Large Scale Protein Identification in Intracellular Aquaporin-2 Vesicles from Renal Inner Medullary Collecting Duct

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Aquaporin-2 (AQP2) is the vasopressin-regulated molecular water channel of the renal collecting duct where it constitutes the major route of water movement across the apical plasma membrane. Vasopressin rapidly increases the water permeability of the collecting duct epithelium by binding to V2 receptors in the basolateral plasma membrane and inducing the cAMP-dependent trafficking of AQP2-containing vesicles from throughout the cytoplasm to the apical region of collecting duct principal cells followed by fusion of these vesicles with the apical membrane of collecting duct cells (1). Although this fundamental mechanism is well established, there is little information about the specific intracellular protein trafficking pathways involved and the nature of the intracellular compartments in which AQP2 resides.

Until now, studies to identify the intracellular localization of AQP2 in collecting duct cells have depended largely on two fundamental approaches, namely immunoelectron microscopy and immunofluorescence immunocytochemistry with confocal microscopy. Immunoelectron microscopy (1, 2) has demonstrated that aquaporin-2 resides in intracellular vesicles distributed throughout the cytoplasm of collecting duct cells. However, it has not been feasible to identify the specific intracellular compartments that contain aquaporin-2 by immunoelectron microscopy in part because fixatives needed for high quality structural preservation markedly decrease the ability of aquaporin-2 antibodies to recognize the target protein. The second approach, viz. immunofluorescence immunocytochemistry with confocal microscopy (3–5), lacks sufficient spatial resolution to identify aquaporin-2 localization in subcellular compartments with certainty even with double labeling using antibodies to compartment-specific marker proteins.

Studies in other experimental systems have identified several alternative pathways for trafficking to the plasma membranes of cells (6, 7). First, transport vesicles from the trans-Golgi network (TGN) are directed to the plasma membrane by a pathway that does not require the endocytic machinery; these vesicles contain transmembrane proteins and associated membrane proteins (TGN, transferrin receptor). In the renal collecting duct, AQP2 resides in a subpopulation of vesicles that fuse directly with the basolateral membrane. This alternative pathway for aquaporin-2 localizes to the apical region of collecting duct cells (1). However, it is not clear whether the basolateral AQP2 pool is entirely separate from the apical pool or if it fuses with the apical membrane as part of the same vesicle trafficking pathway.

The first approach, viz. transmission electron microscopy (TEM) with immunogold labeling, is able to resolve subcellular compartments with nanometer resolution; it has been used to identify aquaporin-2 localization in renal collecting duct principal cells (6). These results are consistent with a model in which AQP2 resides in intracellular vesicles that fuse with the plasma membrane via cAMP-dependent trafficking. However, vesicle labeling using antibodies to compartment-specific marker proteins indicates that vesicles containing aquaporin-2 may fuse with other intracellular organelles as well (7). These results suggest that AQP2 resides in multiple intracellular compartments in collecting duct cells, and that these compartments may fuse with each other or with the plasma membrane.

The vesicles distributed throughout the cytoplasm of collecting duct cells demonstrate that aquaporin-2 resides in intracellular vesicles by immunoelectron microscopy (1, 2) and immunoconfocal microscopy (6, 7). These results are consistent with a model in which AQP2 resides in intracellular vesicles that fuse with the plasma membrane via cAMP-dependent trafficking. However, vesicle labeling using antibodies to compartment-specific marker proteins indicates that vesicles containing aquaporin-2 may fuse with other intracellular organelles as well (7). These results suggest that AQP2 resides in multiple intracellular compartments in collecting duct cells, and that these compartments may fuse with each other or with the plasma membrane.
Proteomics of AQP2 Vesicles

Golgi network (TGN) can travel directly to the plasma membrane in the secretory pathway as "secretory vesicles." Second, membrane proteins can be delivered to the apical plasma membrane via so-called "recycling endosomes." Recycling endosomes can receive membrane traffic directly from the TGN or from early endosomes formed as a result of endocytosis. Third, proteins initially targeted to the basolateral plasma membrane via the exocyst complex may travel to the apical plasma membrane by transcytosis (8). AQP2 trafficking to the apical plasma membrane of collecting duct cells may utilize one or more of these pathways.

