Heat Shock Protein 70 Interacts with Aquaporin-2 and Regulates Its Trafficking*

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The trafficking of aquaporin-2 (AQP2) involves multiple complex pathways, including regulated, cAMP-, and cGMP-mediated pathways, as well as a constitutive recycling pathway. Although several accessory proteins have been indirectly implicated in AQP2 recycling, the direct protein-protein interactions that regulate this process remain largely unknown. Using yeast two-hybrid screening of a human kidney cDNA library, we have identified the 70-kDa heat shock proteins as AQP2-interacting proteins. Interaction was confirmed by mass spectrometry of proteins pulled down from rat kidney papilla extract using a GST-AQP2 C-terminal fusion protein (GST-A2C). The direct interaction of AQP2 with hsc70 is partially inhibited by ATP, and the Ser-256 residue in the AQP2 C terminus is important for this direct interaction. Vasopressin stimulation in cells enhances the interaction of hsc70 with AQP2 in IP assays, and vasopressin stimulation in vivo induces an increased co-localization of hsc70 and AQP2 on the apical membrane of principal cells in rat kidney collecting ducts. Functional knockdown of hsc70 activity in AQP2 expressing cells results in membrane accumulation of AQP2 and reduced endocytosis of rhodamine-transferrin. Our data also show that AQP2 interacts with hsp70 in multiple in vitro binding assays. Finally, in addition to hsc70 and hsp70, AQP2 interacts with several other key components of the endocytotic machinery in co-IP assays, including clathrin, dynamin, and AP2. To summarize, we have identified the 70-kDa heat shock proteins as AQP2 interactors and have shown for hsc70 that this interaction is involved in AQP2 trafficking.

Aquaporin 2 is expressed in principal cells of the kidney collecting duct and mediates water absorption and urinary concentration in response to vasopressin (VP). Upon stimulation by VP, AQP2 accumulates on the plasma membrane of collecting duct principal cells (1–4). Activation of both cAMP-dependent, VP/forskolin-stimulated and cAMP-independent, cGMP-responsive signaling pathways stimulates membrane accumulation of AQP2 (5). Recent evidence also indicates that cAMP- and cGMP-independent membrane accumulation of AQP2 can also occur in the absence of hormonal stimulation (6–8), such as by blocking endocytosis (6, 8) and by disrupting the actin cytoskeleton (9, 10).

Although the complicated pathways of intracellular AQP2 trafficking remain to be completely elucidated, even less is known about the direct interaction of AQP2 or AQP2-containing vesicles with a host of accessory factors that must be involved in these processes. Understanding these interactions is a critical step in dissecting the processes of exocytosis, endocytosis, intracellular translocation, as well as degradation and shedding of AQP2 that all contribute to the final physiological response to VP in vivo. Many of the cytosolic proteins known to be involved in vesicular trafficking in general have also been implicated in AQP2 trafficking, including cytoskeletal components such as actin filaments, myosins, and microtubules, SNARE proteins, Rab proteins, and heterotrimeric G proteins, as well as protein kinases and their associated proteins (1, 11–15). However, direct evidence regarding the interaction of AQP2 with these components of the trafficking machinery remains scarce, although more information is slowly emerging in this area. For example, a recent study has identified AQP2 as a component of a multiprotein “motor” complex, including actin and the rho GTPase regulatory protein SPA1 (16). Even before AQP2 was identified, several studies had suggested that water channels in the collecting duct are internalized via a clathrin-mediated endocytic process (17). More recently, we have shown that AQP2 traffics via this clathrin-mediated endocytic pathway in either a regulated or a constitutive manner. Blocking the process of clathrin-mediated endocytosis either with dominant-negative dynamin or pharmacologically using methyl-β-cyclohexdrin results in hormone-independent and phosphorylation-independent plasma membrane accumulation of AQP2 in transfected cell models (6, 8) and in the isolated, perfused rat kidney (18).

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4 The abbreviations used are: VP, vasopressin; AQP2, aquaporin-2; GST, glutathione S-transferase; co-IP, co-immunoprecipitation; PBS, phosphate-buffered saline; NBD, nucleotide binding domain; WT, wild type.
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The clathrin-mediated endocytotic pathway is one of the major routes of endocytosis in eukaryotic cells and is characterized by the selective internalization of specific proteins from the cell surface. A complex series of protein-protein interactions is involved in the regulation and selectivity of this process, which is highly regulated and dynamic, and involves rapid assembly or disassembly of transient protein complexes that involve the activity of both ATPases and GTPases (19, 20). Of particular interest, recent studies have expanded the many well known functions of heat shock protein 70, such as participating in protein synthesis, folding and assembly, translocation, degradation, and surface antigen presentation, to a novel role in mediating endocytosis (21–24). hsc70 plays an important role together with auxillin in the ATPase-dependent uncoating of clathrin-coated vesicles during endocytosis in vitro and in vivo (24–27).

hsc70 has been reported to bind directly to membrane channel proteins, such as K+ channels and cystic fibrosis transmembrane regulators, and to mediate their trafficking/recycling at various steps (28–32). Many data have also shown that heat shock protein 70 is highly concentrated in the papilla of mammalian kidney, and a hypertonicity-responsive element that regulates hsp70 expression was identified in cultured kidney cells (33). Microarray analysis of the mouse kidney after water deprivation reveals an up-regulation of hsp70 expression, and a more recent proteomic analysis of the inner medullary collecting duct of Brattleboro rats after vasopressin treatment revealed that the level of heat shock protein 70 is increased in response to [deamino-Cys, d-Arg8] vasopressin (34, 35). All these data and the emerging appreciation of the role hsc70 in mediating channel trafficking raised the possibility that the 70-kDa heat shock proteins may also play a role in mediating AQP2 recycling. In this study, we use convergent techniques to show that both hsc70 and hsp70 interact with AQP2 directly and that the interaction with hsc70 is important for AQP2 trafficking/recycling.

MATERIALS AND METHODS

Chemicals and Antibodies—Lysine VP was obtained from Sigma. Tetramethylrhodamine transferrin conjugate was supplied by Molecular Probes (Eugene, OR). GST, glutathione S-transferase-agarose, and protein A-Sepharose were obtained from Amersham Biosciences. Purified, biotinylated hsc70 and purified hsp70 were purchased from StressGen (Victoria, British Columbia, Canada). Purified hsp70 was biotinylated using a biotinylation kit from Pierce.

