with small (10-nm) gold particles (AQP2; indicated by arrows). Original magnification (× 20,000).

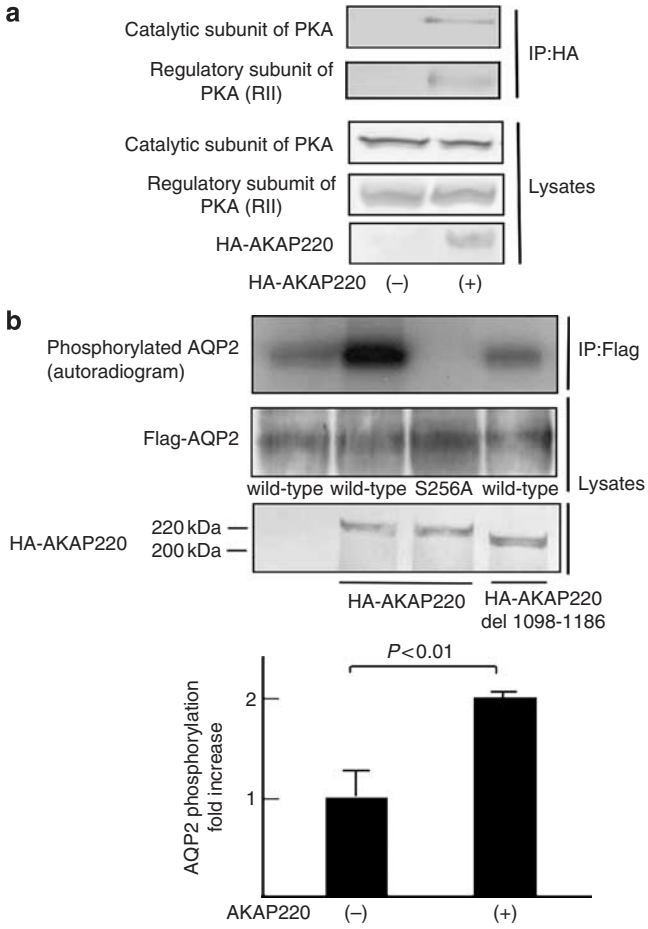

Figure 3 | AKAP220 regulates the phosphorylation of AQP2 in COS cells. (**a**) Total cell lysates from COS cells transfected with either an empty vector or an HA-AKAP220 expression vector were immunoprecipitated with anti-HA antibody. The immunoprecipitates and total lysates were separated by SDS–PAGE and transferred to a nitrocellulose membrane that was then probed with antibodies to the catalytic and regulatory subunits of PKA. (**b**) Increased AQP2 phosphorylation by AKAP220. Demonstrable autoradiogram of phosphorylated AQP2 in COS cells with or without AKAP220 coexpression (upper panels). In the third lane from the left, AQP2 phosphorylation was not observed when serine 256 was mutated to alanine, indicating that Ser256 was the only phosphorylation site by PKA in this assay system. Full-length AKAP220 significantly increased AQP2 phosphorylation (second lane), whereas the mutant AKAP220 lacking the binding site to AQP2 did not increase AQP2 phosphorylation (fourth lane). Signal intensities of AQP2 phosphorylation measured by Image Gauge software (Fuji Film, Tokyo, Japan) were compared with the paired *t*-test (bar graph; $P < 0.01$, $n = 4$).

phosphorylation was shown. In the present study, a new yeast two-hybrid assay developed for membrane proteins identified AKAP220 as a protein that binds to AQP2. This finding may be physiologically relevant, as AKAP220 was colocalized with AQP2 in rat kidney collecting ducts and AKAP220 enhanced forskolin-induced AQP2 phosphorylation. Accordingly, at least two AKAPs (AKAP18δ and AKAP220) and possibly several AKAPs may be involved in the regulated AQP2 trafficking by PKA stimulation.