The objective of the present studies was to identify the intracellular compartments in which AQP2 resides in unstimulated inner medullary collecting duct (IMCD) cells freshly isolated from rat kidneys. To do this, we devised a proteomics-based strategy to identify the proteins associated with AQP2 in immunoisolated intracellular vesicles using LC-MS/MS for large scale protein identification. The identified proteins were expected to include markers of specific intracellular compartments (e.g., members of the Rab family of small GTPases and SNARE proteins), pinpointing the location of AQP2 in IMCD cells during the unstimulated steady state.

MATERIALS AND METHODS

The general protocol for immunoisolation of AQP2 vesicles from rat IMCD is summarized in Fig. 1. Details of the procedures involved are described in the following.

Production and Biotinylation of Chicken Anti-AQP2 Antibody—An anti-peptide antibody against the COOH-terminal 15 amino acids of rat AQP2 was raised in chickens (Aves Labs, Inc., Tigard, OR). The sequence of the immunizing peptide was CVELHSQSLPRGSKA including an N-terminal cysteine to allow facile linkage to the carrier protein keyhole limpet hemocyanin. This antibody was extracted and affinity-purified from the egg yolks by the supplier. We biotinylated it using the EZ-Link sulfo-NHS-LC biotinylation kit (Pierce) according to the manufacturer’s instructions. Briefly a 12-fold molar excess of sulfo-NHS-LC-biotin was added to the antibody in PBS and incubated at room temperature for 30 min. The solution was then run through a 10-ml desalting column to remove excess biotin. Ten 1-ml fractions were collected.

Preparation of an Intracellular Membrane Fraction by Differential Centrifugation—Animal experiments were conducted under the auspices of approved NHLBI, National Institutes of Health animal protocol 2-KE-3. Five Sprague-Dawley rats, weighing between 200 and 250 g, were injected intraperitoneally with furosemide (5 mg/rat) 20 min before decapitation and removal of both kidneys. Furosemide dissipates the medullary osmolality gradient and thereby prevents osmotic shock to the cells (9). Inner medullas were dissected from the kidneys, minced with a razor blade, and homogenized using a Dounce homogenizer in 1 ml of isolation fluid (10 mM triethanolamine, 250 mM sucrose, pH to 7.6, plus 8 mg/liter PMSF and 0.08 mg/liter leupeptin). The homogenate was centrifuged at 4,000 × g for 10 min at 4 °C (Tomy, MTX-150). The pellet was resuspended in 1 ml of isolation fluid and then rehomogenized and recentrifuged under the same conditions. The pellets were discarded, and the supernatants were pooled and centrifuged at 17,000 × g for 20 min at 4 °C (Sorvall RC-2B centrifuge with SS34 rotor). The supernatant was collected and centrifuged at 200,000 × g for 1 h at 4 °C (Beckman ultracentrifuge with Ti-80 rotor). The rotor was resuspended in 500 μl of PBS and was centrifuged for another hour under the same conditions. The resulting pellet was resuspended in 520 μl of PBS. This low density membrane suspension was used for immunoisolation of AQP2-containing vesicles.

Previous studies have concluded that the 200,000 × g pellet from this procedure is virtually devoid of plasma membranes based on immunoblotting with antibodies to plasma membrane marker proteins (10, 11). To verify this conclusion, we carried out a control experiment in inner medullary collecting duct (IMCD) cell suspensions using surface biotinylation to label plasma membrane proteins (12). An IMCD suspension was prepared from two rats as described previously (13). Working at 4 °C to prevent endocytosis, the IMCD suspension was first gently pelleted via centrifugation (50 × g for 10 s) and then washed three times by resuspension in 1 ml of cold biotinylation solution (215 mM NaCl, 4 mM KCl, 2.5 mM Na2HPO4, 2 mM CaCl2, 1.2 mM MgSO4, 5.5 mM glucose, and 10 mM triethanolamine, pH 7.4). The resulting pellet containing the IMCD cells was resuspended in 1 ml of biotinylation solution plus 2 mg of sulfo-NHS-LC-biotin (Pierce) and incubated for 1 h. The IMCD cells were then washed two times with 1 ml of biotin quenching solution (0.1 mM CaCl2, 1 mM MgCl2, and 260 mM glycine in PBS, pH 7.4) followed by incubation with the same solution for 20 min. The biotin quenching solution was subsequently replaced with sucrose-based isolation fluid (described above), and the IMCD cells were homogenized and subjected to differential centrifugation as described in the previous paragraph. Detection of biotinylated proteins in the 17,000 × g and 200,000 × g pellets was accomplished by SDS-PAGE followed by electroblotting to nitrocellulose membranes and exposure of the resulting nitrocellulose blots to a streptavidin-horseradish peroxidase (HRP) conjugate (Pierce). Biotinylated proteins were then visualized by chemiluminescence (see "Immunoblotting" below). If plasma membranes had been successfully excluded from the 200,000 × g pellet (prior to AQP2 vesicle immunoisolation), biotinylated proteins should be absent from this pellet.