Anti-c-Myc monoclonal antibody was obtained from the supernatant of the 9E10 hybridoma cell line, which was purchased from the ATCC. Polyclonal antibodies against clathrin heavy chain, AP2, and dynamin 2 were kindly provided by Dr. Sanja Sever (36). Our affinity-purified rabbit polyclonal antibody against the AQP2 C terminus has been described previously (37). Antibodies against hsc70 (rat monoclonal, SPA815) and hsp70 (mouse monoclonal, SPA 810, and rabbit polyclonal, SPA 812) were purchased from StressGen. Secondary fluorescent isothiocyanate or CY3-conjugated antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Secondary horseradish peroxidase-conjugated antibodies were purchased from Santa Cruz Biotechnology.

Stable Cell Lines, DNA Constructs, and Recombinant Adenovirus—The generation of LLC-PK1 cell lines stably expressing c-Myc-tagged wild type AQP2, referred to as W2 in Figs. 3 and 4 and LLC-AQP2 in the remainder of the text and figures, has been described previously (7, 38). All cell lines were routinely maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C with 5% CO2.

The rat aquaporin-2 coding sequence was cloned into a GAL4:DBD vector pAS2-1 (Clontech) as a bait construct for yeast two-hybrid screening. This bait protein was engineered as a single fusion peptide consisting of GAL4:DBD, plus the sequence coding for amino acids Met1–Glu16 from the N terminus of AQP2, Ala147–Ala161 from the second intracellular loop of AQP2, and Asn220–Ala271 from the C-tail of AQP2. The coding sequence of this C-terminal chimera construct was also subcloned into the bacterial expression vector pET41a (Novagen) as a fusion protein with a GST tag on its N terminus (39). All the constructs were sequenced to confirm their reading frame and predicted composition. The GAL4 AD:human kidney cDNA library constructed in the yeast plasmid pACT2 was purchased from Clontech. Recombinant adenoviruses expressing wild type hsc70 (Ad-hsc70), an ATPase-deficient hsc70 mutant (Ad-hscT204V), and a GTPase-deficient dynamin mutant (Ad-Dyn/K44A) were kindly provided by Dr. Sandra Schmidt (27).

Details of the infection of epithelial cells with adenovirus were described in our previous publication (6). Briefly, recombinant adenovirus of ~5 multiplicity of infection was used to infect LLC-AQP2 stably transfected cells. After incubation for 36 h, cells were harvested and used for immunocytochemistry or endocytosis assays with rhodamine-transferrin.

Yeast Two-hybrid Screening and Yeast Two-hybrid Assay—The yeast two-hybrid screening was performed in AH109 Saccharomyces cerevisiae (Clontech) that contains three reporters, ADE2, HIS3, and MEL1. The BD-expressing plasmid contains TRPI, and the AD-expressing plasmid contains LEU2. The medium stringency selection medium lacks tryptophan, leucine, and histidine; the high stringency selection medium lacks tryptophan, leucine, histidine, and adenine but contains X-β-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (20 mg/ml). 3-Aminotriazole (2.5 mM) was added to both medium and high stringency selection media to inhibit HIS3 leakage. The AH109 containing the pAS2-1/ALCT plasmid was transformed with 0.2 mg of AD:cDNA plasmid library using a modified lithium acetate method. The transformed cells were plated onto medium stringency selection medium and incubated at 30 °C for 14 days. Positive colonies were patched onto high stringency selection medium plates and incubated at 30 °C until sufficient growth was achieved and colonies turned blue. Colonies that activated all of the reporter genes in the AH109 stain were further analyzed. The AD:cDNA plasmid encoding the interacting protein was isolated from yeast cells and transformed to KC8 Escherichia coli. The colonies containing AD:cDNA plasmid were rescued on M9/-Leu selection
medium plates, and the plasmid DNA was isolated and sent for sequencing.

Yeast two-hybrid assays were performed to further confirm the positive interaction of pAS2-1/ALCT and AD:cDNA plasmids as follows. AD:cDNA plasmids isolated from the primary screen were used to co-transform AH109 with pAS2-1/ALCT to confirm activation of reporters. The potential interacting clones were also co-transformed to AH109 with BD vector alone to test for false positivity. Transformed cells were plated on high stringency selection medium. AD:cDNA clones that were positive with pAS2-1/ALCT but negative with pAS2-1 were further characterized by DNA sequencing.

Preparation of Cell Culture Lysates and Rat Kidney Papilla Extract—Cells were grown in 10-cm culture dishes in complete Dulbecco’s modified Eagle’s medium (with 10% fetal bovine serum) to 95% confluence. After various treatments, cells were washed three times with cold PBS and were scraped into lysis buffer (PBS buffer, pH 7.4, NaF 20 mM, Na$_3$VO$_4$ 2 mM, 0.5% serum) to 95% confluence. After various treatments, cells were washed three times with cold PBS and were scraped into lysis buffer (PBS buffer, pH 7.4, NaF 20 mM, Na$_3$VO$_4$ 2 mM, 0.5% serum) to 95% confluence. After various treatments, cells were washed three times with cold PBS and were scraped into lysis buffer (PBS buffer, pH 7.4, NaF 20 mM, Na$_3$VO$_4$ 2 mM, 0.5% serum) to 95% confluence. After various treatments, cells were washed three times with cold PBS and were scraped into lysis buffer (PBS buffer, pH 7.4, NaF 20 mM, Na$_3$VO$_4$ 2 mM, 0.5% serum) to 95% confluence. After various treatments, cells were washed three times with cold PBS and were scraped into lysis buffer (PBS buffer, pH 7.4, NaF 20 mM, Na$_3$VO$_4$ 2 mM, 0.5% serum) to 95% confluence. After various treatments, cells were washed three times with cold PBS and were scraped into lysis buffer (PBS buffer, pH 7.4, NaF 20 mM, Na$_3$VO$_4$ 2 mM, 0.5% serum) to 95% confluence. After various treatments, cells were washed three times with cold PBS and were scraped into lysis buffer (PBS buffer, pH 7.4, NaF 20 mM, Na$_3$VO$_4$ 2 mM, 0.5% serum) to 95% confluence.

Kidney papilla extract was prepared from Sprague-Dawley rat kidney. The papillae from two kidneys were washed three times with cold PBS and homogenized in a Teflon pestle homogenizer (Thomas Scientific). After passing through a syringe with a 25-gauge needle, the homogenates were resuspended in cold lysis buffer containing PBS, pH 7.4, 20 mM NaF, 2 mM Na$_3$VO$_4$, 0.5% Nonidet P-40, and 0.1% Triton X-100, and a mixture of protease inhibitors (Roche Applied Science). Cell lysates were incubated on ice for 20 min and then passed 5–10 times through a 27-gauge syringe and centrifuged at 14,000 × g for 5 min at 4 °C to remove cell debris. The supernatants were used for pull down and co-immunoprecipitation assays.