A-kinase anchoring protein 220 was initially identified as a protein binding to the regulatory subunit (RII) of PKA.[16–19] Its mRNA was detected in heart, brain, lung, testis, and kidney by northern blot. However, the types of cells in each organ that express AKAP220 have not been identified. The present study clearly shows that AKAP220 is abundant in the papilla in kidney, especially in the collecting ducts. As the C-terminal sequence of AKAP220 (Cys-Arg-Leu) conforms to a peroxisomal targeting signal motif,[16] AKAP220 was

speculated to be a peroxisome AKAP; but the intracellular localization of AKAP220 has not been fully investigated. We performed a double immunofluorescence assay and found that AKAP220 colocalized with AQP2 in the cytosol near the apical membranes, suggesting that AKAP220 may be an endosomal AKAP. Very recently, AKAP11 (=AKAP220) was identified as the most abundantly expressed AKAP in the inner medullary collecting ducts among various AKAPs;[20] these results further support the concept that AKAP220 is important for PKA signaling in the collecting ducts.

The previously identified binding partner of AKAP220 is glycogen synthase kinase-3β.[21] PKA, type 1 protein phosphatase 1, glycogen synthase kinase-3β, and AKAP220 form a quaternary complex. PKA and protein phosphatase 1 regulation of glycogen synthase kinase-3β activity was more efficient when glycogen synthase kinase-3β was bound to AKAP220. AKAP220 also coordinated the location of the PKA RII and the type 1 protein phosphatase catalytic subunit, which is important for regulating protein phosphatase catalytic activity.[16,22]

The binding sites on both proteins were investigated by using deletion mutants of AKAP220 and AQP2 in the yeast two-hybrid assay. We confirmed that the carboxy cytoplasmic region of AQP2 and the middle portion of AKAP220 (residues 1085–1174) near the binding site to PP1 were involved in their interaction. Thus, the binding of AQP2 and AKAP220 was clearly demonstrated in yeast. However, we could not successfully demonstrate their binding by immunoprecipitation in COS cells and in rat kidney. The failure of coimmunoprecipitation in rat kidney tissues may be due to the lack of suitable AKAP220 antibodies for immunoprecipitation. In COS cells, however, AKAP220 and AQP2 were overexpressed, and immunoprecipitation was performed with high-affinity antibodies to the tag (HA and 3 × FLAG). Accordingly, we speculate that the interaction of AQP2 and AKAP220 can be easily disrupted by detergents. In the case of AKAP18δ, its direct binding to AQP2 was not demonstrated, although the proteins appeared to be colocalized.[14,15] As mentioned by Henn *et al.*, AQP2 was not included in protein complexes isolated by cyclic AMP-agarose gel. This finding suggests that the interaction of the PKA signaling complex and AQP2 may be weak, consistent with the results of the present study. Although the binding may be weak, the effect of AKAP220 on AQP2 phosphorylation was clearly demonstrated in Figure 3. As previously shown, AKAP220 formed a complex with the regulatory and catalytic subunits of PKA (as shown in Figure 3a). Under this condition, AQP2 phosphorylation by forskolin with AKAP220 was stronger than in the condition without AKAP220 or with AKAP220 lacking the binding domain to AQP2. This result also supports the notion that AKAP220 has a binding affinity to AQP2 and recruits AQP2 in close vicinity to PKA.

In summary, AKAP220 binds to AQP2 and may regulate the phosphorylation of AQP2 *in vivo*.

MATERIALS AND METHODS

Yeast two-hybrid system that uses split ubiquitin

We performed the screening procedures according to the manufacturer's (Dualsystems Biotech AG, Switzerland) recommendations. Briefly, whole human AQP2 cDNA amplified by PCR was cloned into the bait vector, pCCW-STE, transformed into the NMY32 yeast strain, and selected on SD/-Leu plates at 30 °C for 3–4 days. Several colonies were tested for autonomous activation on the selection plates, SD/-tryptophan, -leucine, and -histidine. To create a more stringent screening condition, 10 mM 3-amino-1,2,4-triazol was added to the selection plates. A human kidney cDNA library prepared in pDL2 × N-STE vector (Dualsystems Biotech AG) was transformed into the bait vector-transformed yeast cells, which were then incubated on the selection plates for 3–4 days. The plasmids in the selected colonies were recovered according to the manufacturer's protocol, electroporated into *Escherichia coli* (DH 10B), and sequenced.