Imunoisolation of AQP2-containing Vesicles—Dynal M-280 streptavidin-coated magnetic beads were mixed with biotinylated anti-AQP2 antibody on a Dynal sample mixer (15 mg of beads with...
38.8 μg of biotinylated anti-AQP2 antibody. An excess of free biotin (0.075 μmol) was added to each mixture and mixed for 30 min at 22 °C to bind any streptavidin sites not occupied by the antibody. Control beads were handled in the same way except that the chicken anti-AQP2 was replaced with 38.8 μg of chicken non-immune IgY. Beads were separated with a magnet, and the supernatant was discarded. The beads were then resuspended with 1% BSA in wash solution (5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 mM NaCl) and mixed for 30 min to block nonspecific binding. The beads were then washed in a 0.1% BSA solution in PBS four times for 5 min each. Low density membranes (200,000 × g pellet as described in previous section, 750 μg of protein) were added to each bead mixture and mixed for 17 h at 4 °C. The supernatant was then discarded, and the beads were then washed in 0.1% BSA in PBS three times for 5 min each. The supernatant was discarded, and proteins were eluted and solubilized by addition of 100 μl of Laemmli sample buffer (10 mM Tris, pH 6.8, 1.5% SDS, 6% glycerol) followed by heating to 100 °C for 10 min. Protein concentration in the supernatant was assessed using the BCA protein assay (Pierce).

Separation by 1-D SDS-PAGE and Trypsinization—Immunolocated AQP2 vesicle proteins were separated by SDS-PAGE using 12% polyacrylamide minigels (Bio-Rad). Gels were then stained for 15 min with Coomassie Blue to visualize the proteins. The entire sample lane was cut into 35 sequential slices of ~1–2-mm thickness. Each of the 35 slices was then destained with 25 mM NH₄HCO₃, 50% ACN for 10-min intervals until entirely destained. Gel slices were dried and then reduced with 10 mM DTT in 25 mM NH₄HCO₃ for 1 h at 56 °C. The supernatant was removed, and an aqueous solution containing 55 mM iodoacetamide in 25 mM NH₄HCO₃ was added for 45 min in darkness at 22 °C to alkylate the reduced cysteine residues. The supernatant was then removed, and gels were washed with 25 mM NH₄HCO₃ for 10 min. Gel pieces were dehydrated with 25 mM NH₄HCO₃, 50%ACN and dried. Proteins were trypsinized using 12.5 ng/μl sequencing grade modified trypsin (Promega, Madison, WI) diluted in 25 mM NH₄HCO₃ and incubated at 37 °C for 16 h. Peptides were extracted from the gel by sonication in a 50% ACN, 0.5% formic acid solution, then dried, and reconstituted with 0.1% formic acid.

LC-MS/MS—Tryptic peptides from each gel block were analyzed by one-dimensional LC-MS/MS using a modified configuration of the Proteomex 2D LC/MS work station (Thermo Finnigan, San Jose, CA). Chromatographic separation of peptides was accomplished using two Zorbax 300SB C₁₈ peptide traps (Agilent Technologies, Wilmington, DE) working in alternating fashion (replacing the standard strong cation exchange and reverse phase columns), while the standard ESI ion source was replaced by a nanospray ionization source and a reverse phase PicoFrit™ column (BioBasic C₁₈, 75 μm; New Objective, Woburn, MA). The peptides were loaded onto the traps in alternating fashion using an autosampler (Surveyor, Thermo Electron, San Jose, CA). After washing with 0.1% formic acid, the peptides were eluted by 0–60% solvent B in solvent A (A = 0.1% formic acid; B = acetonitrile) in 30 min at a flow rate of about 200 nl/min (75 μl/min prior to splitting).