Expression and Purification of GST AQP2 Fusion Protein (GST-A2C) — *Escherichia coli*, BL21 (DE3) (Novagen), was used for GST fusion protein expression. DNA constructs encoding various GST-AQP2 C-terminal fusion proteins were transformed into bacteria using electroporation. Expression of GST-A2C in *E. coli* was induced by isopropyl 1-thio-β-D-galactopyranoside (1 mM) at 37 °C for 4 h. Bacteria were lysed by lysozyme (1 mg/ml) and 0.5% Nonidet P-40 in STE buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, EDTA 1 mM). Bacterial supernatant was allowed to bind to the glutathione-agarose column at 4 °C for 40 min with gentle rocking. After washing twice with STE buffer with 500 mM NaCl, 0.5% Nonidet P-40, and 0.1% Triton X-100 and three times with normal STE buffer, the GST fusion protein was eluted with elution buffer (5 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0) and further dialyzed against PBS buffer at 4 °C. Purified GST and GST-AQP2 fusion proteins were subjected to SDS-PAGE and immunoblot analysis.

Pulldown Experiments—Cell lysates and papilla extracts were pre-cleared on a glutathione column at 4 °C for 40 min. Approximately 20 μg of the GST or GST-A2C protein was incubated with 40 μl of glutathione beads and 500 μg to 1 mg of pre-cleared cell lysate or rat kidney papilla extract at 4 °C with gentle rocking for 2 h. Then the glutathione beads were washed four times with PBS containing Nonidet P-40 (0.5%), Triton X-100 (0.5%), NaF (20 mM), and protease inhibitors to remove nonspecifically bound proteins. Finally, the pulled down material was analyzed by SDS-PAGE and immunoblot analysis. For mass spectrometry, the pulled down proteins from rat kidney papilla extracts were further processed with thrombin digestion (Novagen) and gel purification and finally sent for mass spectrometry analysis by MDS proteomics (Toronto, Canada). Meanwhile, purified GST-A2C fusion proteins were also used to pull down purified biotinylated hsc70 (StressGen) as mentioned above. Briefly, 20 μg of GST-A2C (wild type, S256A, and S256D constructs) was incubated with 40 μl of glutathione beads and 1 μg of biotinylated hsc70 at 4 °C for 2 h. After washing, the pulled down material was subjected to SDS-PAGE and Western blot analysis using horseradish peroxidase-conjugated streptavidin as a probe to detect the biotinylated hsc70 (Pierce). The immunoblots from five independent experiments were scanned, and mean signal intensity was calculated using IPLab Spectrum software. The percentages of the reduction of the ratio of biotinylation signal over AQP2 signal were calculated.

Similar pulldown experiments were performed using purified wild type GST-A2C with purified, biotinylated hsc70 or hsp70, respectively, in the presence or absence of ADP (10 mM) or ATP (10 mM). Data were obtained from at least three independent experiments, analyzed using IPLab Spectrum software. As mentioned above, the percentages of the reduction of the signal intensity ratio of hsc70 (or hsp70) over AQP2 were calculated.

Co-immunoprecipitation Procedure—Lysates from LLC-PK1, LLC-AQP2 (W2) cells, and rat kidney papilla extract were pre-cleared on protein A beads at 4 °C for 40 min prior to co-IP studies. Protein A beads (40 μl) were incubated with 0.5 μg of antibody and 500 μg of cell lysate or rat kidney papilla extract with gentle rocking for 2 h at 4 °C. After washing five times with washing buffer (PBS buffer, protease inhibitors, 0.5% Nonidet P-40, and 0.1% Triton X-100), the co-IP samples were subjected to SDS-PAGE and immunoblot analysis using a variety of antibodies. Cell lysates incubated with protein A beads alone were routinely used as a negative control. Co-IP using lysates from nontransfected cells was also used as a negative control in each experiment. Studies were repeated at least three times.

Immunocytochemistry and Electron Microscopy of Tissue and Cultured Cells—Kidney tissue was fixed by cardiac perfusion with paraformaldehyde lysine periodate as described previously (39) with or without pre-exposure to [deamino-Cys, d-Arg]$^8$ vasopressin administered for 3 days via Alzet osmotic minipumps implanted subcutaneously in the nape of the neck, and delivering about 1.2 μg or DDAVP per day. Tissues were rinsed three times for 5 min in PBS, cryo-protected in 30% sucrose in PBS, frozen, and sectioned at 5 μm for immunostaining. The kidney sections were treated with 1% SDS for 4 min to improve antigenicity (40) and then blocked with 1% bovine serum albumin in PBS buffer before immunostaining. Primary antibodies against AQP2 (1:100) or hsc70 (or hsp70) (1:100) were added to kidney sections and incubated at room temperature for 90 min. After washing with PBS, fluorescein isothiocyanate- and CY3-conjugated secondary antibodies were applied for 60 min at room temperature. After final washes with PBS, sections were mounted in Vectashield (Vector Laboratories, Burlingame CA).
and examined by conventional immunofluorescence microscopy (Nikon Eclipse 800) and confocal microscopy (Bio-Rad Radiance 2000).

LLC-PK1 or LLC-AQP2 cells were seeded onto coverslips and treated as described below. Cells grown on coverslips were fixed with 4% paraformaldehyde in PBS for 20 min, washed three times for 5 min with PBS, permeabilized with 1% Triton X-100 for 4 min, and then subjected to routine immunostaining for AQP2 or hsc70 as described previously (39). An estimate of translocation of AQP2 to the plasma membrane was obtained by quantitative analysis of AQP2 labeling in three independent experiments from cells infected with empty adenovirus (control), and cells infected with adenovirus expressing wild type hsc70, ATPase-deficient hsc70 mutant, and the GTPase-deficient K44A dynamin mutant. The percentage area occupied by AQP2 staining in a region of interest of the cytoplasm in each of at least 20 cells per experimental condition was quantified using IPLab Spectrum software, as described previously (39), and was compared with the control cells. The loss of cytoplasmic staining was shown previously to parallel an increase in membrane staining during the AQP2 trafficking process.

For electron microscopy, kidneys were fixed by perfusion with paraformaldehyde lysine periodate. Small blocks of tissue were dehydrated through a graded series of ethanol and embedded in LR white resin (EMS, Hatfield, PA) at 50 °C overnight. Thin sections were cut on a Reichert Ultracut E ultramicrotome and collected on Formvar-coated gold grids. Then the sections were blocked for 10 min with 5% normal goat serum (Sigma) and incubated for 1 h at room temperature with rabbit anti-AQP2 antibody at a final concentration of 1:10 plus rat anti-hsc70 (SPA815) at a final concentration of 1:10, in DAKO diluent (DAKO Corp). Subsequently, the secondary antibodies labeled with goat anti-rabbit and goat anti-rat IgG gold, 10 and 15 nm, respectively, were added (Ted Pella, Redding, CA) at final concentrations of 1:20 each in DAKO diluent for 1 h at room temperature. Following several rinses with distilled water, the grids were stained on 2% aqueous uranyl acetate for 5 min and rinsed with distilled water and dried. Finally, sections were examined in a JEOL 1011 TEM and imaged digitally.