Immunoblot and immunofluorescence of AKAP220 in rat kidney

We divided the rat kidneys into the cortex, outer medulla, and papilla under a dissecting microscope. Each section of rat kidney was homogenized in a solution, which consisted of 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), and Complete (Roche, Indianapolis, IN) on ice, and then centrifuged at 600 *g* at 4 °C for 15 min. The supernatants were recovered and NP40 was added at the final concentration of 0.5% volume. The samples were mixed with 2 × SDS sample buffer, denatured, resolved on SDS–polyacrylamide gel electrophoresis, and transferred to Hybond-ECL membranes (Amersham, Uppsala, Sweden). Anti-mouse AKAP220 antibody (BD Biosciences, San Jose, CA) at a 1:200 dilution and anti-rabbit actin antibody (Cytoskeleton, Denver, CO) at a 1:100 dilution were used.

For immunofluorescence, rat kidneys were obtained after perfusion with 4% paraformaldehyde and 6 h of immersion in the same liquid at 4 °C Then, the tissues were immersed overnight in 20% sucrose/phosphate-buffered saline at 4 °C and snap frozen in liquid nitrogen. Cryostat sections of 10-μm thickness were prepared on slide glasses. After blocking with 1% bovine serum albumin, the sections were incubated overnight with anti-AKAP220 antibody and goat anti-AQP2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C, washed with phosphate-buffered saline several times, and then incubated with Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin G and Alexa Fluor 546-conjugated donkey anti-goat immunoglobulin G (diluted 1:200) at 37 °C for 30 min. Fluorescent images were obtained by LSM510 Meta confocal microscopy.

Double immunoelectron microscopy was performed as described previously.[23] The secondary antibodies were rabbit anti-goat immunoglobulin G(H + L), 10-nm gold (British Biocell International, London, UK) and rabbit anti-mouse immunoglobulin G, 20-nm gold (EY Laboratories, San Mateo, CA).

Phosphorylation of AQP2

Full-length human AKAP220 cDNA with an N-terminal HA tag was cloned into the pCGN vector.[21] HA-tagged AKAP220 lacking amino acids 1098–1186 was generated by PCR. Human AQP2 with N-terminal 3 × FLAG tag expression vector was prepared by PCR. An S256A mutation was introduced by using a QuikChange kit (Stratagene, La Jolla, CA). Both plasmids were cotransfected into COS7 cells with Lipofectamine 2000. At 24 h after transfection, the medium was replaced with phosphate-free Dulbecco's modified Eagle's medium with 0.5% bovine serum albumin and 10 mM Hepes

(pH 7.4) and incubated for 2 h, followed by further incubation for 2 h in the same medium containing ^{32}[P]orthophosphate (1 mCi/ml). These cells were then treated with 100 μM forskolin, 1 μM 3-isobutyl-1-methyl-xanthine, and 200 μM 8-(4-chlorophenylthio) adenosine 3′,5′-cyclic monophosphate for 30 min before harvest. The cells were lysed for 1 h on ice in 150 mM NaCl, 15 mM Tris-HCl, 25 mM NaF, and 1 mM Na_3VO_4 (pH 8.0) and were immunoprecipitated with anti-FLAG M2 affinity gel. Immunoprecipitated AQP2 was resolved by SDS–polyacrylamide gel electrophoresis, and the phosphorylation was visualized by autoradiography. Expression of HA-AKAP220 and 3 × FLAG AQP2 was confirmed by immunoblot of the lysates with anti-HA and anti-FLAG antibodies, respectively.

Immunoprecipitation of HA-AKAP220 was performed by using anti-HA affinity gel (Roche). Antibodies to catalytic and regulatory subunits of PKA were purchased from BD Biosciences.

DISCLOSURE

All the authors declared no competing interests.

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