Inclusion Criteria for Identified Peptides—The m/z ratios of peptides and their fragmented ions were recorded by a method that allows the acquisition of three MS² scans (i.e., for the three highest intensity peaks in MS¹ scans) following each full MS scan. The raw data files were searched against the rat protein data base from the National Center for Biotechnology Information (NCBI) and rat ab initio protein data base from Ensembl using BioWorks (Version 3.1) software (Thermo Finnigan) based on the Sequest algorithm. The search parameters included the following: precursor ion mass accuracy = 3 amu, fragment ion mass accuracy = 1 amu, modification allowed for carboxypyrrolidomethylation, and two missed cleavages allowed. After the peptide sequence and protein identification from BioWorks software was carried out, the identified peptide sequences were initially qualified and filtered using the cross correlation score (Xcorr) at the following thresholds: Xcorr > 1.5 for 1+ ion, 2.0 for 2+ ion, and 2.5 for 3+ ion. For each identified peptide sequence that passed the filter threshold, proteins identified from two or more different peptides were selected if they achieved the following criteria: 1) peptide sequence had the highest Xcorr score for a particular CID spectrum, 2) peptide sequence had a Δ normalized correlation (ΔCn) score > 0.1, and 3) peptide sequence had good quality CID spectra by visual inspection. In addition, manual inspections were carried out for identifications based on a single peptide if such peptides corresponded to proteins involved in endosomal trafficking, cytoskeletal organization, or various functions at the plasma membrane (putative cargo proteins), according to gene ontology classifications obtained using the Rat Genome Database (rdg.mcw.edu) and Harvester software (European Molecular Biology Laboratory (EMBL), harvester.embl.de). All identified peptide sequences were searched using BLAST to obtain the best possible unique protein ID, thus eliminating redundant annotations.

A full list of proteins whose identifications were validated by identification of two or more component peptides or one peptide and manual inspection of spectrum is given in Supplemental Table 2. Supplemental Tables 3 and 4 give a summary of the raw (unvalidated) data.

Immunoblotting—Immunoblotting was performed as described previously (14). Briefly 10–20 μg of protein was resolved by SDS-PAGE on 10–12% polyacrylamide gels and transferred electrophoretically onto nitrocellulose membranes. The membranes were then blocked with 5% nonfat dry milk in immunoblot wash buffer (42 mM Na₂HPO₄, 8 mM NaH₂PO₄, 150 mM NaCl, and 0.05% Tween 20, pH 7.5), rinsed, and probed with primary antibody overnight at 4 °C. After washing, blots were incubated with species-specific secondary antibody conjugated to horseradish peroxidase. After the final wash, antibody binding was visualized by chemiluminescence (LumiGLO, Kirkegaard & Perry Laboratories, Inc. (KPL), Gaithersburg, MD).

Immunoelectron Microscopy—Vesicle suspensions were prepared by eluting the immunolocated vesicles from magnetic beads with PBS titrated to pH 3 with 50 mM HCl. Then the suspension was titrated back to pH 7.5 with 50 mM NaOH. Vesicle suspensions were mixed 1:1 with 4% paraformaldehyde and then applied to 200 mesh nickel grids. After blocking with 1% BSA and washing, the grid was incubated with primary antibody for 45 min at room temperature. Groups were exposed to primary antibodies recognizing AQP2, Rab5, Rab7, or Rab11 followed by exposure to species-specific anti-IgG antibodies conjugated to colloidal gold particles (5 or 12 nm, Jackson Immunoresearch Laboratories, West Grove, PA). After washing, vesicles underwent negative staining with 1% uranyl acetate. After drying, the grids were examined with a JOEL 1200 EX electron microscope operated at 60 kV. Control labeling was performed identically, but non-immune IgY was substituted for the primary antibody.