**Endocytosis Assays in LLC-PK1 Cells**—An endocytosis assay using tetramethylrhodamine transferrin conjugate as a marker of clathrin-mediated endocytosis was performed in a similar manner as we described previously for a fluid phase endocytosis assay using fluorescein isothiocyanate-dextran (8). Briefly, cells grown on coverslips were infected with adenoviral constructs for 28–30 h, then cells were incubated with 30 μM rhodamine-transferrin at 37 °C for 20 min followed by four washes with cold PBS. Immediately after washing, cells were fixed with 4% paraformaldehyde in PBS for 20 min. After washing again with PBS, coverslips were mounted and viewed. The uptake of rhodamine transferrin was digitally monitored using a Nikon E800 microscope and Hamamatsu Orca CCD camera.

**Data Analysis**—Quantification of the change of signal intensity in the in vitro binding assays, i.e. co-IP and pulldown assays, was performed as follows: the intensity of each band on each immunoblot was obtained using densitometry and IPLab Spectrum software. On each individual blot, the ratios of signal intensity of hsc70 (hsp70) over the signal intensity of its corresponding input AQP2 (or GST-AQP2) in the same blot were calculated. Then the percentage change (either an increase or a decrease from the WT level) for each mutant (or different treatment) was calculated for each experiment. The percentage of relative signal intensity obtained from at least three experiments was analyzed using a program called Prism. Comparisons of data from each treatment group were performed using analysis of variance. Finally, the relative signal intensity of hsc70 (hsp70) over AQP2 was presented in histogram form in the respective figures; the error bars indicate S.E., and the asterisk indicates p < 0.05.
RESULTS

Heat Shock Protein 70 Is an AQP2-interacting Protein; Yeast Two-hybrid Screening and Mass Spectrometry Analysis of the Pulldown Complex by GST-A2C—Yeast two-hybrid screening was performed to search for potential AQP2-interacting proteins as described above. After lengthy screening, almost 200 positive colonies were identified initially, representing ~40 different binding candidates after searching the data base in Gen-Bank™ using BLAST (data not shown). Two cDNA clones, clone 130 and 157, were further characterized. Clone 130 spans an amino acid sequence of both human hsp70 and hsc70 from amino acids 146–326. Clone 157 spans an amino acid sequence of both human hsp70 and hsc70 from amino acids 166–385. The amino acid sequence of these two cDNA clones fell within the 44-kDa nucleotide-binding domain (NBD from amino acids 1–383 (Fig. 1) of heat shock protein 70 and is more similar to hsp70 than to the hsc70 sequence in this highly homologous region.

Simultaneously, purified GST-A2C was used to pull down potential AQP2-binding partners from rat kidney papilla extract. The purified GST-A2C wild type fusion protein was examined by SDS-PAGE and by immunoblots using antibodies against AQP2 (Fig. 2, panel A). The AQP2 antibody recognized only the GST-A2C fusion protein (Fig. 2, panel A, 4th lane). GST-bound A2C and its interacting proteins were released by thrombin cleavage, and Coomassie-stained gels showed the profiles of the released proteins (Fig. 2, panel B). Some bands were also present in control lane of GST with kidney extract, but several proteins were detected only in the GST-A2C lane and not in the control lane. The most prominent band at about 70 kDa (Fig. 2, panel B) was identified by mass spectrometry as hsc70.

Interaction of hsc70 and AQP2 Is Confirmed by Pulldown and Co-IP Studies—A pulldown experiment was performed using purified GST-A2C and GST alone, with pre-cleared cell lysates and rat kidney papilla extracts. Fig. 3 shows a Western blot of the pulled down material probed with an antibody against hsc70 (Fig. 3, panel A) and hsp70 (panel B). Results show that hsc70 is present in whole lysates from LLC-AQP2 (W2) cells and kidney papilla. GST-A2C is also able to pull down hsp70 from W2 lysate (Fig. 3, panel B). Neither hsc70 nor hsp70 was detected in pull down experiments using beads or GST with kidney or W2 cell lysates.

The interaction of AQP2 and hsc70/hsp70 was further tested by co-IP experiments using antibodies against AQP2 and hsc70 (or hsp70), respectively (Fig. 4). Fig. 4, panel A, shows that antibody against the AQP2 C terminus was able to immunoprecipitate AQP2 and hsc70 from LLC-AQP2 (W2) cells, but not from untransfected LLC-PK1 (LLC) cells, or from lysates incubated with protein A beads alone (data not shown). In addition, anti-hsc70 antibody was able to co-immunoprecipitate AQP2 from LLC-AQP2 (W2) cells but not from untransfected LLC-PK1 (LLC) cells (Fig. 4, panel B). A similar co-IP study was also performed using rat kidney papilla extract (Fig. 4, panel C). Results show that AQP2 co-immunoprecipitates not only with hsc70, but also with the homologous protein hsp70 from kidney papilla extract. Both nonglycosylated (with molecular size around 25 kDa) and glycosylated AQP2 (with molecular size around 37 kDa) were detected in this co-immunoprecipitation study, in contrast to transfected cells that express mainly the nonglycosylated AQP2 (with molecular size around 25 kDa). A similar result was observed by mass spectrometry analysis of proteins pulled down by GST-A2C but not GST is able to pull down hsc70 from both rat kidney papilla extract and GST-A2C lane 6 (Fig. 3, panel B). Results show that hsc70 and hsp70 were detected in the control samples using either beads (data not shown) or GST with kidney or W2 cell lysate. Panel A, lane 1, W2 cell lysate; lane 2, kidney lysate (Kid Lys); lane 3, GST alone with W2 lysate; lane 4, GST alone with kidney lysate; lane 5, GST-A2C; lane 6, GST-A2C with kidney lysate; lane 7, GST-A2C with W2 lysate. Arrow indicates hsc70. Panel B, lane 1, W2 lysate; lane 2, GST-A2C alone; lane 3, GST and W2 lysate; lane 4, GST-A2C with W2 lysate. Arrow indicates hsp70.
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FIGURE 4. AQP2 co-immunoprecipitates with hsc70 and hsp70. Panel A, co-IP of AQP2 and hsc70 from AQP2 stably transfected LLC-AQP2 (W2) cells using an antibody against AQP2. Both cell lysate and kidney papilla extract were pre-cleared by protein A beads prior to co-IP experiments. hsc70 is detected in the co-IP complex pulled down with the anti-AQP2 antibody; AQP2 was also detected using anti-AQP2 antibody as expected (lower panel). Panel B shows co-IP of hsc70 and AQP2 using anti-hsc70 antibody. Both panels A and B show that antibodies against AQP2 or hsc70 are able to co-IP their binding counterparts from cell lysates, and this two way co-IP assay suggests an interaction between AQP2 and hsc70. Panel C shows that AQP2 is not only able to co-IP with hsc70 but also co-IP with hsp70 from rat kidney (Kid) papilla extract. Antibodies against hsc70 and hsp70 were used in this co-IP experiment. The molecular weight of AQP2 from LLC-AQP2 (W2) cells is greater than that from kidney papilla extract because of the presence of c-Myc tag in AQP2-transfected cells. Arrows indicate proteins being detected. WB, Western blot.