Antibodies—Aside from the chicken anti-AQP2 antibody described above, primary antibodies were obtained from either commercial sources or from independent investigators. The affinity-purified rabbit polyclonal aquaporin-2 (14) and VAMP2 (15) antibodies were produced in our laboratory. The ADP-ribosylation factor (ARF) 6 antibody was kindly provided by Dr. J. Donaldson (NHBLI, National Institutes of Health, Bethesda, MD). The anti-myosin II A rabbit polyclonal was a gift of Dr. Robert Adelstein (NHBLI, National Institutes of Health, Bethesda, MD). The myosin 1C rabbit polyclonal (M3567) and myosin VI mouse monoclonal (M0691) antibodies were obtained from Sigma. The following rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology: Rab7 (H-50, sc-10767), Rab4 (D-20, sc-312), and Rab5a (A-20, sc-598). The Rab11 mouse monoclonal (610656) was obtained from BD Transduction Laboratories. The
Centrifugation of the 17,000 g low density membrane fraction of rat inner medulla (200,000 g supernatant) was performed (see “Materials and Methods”). The IMCD cells were homogenized and then subjected to differential centrifugation to obtain the 17,000 g pellets. Detection of biotinylated proteins in these two fractions was accomplished by SDS-PAGE followed by electroblotting and probing the blots with a streptavidin-HRP conjugate. Three micrograms of protein were stained gels were sliced into blocks designated A through I at points defined by the molecular weight markers, and then each block was further cut into 1–2-mm thick slices, each of which was subjected to in-gel trypsinization and elution of the resulting peptides. To confirm the specificity of the AQP2 vesicle immunoisolation, an immunoblot was performed using a rabbit anti-AQP2 antibody (Fig. 3A). AQP2 band density was ~50-fold greater in AQP2-immunoisolated vesicle sample than in the control sample. Fig. 3C shows immunogold labeling of AQP2 in the immunoisolated vesicles, confirming that AQP2 is associated with discrete vesicular structures.

Following trypsinization and extraction from each gel slice (Fig. 1), the peptides were analyzed by LC-MS/MS. The associated proteins were identified by comparison with the rat protein data bases (standard data base from NCBI and ab initio data base from Ensembl) using BioWorks Version 3.1 software. Fig. 4 and Supplemental Table 1 report the 181 proteins identified for which at least two distinct peptides were found. Table I shows a summary of the proteins identified for which at least two distinct peptides were found. Table I shows a summary of the proteins identified based on a single peptide with manual inspection of the spectra. The identification of Rab GTPases 4, 5, 18, and 21 (associated with early endosomes), Rab7

### RESULTS

An anti-AQP2 antibody was prepared in chickens and biotinylated for these studies. The ability of the biotinylated chicken antibody to recognize AQP2 was tested by probing an immunoblot prepared from a homogenate of rat renal inner medulla (Fig. 2A). The biotinylated chicken anti-AQP2 antibody (right lane) labeled the same bands as did a previously characterized rabbit anti-AQP2 antibody (left lane).

The biotinylated chicken anti-AQP2 antibody was used to immunoisolate AQP2-containing intracellular vesicles from a low density membrane fraction of rat inner medulla (200,000 g centrifugation of the 17,000 g supernatant). By immunoblotting with antibodies to plasma membrane marker proteins, we showed previously that this low density membrane fraction is virtually devoid of plasma membranes (10, 11). To confirm that conclusion, we carried out surface biotinylation of rat IMCD cells in suspension and used these cells as input for the same differential centrifugation procedure (Fig. 2B). Although the high density membrane fraction (17,000 g pellet) contained abundant quantities of biotinylated (plasma membrane) proteins, the low density membrane fraction (200,000 g pellet) contained only very small amounts of biotinylated proteins. Hence we conclude that the low density membrane fraction used for AQP2 vesicle isolation does not contain substantial amounts of plasma membranes.

Intracellular membrane vesicles containing AQP2 were immunoisolated from the low density membrane fraction of rat inner medulla using the chicken anti-AQP2 antibody, and the associated proteins were separated initially by 1-D SDS-PAGE (Fig. 3A). A control sample was obtained by carrying out the same procedure with substitution of non-immune chicken IgY for the anti-AQP2 antibody. The Coomassie-stained gels were sliced into blocks designated A through I at points defined by the molecular weight markers, and then each block was further cut into 1–2-mm thick slices, each of which was subjected to in-gel trypsinization and elution of the resulting peptides. To confirm the specificity of the AQP2 vesicle immunoisolation, an immunoblot was performed using a rabbit anti-AQP2 antibody (Fig. 3B). AQP2 band density was ~50-fold greater in AQP2-immunoisolated vesicle sample...
(associated with late endosomes), and Rab11 and -25 (associated with recycling endosomes) points to the conclusion that a large component of intracellular AQP2 is located in endosomes (16). In addition, several endosome-associated SNARE proteins were identified including syntaxin-7 (present in late endosomes (17)), syntaxin-12 and -13 (present in recycling endosomes (18)), Vti1a (present in TGN (19)), and VAMP3. Additionally we identified actin and several actin-related proteins as well as actin-based motor proteins (myosin 1C, non-muscle myosins IIa and IIb, and myosin VI), annexins, tetraspan proteins, coat proteins, adaptor proteins, and heterotrimeric G-proteins as summarized in Table I.