FIGURE 5. hsc70 and hsp70 directly interact with AQP2 C-terminal construct in vitro. Both purified, biotinylated hsc70, hsp70, and purified GST-A2C were used to perform pulldown experiments to test for direct interaction. Panel A shows that biotinylated hsc70 is detected in pulldown complexes using GST-A2C but not using GST or glutathione beads alone. In the presence of ATP but not ADP, the binding of biotinylated hsc70 is attenuated (*, p < 0.05). Lane 1, biotinylated hsc70 control; lane 2, biotinylated hsc70 with glutathione beads; lane 3, biotinylated hsc70 with GST; lane 4, biotinylated hsc70 with GST-A2C; lane 5, biotinylated hsc70 with A2C + ADP 10 mM; lane 6, biotinylated hsc70 with A2C + ATP 10 mM. Panel B shows that biotinylated hsp70 is pulled down by GST-A2C but not by GST. Similarly, the presence of ATP attenuates the interaction of GST-A2C and hsp70 (*, p < 0.05). In panel B, biotinylated hsp70 was incubated without ATP/ADP in lane 1, with ADP 10 mM in lane 2, with ATP 10 mM in lane 3, and with GST in lane 4. Biotinylated hsc70 and hsp70 were detected using horseradish peroxidase-streptavidin (upper panel), and GST fusion proteins were detected using anti-GST antibody (lower panel in panel A) and AQP2 in panel B. The percentage of the reduction of the signal intensity ratio hsc70 (or hsp70) over GST (or AQP2) was analyzed statistically and is presented in the lower panels. Data were obtained from at least three experiments (* indicates p < 0.05 compared with no nucleotide controls). WB, Western blot.

A). This experiment shows that biotinylated hsc70 is pulled down by GST-A2C (Fig. 5, panel A, lane 4), but not by GST (lane 3) or glutathione beads alone (lane 2).

hsc70 has intrinsic ATPase activity, and it interacts with its various binding partners in an ATP-dependent manner. Therefore, the interaction of hsc70 and AQP2 C terminus was investigated in the presence of ADP and ATP (Fig. 5). In the presence of ADP, the binding of biotinylated hsc70 and GST-A2C was similar to that seen in the absence of nucleotide (Fig. 5, panel A, lane 5). However, in the presence of ATP, the binding of biotinylated hsc70 to GST-A2C was significantly reduced (Fig. 5, panel A, lane 6; *, p < 0.05). The percentage of reduction of the signal intensity ratios of hsc70 over GST-A2C is presented in Fig. 5, panel A, lower panel, and shows that there is a 50% reduction of the binding of hsc70 to GST-A2C in the presence of ATP. These data suggest that the interaction of hsc70 and AQP2 in vitro is ATP-dependent; specifically, ATP attenuates the interaction of hsc70 and AQP2. A similar experiment was also performed using purified, biotinylated hsp70 and GST-A2C (Fig. 5, panel B). Results showed that similar to hsc70, hsp70 can bind directly to AQP2 in vitro, and this interaction is also ATP-dependent, i.e. ATP attenuates the interaction of hsp70 and AQP2 (Fig. 5, panel B, lane 3; *, p < 0.05). ADP does not show any significant effect on the binding of hsp70 with GST-A2C. Data were obtained from three independent experiments.

To investigate the impact of the phosphorylation state of Ser-256 on the interaction of AQP2 with hsc70, GST-A2C wild type, and S256D and S256A mutants that mimic differential phosphorylation states of Ser-256 were generated and purified (Fig. 6). Interestingly, all GST-AQP2 fusion proteins show doublets at a 37–40-kDa range in Fig. 6, panel A, and likely represent post-translational modification such as nonenzymatic glycosylation (41) or nonspecific proteolysis and truncation of recombinant protein. The pulldown experiment was performed using purified, biotinylated hsc70 with various GST-AQP2 mutant proteins (Fig. 7, panel A).

Interestingly, introducing point mutations at Ser-256 either with Asp, mimicking the constitutive phosphorylated form, or with Ala, mimicking a constitutive de-phosphorylated state, attenuated the interaction of AQP2 and hsc70. The reduction of binding of biotinylated hsc70 by various GST-AQP2 constructs was further quantified in Fig. 7, panel B. Data were obtained from five experiments and showed a greater than 50% reduction in binding of hsc70 when Ser-256 was mutated to either alanine (Fig. 7, panel A, lane 5, p < 0.05) or aspartic acid (Fig. 7, panel A, lane 3, p < 0.05). This result suggests that an intact Ser-256 residue is important for the interaction of AQP2 with hsc70 in vitro.

Interaction of AQP2 with hsc70 Is Modified by Vasopressin—It has been well documented that vasopressin regulates AQP2 trafficking both in vitro and in vivo. We have demonstrated so far using various techniques that AQP2 interacts with hsc70, and we therefore asked whether VP affects the interaction of AQP2 and hsc70 in intact cells. The interaction of AQP2 and hsc70 in the presence and absence of VP
stimulation was investigated by co-IP using LLC-AQP2 cells incubated with VP for various times (Fig. 8, panel A). The results show an increased intensity of hsc70 signal in the co-IP complex after 30 min to 1 h of treatment with VP, which became even more intense after 2 h (Fig. 8, panel C; p < 0.05). This experiment suggests that the interaction of AQP2 and hsc70 is progressively enhanced after VP treatment. The level of hsc70 in the presence of VP stimulation was also examined using Western blot, respectively, and showed that there is no alteration of the expression of hsc70 after VP treatment within the same time frame (Fig. 8, panel E). Similar experiments were performed using anti-hsp70 antibody (Fig. 8, panel B) and show that there is also a markedly increased amount of hsp70 in co-IP samples treated with VP for 1 and 2 h as shown in a graph (Fig. 8, panel D; *, p < 0.05). Again, VP treatment does not alter the expression of hsp70 within the same time frame (Fig. 8, panel F).