Table II shows a summary of putative cargo proteins that were identified in association with the immunoisolated AQP2 vesicles. For the purposes of this study we define putative cargo as “proteins that are putatively carried in or attached to vesicles but are not part of the trafficking apparatus.” In most cases, these are proteins that function as components of the plasma membrane. Furthermore there were several membrane receptors, adhesion molecules, cell surface enzymes, and transport proteins including AQP2. These studies, however, did not demonstrate in which intracellular subcompartment these proteins are present.

The immunoisolated AQP2 vesicles also contained many ribosomal and endoplasmic reticulum-resident proteins (Fig. 4 and Supplemental Table 1) consistent with the presence of a substantial amount of intracellular AQP2 in the rough endoplasmic reticulum. Thus, we conclude that AQP2-containing intracellular vesicles are heterogeneous and include rough endoplasmic reticulum as well as various endosomal compartments.

The protein yield for the control isolation with non-immune chicken IgY was much lower than the yield for the AQP2-specific isolation (Fig. 3A). Much of the protein isolated appears to be BSA used for blocking the beads. LC-MS/MS analysis of proteins eluted from the control beads did not recapitulate the protein list found in analysis of those isolated with the AQP2 antibody (Supplemental Table 4). Nevertheless a few abundant proteins did appear on both lists including α-B crystallin, aldehyde reductase 1, α1 and α2 Na/K-ATPase, annexin A2, myosin 1C, Rab1, valosin-containing protein, hydroxyysteroid 11β-dehydrogenase 1, β- and γ-ac- tin, band 7 protein, and GTP-binding protein (Gαo). The association of these proteins with AQP2 intracellular vesicles, therefore, must be held as uncertain. Among all proteins identified from the control sample (no AQP2 antibody), only 15 proteins were identified from distinct spectra corresponding to two or more peptides. All others were based on single peptides, and most of these spectra were of relatively low quality and did not in general satisfy the acceptance criteria applied for the AQP2-immunoisolated proteins.

We carried out immunoblotting of additional immunoiso-
lated AQP2 vesicle preparations to confirm the presence of selected proteins identified by LC-MS/MS. Fig. 5A shows confirmatory immunoblots of Rab GTPases associated with endosomes including Rab4 (early endosomes), Rab5 (early endosomes and clathrin-coated endocytic vesicles), Rab7 (late endosomes), and Rab11 (recycling endosomes). All of these Rab proteins were enriched in the AQP2 vesicle sample relative to the whole IM sample and were of decreased abundance in the control sample. These results lend further support to the conclusion that substantial quantities of AQP2 are present in endosomal subcompartments including early endosomes, late endosomes, and recycling endosomes as well as the TGN (see “Discussion”).

Secretory vesicles typically contain a different Rab protein, namely Rab3. The LC-MS/MS analysis did not identify Rab3 isoforms in AQP2 vesicles. To look further, we carried out immunoblotting for Rab3, which has been reported previously to be present in rat inner medullary intracellular vesicles (20) (Fig. 5B). Although a clear cut band was seen in the whole brain homogenate, no evidence of Rab3a was found in inner
medulla or AQP2-isolated vesicles. Thus, Rab3a-positive vesicles do not appear to be abundantly represented among the AQP2-containing intracellular membrane compartments in the IMCD.

The presence of Rab5, Rab7, and Rab11 in AQP2 vesicles was further confirmed by immunogold labeling (Fig. 6A). Fig. 6A shows labeling for Rab5 with double labeling for Rab5 (small gold particles) and AQP2 (large gold particles) in the inset. Fig. 6B shows double labeling for Rab7 (small gold particles) and AQP2 (large gold particles). Single immunolabeling is shown for Rab11 in Fig. 6C. In general, each of these three Rab proteins appears to be present in a subset of AQP2-immunoisolated vesicles.

Several members of the ARF family of small GTP-binding proteins were also identified by mass spectrometry of immunoisolated AQP2 membranes, viz. ARF1–6. These proteins are important in clathrin-mediated vesicle budding but have other roles in membrane trafficking (21). Fig. 7 depicts a confirmatory immunoblot for ARF6, showing a single band at ~18 kDa in both the IM and AQP2-immunoisolated samples but not the control sample.

Immunoblotting was also carried out for the SNARE proteins syntaxin-13, VAMP2 (synaptobrevin II), and VAMP3 (cellubrevin), all of which were identified by LC-MS/MS analysis in AQP2-immunoisolated vesicles (Fig. 8). All of these SNARE proteins were enriched in the AQP2-isolated sample relative to the whole IM sample and were of decreased abundance in the control sample. Syntaxin-13 was shown previously to be localized to early and recycling endosomes (18, 22, 23). VAMP2 (synaptobrevin-II) has been shown previously to be associated with endosomal vesicles (24). It has been shown to be present in intracellular vesicles from rat inner medulla (15) and to colocalize with AQP2-labeled intracellular vesicles via immunoelectron microscopy (25). VAMP3 has also been shown previously to be associated with AQP2 vesicles (26).
Again these data support the idea that the AQP2 immunoisolation procedure yields intracellular vesicles that are largely of endosomal origin.

The myosin isoforms identified by LC-MS/MS included myosin regulatory light chain and conventional non-muscle myosins IIA and IIB as well as unconventional myosins 1C, VI, and IXB (Table I). Fig. 9 shows confirmatory immunoblots for myosin 1C, myosin IIA, and myosin VI, all of which were identified in the AQP2-immunoisolated sample. Note the modest decrease in mobility in myosin IIA and myosin VI proteins attached to AQP2 vesicles, suggesting the presence of an unknown post-translational modification.

Ubiquitination is a signal for endocytosis and/or degradation of plasma membrane proteins. Ubiquitin was identified by LC-MS/MS in the SDS-polyacrylamide gel blocks representing molecular masses greater than 75 kDa (Supplemental Table 2). Immunoblotting with an anti-ubiquitin antibody confirmed the presence of ubiquitin in the AQP2-immunoisolated vesicles (Fig. 10). The anti-ubiquitin antibody labeled proteins throughout the molecular mass range from 25 to over 250 kDa. The broad smear suggests that ubiquitin is attached to a variety of proteins present in the AQP2 vesicles.

Proteins initially targeted to the basolateral plasma membrane via the exocyst complex may travel to the apical plasma membrane by transcytosis (8). In the LC-MS/MS analysis, we did not identify any components of the exocyst complex. Because a negative result using mass spectroscopy does not necessarily imply the absence of a particular protein, we tested for exocyst complex components by immunoblotting (Fig. 11). Two components, Sec6 and Sec8, were found to be associated with AQP2 vesicles. Furthermore a small GTP-binding protein known to be associated with the exocyst complex, RalA, was also identified in the AQP2 vesicles. The exocyst is known to function as an effector for the Rab-like small GTPase Sec4 in yeast (16). The mammalian Rab with the highest similarity to Sec4 is Rab8 (BLAST analysis), which was not identified in AQP2 vesicles by mass spectrometry. These results give preliminary support to the view that AQP2 is present in an intracellular structure that contains the exocyst complex, possibly recycling endosomes or TGN (16).

To investigate whether vasopressin may affect the ability to detect Rab proteins in AQP2 vesicles, we carried out the studies shown in Fig. 12. Here we infused vasopressin-deficient Brattleboro rats with either vehicle or dDAVP for 24 h and immunoblotted inner medullary samples for markers of the early endosomes (Rab4 and -5), late endosomes (Rab7), recycling endosomes (Rab11), and secretory vesicles (Rab3). Again Rab4, Rab5, Rab7, and Rab11 were readily detectable, and we could find no evidence for Rab3 under either condition. dDAVP infusion appeared to increase the amount of Rab11 (recycling endosomes) while decreasing the amount of Rab7 (late endosomes) associated with AQP2-immunoisolated intracellular membranes.

**DISCUSSION**

Advances in proteomics and mass spectrometry applied to proteins have enabled large scale identification of proteins in specific tissues, cells, or subcellular fractions (27, 28). Here we utilized LC-MS/MS to enumerate proteins in inner medul-
lary collecting duct cells that are contained in or attached to AQP2-bearing intracellular membranes. These “AQP2 vesicles” were purified by differential centrifugation followed by immunoisolation using a biotinylated chicken anti-AQP2 antibody. One-dimensional SDS-PAGE was used to pre-fractionate the proteins prior to trypsinization and one-dimensional LC-MS/MS.