Immunolocalization of the hsc70 and AQP2 Interaction—The functional significance of the interaction of hsc70 and AQP2 was next investigated. First, we showed that hsc70 is co-expressed with AQP2 in principal cells of kidney collecting ducts by immunofluorescence staining of rat kidney sections using antibodies against AQP2 and hsc70. Without VP treatment, hsc70 was distributed diffusely throughout the cytoplasm, whereas staining of AQP2 was on apical, subapical, and even basolateral membranes (Fig. 9, panel A). After vasopressin treatment of rats for 3 days, there was an intense accumulation of AQP2 on the plasma membrane as expected, but there was also a dramatically increased apical membrane staining for hsc70 in the principal cells of the collecting duct in the inner medullary region. There was a clear partial co-localization of hsc70 and AQP2 in this apical region after VP treatment (Fig. 9, panel A). Interestingly, this co-localization was not seen in the immunofluorescence staining with hsp70 and AQP2 using the same VP-treated kidney tissue (Fig. 9, panel B). This suggests that hsc70, but perhaps not hsp70, redistributes and partially co-localizes with AQP2 upon chronic (3 days) VP stimulation. The VP-induced recruitment of hsc70 to the apical membrane was also observed by electron microscopy using double immunogold labeling for AQP2 and hsc70 (Fig. 10). Clusters of gold particles representing AQP2 and hsc70 antigenicity were co-localized on the apical plasma membrane of principal cells from VP-treated animals (Fig. 10, panel B), but not in tissues without treatment with VP (Fig. 10, panel A). A similar control EM study using double immunogold labeling for glucose transporter 1 (Glut1) and hsc70 in this VP-treated kidney tissue did not reveal any clustering of double-labeled gold particles (representing hsc70 and Glut1) anywhere in cells or on the apical membrane (Fig. 10, panel C).

Disruption of hsc70 ATPase Activity Causes Membrane Accumulation of AQP2 and Reduces Endocytosis in LLC-
hsc70 Interacts with AQP2 and Regulates Its Trafficking

**FIGURE 8.** The interaction of AQP2 and hsc70/hsp70 is increased after VP treatment. Co-IP was performed in AQP2 stably transfected cells at various times after VP treatment. Anti-c-Myc antibody or anti-AQP2 antibody was used for immunoprecipitation. Anti-c-Myc, anti-AQP2, and anti-hsc70 antibodies were used for Western blot (WB). Panel A shows that an increased signal of hsc70 detected in the co-IP complex occurs between 30 and 60 min of treatment with VP \( (p < 0.05) \), and increases more after 2 h of treatment with VP \( (p < 0.05) \). The ratios of signal intensity of hsc70 over AQP2 were obtained at different times. The relative signal intensity \( (\%\) with and without VP treatment is presented in panel C. Data were obtained from three independent experiments. Similar experiments were also performed using anti-hsp70 antibody as shown in panel B. Panel B suggests that there is a similar increased interaction of hsp70 and AQP2 after VP treatment from 1 to 2 h. The relative signal intensity \( (\%\) with and without VP treatment is shown in the graph (panel D). Panel E is the Western blot of cell lysates with or without VP treatment and shows that the level of hsc70 in cells was not affected by VP treatment within the same time frame. A similar finding was shown in panel F and suggested that the expression of hsp70 was not affected by VP within the same time frame. Loading control was performed using anti-actin antibody (* indicates \( p < 0.05 \)).

**AQP2 Cells**—To demonstrate the functional significance of hsc70 activity on AQP2 trafficking, we examined the trafficking of AQP2 after competitively knocking down wild type hsc70 function by overexpressing an ATPase-deficient recombinant adenoaviral construct. LLC-AQP2 cells were infected with control adenovirus, wild type hsc70 (Ad-hsc70), ATPase-deficient hsc70 (hsc70/T204V), GT-Pase-deficient dynamin adenovirus (K44A mutation), and then fixed and stained with anti-c-Myc antibody to detect AQP2 (Fig. 11). A marked membrane accumulation of AQP2 occurred in cells infected with virus expressing the ATPase-deficient hsc70, and a similar effect with virus expressing the GT-Pase-deficient dynamin mutant was observed as we have reported previously (6). There was no alteration of AQP2 trafficking in cells infected with wild type hsc70. Quantification of the distribution of AQP2 in cells infected with ATPase-deficient hsc70 or GT-Pase-deficient dynamin virus revealed a significant reduction in cytoplasmic staining, consistent with an increase in membrane staining (Fig. 11, lower panel; *, \( p < 0.05 \)) using a quantitative imaging protocol that we have described previously (39). These data indicate functional disruption of hsc70 activity results in membrane accumulation of AQP2.

To understand whether this membrane accumulation of AQP2 could be due to the involvement of hsc70 in endocytosis, we performed a rhodamine-transferrin endocytosis assay using cells infected with control virus, and adenvirus to express wild type hsc70, ATPase-deficient hsc70, or a dominant interfering dynamin mutant. Our data show that although transferrin is internalized into perinuclear vesicles in cells infected with control virus (Fig. 12, panel A) and cells expressing wild type hsc70 (Fig. 12, panel B), there is considerably reduced uptake and a marked membrane accumulation of the rhodamine-transferrin signal in cells expressing either the dynamin mutant (Fig. 12, panel C) or the hsc70 dominant-negative mutant (Fig. 12, panel D). This indicates that clathrin-mediated endocytosis is inhibited by ATPase-deficient hsc70, although the effect was somewhat greater with the K44A dynamin mutant (Fig. 12, panel C). The level of hsp70 in these virally infected cells was also examined. There is an elevated expression of hsp70 in cells infected with both ATPase-deficient hsc70 and GT-Pase-deficient dynamin virus, but the increase is much more dramatic in cells infected with ATPase-deficient hsc70 (Fig. 13). Interestingly, there was only a slight increase in hsp70 expression in cells infected with the wild type hsc70 virus. This suggests a possible compensatory mechanism in cells in which hsc70 function has been reduced. However, despite the significant up-regulation of endogenous hsp70 expression, the dominant-negative impact of hsc70 on AQP2 trafficking in cells is still detectable. Therefore, functional knockdown of hsc70 activity interferes with endocytosis in a manner similar to the well-documented effect of K44A dynamin on this process (6, 8). The level of hsc70 in cells infected with these recombinant adenoviruses is also shown in Fig. 13. As expected the expression of hsc70 was up-regulated in cells infected with both wild type hsc70 and ATPase-deficient hsc70 viral constructs.

**AQP2 Co-immunoprecipitates with Components of the Clathrin-mediated Endocytic Machinery**—Finally, we performed co-immunoprecipitation experiments to identify additional AQP2-interacting proteins in the clathrin-mediated endocytosis pathway. Fig. 14 shows that in addition to hsc70, AQP2 also co-immunoprecipitates in a complex with clathrin, dynamin, and the clathrin adaptor protein AP2. This suggests the involvement of known endocytic complex proteins in AQP2 trafficking, as expected from our previous data showing that AQP2 is internalized via this pathway (6, 8, 17).

**DISCUSSION**

AQP2 traffics constitutively between intracellular vesicles and the plasma membrane (1), but this pathway can in addition be regulated by a variety of factors, including vasopressin, to achieve the physiological goal of transepithelial water...
transport and urine concentration (1, 8, 10, 39, 42). During its complex recycling itinerary, AQP2 must interact with a host of accessory proteins that are involved in the trafficking process. Some of these have been implicated in AQP2 trafficking directly (e.g. by mutational or inhibitor studies), and some indirectly (e.g. by showing co-localization on AQP2-containing vesicles), but in most instances their ability to interact with AQP2 at the protein level is unknown. We show here that heat shock protein 70 interacts with AQP2. This was achieved using a variety of convergent techniques, including yeast two-hybrid screening, GST pulldown and co-immunoprecipitation assays from cell and tissue lysates, and direct interaction assays using purified proteins.

The cDNA clones that we have identified from the yeast two-hybrid screen share mainly the major C-terminal part of the nucleotide binding domain (NBD) of hsp70 and hsc70. However, despite an extensive search of the data base using various programs, we were not able to detect any conserved domains characteristic for hsp70-and hsc70-binding proteins in our AQP2 C-terminal construct, such as those present in proteins that bind to the NBD of hsc70: hsc70-interacting protein (Hip), Bcl2-associated athanogene (BAG), and other hsc/hsp70 NBD-binding proteins (43–47). Nor did we detect any similar motifs for the hsc70/hsp70 substrate binding domain such as those found in the DnaJ domain, auxillin, the C terminus of hsc70/hsp70 interacting protein (CHIP), and the human glutamine-rich tetra tricopeptide repeat-containing protein (hSGT) in the AQP2 C-terminal construct (48–52).

Even though the exact structural and functional features of the interaction of hsc70 and AQP2 remains to be further characterized, our present data suggest that the Ser–256 residue in the AQP2 C terminus is involved in this interaction.

**FIGURE 9.** Co-localization of AQP2 and hsc70/hsp70 in kidney tissues. Kidney sections were obtained from rats that were treated with VP using Alzet pumps for 3 days. Fixed rat kidney sections were subjected to routine immunohistochemistry staining with antibodies against AQP2 and hsc70 (panel A) and AQP2 and hsp70 (panel B). The images were obtained using a Bio-Rad Radiance confocal microscope. Panel A, in the control without VP treatment, hsc70 is distributed diffusely in principal cells, and AQP2 is present both intracellularly, apically, and subapically in collecting duct principal cells. After VP treatment, AQP2 is distributed apically, together with a redistribution of hsc70 signal, which becomes more intense on the apical membrane of principal cells. The merge panel shows considerable co-localization of AQP2 and hsc70 after chronic vasopressin treatment. Panel B, the staining of hsp70 is distributed diffusely in both AQP2-positive and -negative cells in the collecting ducts (CD) under base-line conditions as well as after treatment with VP. The staining of AQP2 is similar to that seen in panel A. The merge panel shows that, unlike hsc70, there is no increased co-localization of AQP2 and hsp70 after VP treatment. hsp70 staining appears more intense in AQP2-negative intercalated cells than in principal cells. Bar = 20 μm.

**FIGURE 10.** hsc70 co-localizes with AQP2 on apical membrane in VP-treated animal kidney by electron microscopy. Immunogold labeling of AQP2 and hsc70 was performed in both control (panel A) and VP-treated rat kidney tissue (panel B). Panels A and B, hsc70 was labeled with the large gold particles (15 nm) and is indicated by arrows; AQP2 was labeled with small gold particles (10 nm) and is indicated by arrowheads. Without vasopressin treatment, hsc70 and AQP2 are diffusely distributed and are not co-localized in panel A. However, upon VP stimulation (panel B), small gold particles (AQP2) accumulated intensively on or close to the apical membrane, and they often co-localized with a few large gold particles (hsc70) on the membrane. More interestingly, they tend to form clusters on the plasma membrane (as shown as * in panel B). Panel C shows that in the same VP-treated kidney tissue, Glut1 (labeled with small gold particles, arrowhead) does not co-localize with hsc70 (labeled with large gold particles, arrow in panel C). Bar = 250 nm.
Ser-256 is known to be the critical cAMP-dependent protein kinase and protein kinase G phosphorylation site on AQP2, and its phosphorylation is increased upon vasopressin administration to cells and tissues, concomitant with increased plasma membrane accumulation of AQP2 (1, 53). Our in vitro binding data suggest that vasopressin ultimately enhances the interaction of AQP2 and heat shock protein 70 in cells. A key question, therefore, is how this interaction is regulated at the molecular level and what effect this has on AQP2 trafficking?

hsc70 is a clathrin-decoating ATPase and was shown to de-coat internalized clathrin-coated vesicles and thus permit their fusion with a distal endosomal compartment in the internalization pathway (22, 24, 25). Dominant-negative hsc70 mutations have been shown to inhibit the endocytosis of receptors (24, 26, 27), and it is generally accepted that hsc70 is part of the endocytic machinery that regulates clathrin-mediated endocytosis (22, 27). Thus, we hypothesize that hsc70 is crucial for clathrin-mediated AQP2 internalization during the offset of the vasopressin response, as well as during the AQP2 constitutive recycling pathway. We show here that functional knockdown of hsc70 activity induces membrane accumulation of AQP2 in LLC-AQP2 cells and a significant reduction of uptake of rhodamine-

![Figure 11. Functional knockdown of hsc70 induces membrane accumulation of AQP2.](image)

**FIGURE 11. Functional knockdown of hsc70 induces membrane accumulation of AQP2.** Upper panel shows LLC-AQP2 cells infected with recombinant adenoviruses to express wild type hsc70 (Ad-hsc70), ATPase-deficient hsc70 (Ad-hsc/T204V), and dominant interfering dynamin mutant (Ad-DK44A). Immunostaining was performed using an anti-c-Myc antibody. In cells infected with control virus (panel A), there was a baseline perinuclear staining of AQP2; in cells infected with hsc70 ATPase-deficient mutant (panel D), there was a dramatic membrane accumulation of AQP2 ($p < 0.05$) similar to cells infected with dominant interfering dynamin mutant (panel B) ($p < 0.05$). Cells infected with wild type hsc70 did not show any alteration of AQP2 distribution (panel C). Lower panel represents quantification of the c-Myc staining at the plasma membrane after infection by individual adenoviral constructs. Data were obtained from three independent experiments. * indicates $p < 0.05$ compared with controls with empty virus.

![Figure 12. Functional knockdown of hsc70 inhibits endocytosis.](image)

**FIGURE 12. Functional knockdown of hsc70 inhibits endocytosis.** Rhodamine-transferrin was used to monitor clathrin-mediated endocytosis in cells infected with control virus (panel A), virus to express wild type hsc70 (Ad-hsc70) (panel B), GTPase-deficient K44A dynamin (Ad-DK44A) (panel C), and ATPase deficient hsc70 mutant (Ad-hscT204V) (panel D). Endocytosis of transferrin into perinuclear vesicles was observed in control cells (panel A) and was not affected by expression of wild type hsc70 (panel B). In contrast, expression of either dynamin K44A (panel C) or hsc70 T204 (panel D) mutant caused greatly reduced endocytosis of rhodamine-transferrin and significant plasma membrane accumulation of rhodamine-transferrin. Bar = 20 μm.

![Figure 13. Expression of hsp70 and hsc70 in cells infected with adenoviral constructs.](image)

**FIGURE 13. Expression of hsp70 and hsc70 in cells infected with adenoviral constructs.** The level of hsp70 and hsc70 was examined, respectively, in cells infected with adenovirus expressing control virus (lane 1), wild type hsc70 (lane 2), ATPase-deficient hsc70 (lane 3), or GTPase-deficient dynamin virus (lane 4). Lysates of infected cells were subjected to SDS-PAGE and Western blot using anti-hsp70 and anti-hsc70 antibodies, respectively. Loading control was performed using anti-actin antibody. There is a considerably elevated level of hsp70 detected in cells infected with ATPase-deficient hsc70 virus, and a less marked increase in cells infected with GTPase-deficient dynamin virus or wild type hsc70 virus compared with cells infected with control virus. The level of hsc70 was investigated using the same blot after stripping off the previous signals, and found to be elevated in cells infected with adenovirus expressing both wild type and ATPase-deficient hsc70, which was expected.
transferrin, indicating reduced clathrin-mediated endocytosis. One potential regulatory mechanism that could be involved in modulating the hsc70-AQP2 interaction during the recycling process is phosphorylation of residue Ser-256 in the AQP2 C terminus. As mentioned above, we found that VP stimulation enhances the interaction of AQP2 and hsc70, but this occurs only after 30 min of VP treatment, at a time in which the VP response is being down-regulated and intracellular cAMP levels are falling. Increased interaction was not detectable within the first few minutes when AQP2 is rapidly phosphorylated and accumulates on the plasma membrane. Furthermore, immunohistochemistry and EM immunogold labeling data show that VP treatment induces partial co-localization of hsc70 and AQP2 on the apical plasma membrane of collecting duct principal cells in rats chronically treated with VP. We hypothesize that AQP2 in its phosphorylated form accumulates at the cell surface after VP treatment, and subsequently associates with hsc70 during its re-internalization via clathrin-coated pits. This may involve an increased association of hsc70 with nonphosphorylated AQP2 at the cell surface because of local phosphatase activity (54).

In support of this, we found that S256D mutations greatly reduce the interaction of hsc70 and AQP2, as our model predicts. Unexpectedly, however, the S256A mutation was equally effective in preventing AQP2/hsc70 association, implying that a nonmodified serine residue at this position may be critical for optimizing the interaction of hsc70 and AQP2. In transfected cells, AQP-S256D is constitutively present at the cell surface, whereas S256A is mainly intracellular and does not respond to VP treatment. An attractive scenario would be that Ser-256 phosphorylation prevents hsc70 binding to AQP2, and causes membrane accumulation of AQP2 by inhibiting endocytosis. We have shown that inhibition of endocytosis is sufficient to cause membrane accumulation of AQP2 in our previous studies (8). However, because the S256A mutation also inhibits hsc70 binding without causing membrane accumulation of AQP2, other factors must also be involved. Interestingly, it has been reported that impaired constitutive phosphorylation of Ser-256 (S256A) in the Golgi causes AQP2 routing to lysosomes rather than to AQP2-containing vesicles designated for exocytosis (55). Heat shock protein 70 (hsp70) has been well documented to be involved in endoplasmic reticulum-associated degradation (32, 56), and facilitates the degradation of cystic fibrosis transmembrane conductance regulator in yeast (41). It is also possible that the S256D and S256A mutations do not completely reproduce the physiological effect of Ser-256 phosphorylation and dephosphorylation respectively, and further work will be required to address whether Ser-256 phosphorylation in vivo has a similar effect. Finally, it is always possible that other phosphorylation sites in the AQP2 C terminus, including a protein kinase C site, two casein kinase sites, and a putative extracellular signal-regulated kinase (ERK) phosphorylation site (5), could also be involved in heat shock protein 70-AQP2 interaction and regulation of AQP2 trafficking at different steps in the AQP2 biosynthetic and/or recycling pathway.

hsc70 and hsp70 have considerable homology of amino acid sequence, molecular structure, as well as biological function and have been considered to be interchangeable in many circumstances. However, more and more data have shown that they do have some differential functions (57, 58). In our study, both hsc70 and hsp70 were identified by a yeast two-hybrid screen and shown to interact with AQP2 by multiple in vitro assays. Both the interaction of hsc70/AQP2 and hsp70/AQP2 can be modified by ATP/ADP and VP treatment. Even though hsc70 and hsp70 appear to be similar regarding their interaction with AQP2, they may have differential roles on AQP2 trafficking under various physiological conditions. This is supported by our data showing that only hsc70 appears to concentrate along with AQP2 at the plasma membrane of principal cells in response to VP treatment, whereas hsp70 remains diffusely distributed in the cytosol under identical conditions.

hsc70 is an important player mediating protein trafficking of other membrane channels, such as a K+ channel and cystic fibrosis transmembrane regulator, via multiple steps that include intracellular transport, endocytosis and exocytosis (28–31). Despite the possibility that the interaction of AQP2 and hsc70 might occur at multiple sites, our co-localization studies show that at least one important site of interaction is at the plasma membrane. A potential effect on AQP2 endocytosis is reinforced by our co-IP data indicating the presence of other major components involved in clathrin-mediated endocytosis (AP2, dynamin, and clathrin itself) in the AQP2-containing complex. Roles of the newly uncovered AQP2-hsc/hsp70 interaction in cellular events other than endocytosis, for example AQP2 intracellular transport, degradation, recycling, or exocytosis, cannot be excluded, and it is certainly possible that heat shock protein 70 is involved in multiple steps of AQP2 trafficking and recycling.

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