Recent advances in cell biology have led to identification of numerous proteins involved in membrane trafficking including those responsible for budding, fusion, and cytoskeletal interactions. Many of these proteins were identified in this analysis.
(Fig. 4 and Table I). Of particular interest are proteins known to be associated with distinct subcellular membrane domains, which may serve as markers for these discrete membrane compartments. One family of proteins, the Rab family of small GTPases, is an example. Our mass spectrometric analysis identified a number of Rab family members. The Rab GTPases and their effectors orchestrate vesicular trafficking between disparate membrane sub-domains in both the endocytic and exocytic trafficking pathways (16). Thus, it is recognized that specific Rab proteins are associated with secretory vesicles (Rab3 isoforms), with recycling endosomes (Rab11 and Rab25), with early endosomes (Rab4, Rab5, Rab18, and Rab21), and with late endosomes and multivesicular bodies (Rab7). Our identification of Rab4, Rab5, Rab7, Rab11, Rab18, Rab21, and Rab25 supports the conclusion that a substantial component of intracellular AQP2 is contained in endosomal membranes. The presence of Rab5, Rab7, and Rab11 was also confirmed by immunoblotting. Immuno-electron microscopy further confirmed the presence of these proteins and also showed that individual Rab proteins are present in some, but not all immunoisolated vesicles.

SNARE proteins, mediators of membrane fusion between vesicular and target membranes, have been shown to colocalize with markers of distinct membrane domains (29) and can be used as independent markers of specific subcellular compartments. The identification of endosomal syntaxins 7, 12, and 13, as well as VAMP2 and VAMP3 in immunoisolated AQP2 vesicles brings further support to the conclusion that a substantial fraction of intracellular AQP2 is contained in various endosomal compartments.

Although it appears clear that intracellular AQP2 is present in endosomes, analysis of immunoisolated AQP2 vesicles from the IMCD also revealed a large number of ribosomal and endoplasmic reticulum-resident proteins. This result demonstrates that in addition to endosomes, intracellular AQP2 vesicles also include the rough endoplasmic reticulum (RER). Obviously AQP2 and other integral membrane proteins are translated at the RER, and the presence of AQP2 in RER membranes implies that new AQP2 that is being produced has a sufficient residence time in the RER to manifest itself in this analysis. The presence of AQP2 in both endosomes and RER raises doubt about the interpretation of experiments that depend on differential centrifugation alone to assess the distribution of AQP2 between endosomal compartments and the plasma membrane through the determination of the ratio of AQP2 in low density membranes to AQP2 in high density membranes (10, 12). For example, stimuli that increase the production of AQP2 may increase the abundance of AQP2 in the low density membrane fraction of IMCD and result in a reduced high density/low density ratio without any change in trafficking to and from the plasma membrane.

One potential route of AQP2 trafficking from the intracellular compartment to the plasma membrane could be via recycling endosomes. AQP2 can hypothetically move from the TGN directly into recycling endosomes. An additional possibility is that AQP2 can be translocated directly from the TGN to plasma membrane via secretory vesicles as seen with synaptic vesicles. We did not identify Rab3, a secretory vesicle marker, by either mass spectrometry or immunoblotting. However, this result does not rule out some role for the secretory pathway in AQP2 trafficking to the plasma membrane. A negative result could be due to the fact that the secretory vesicles may move very rapidly from the TGN to the plasma membrane as demonstrated by Hirschberg et al. (30) so that a large AQP2 flux could occur despite a low abundance of secretory vesicles. Indeed Rab3a appears to be present in whole inner medullary homogenates (Fig. 12), and previous reports have demonstrated that when sufficient amounts of intracellular membranes are loaded on an immunoblot Rab3 is indeed detectable in inner medulla (20).

Another pathway for translocation of proteins from the TGN to the apical plasma membrane of epithelial cells has been proposed recently. Various membrane proteins may be initially targeted to the basolateral plasma membrane via the exocyst complex and may travel to the apical plasma membrane by transcytosis (8). Our LC-MS/MS analysis did not identify any exocyst component in AQP2 vesicles. However, immunoblotting demonstrated the presence of both Sec6 and Sec8, two exocyst complex proteins in intracellular AQP2 vesicles (Fig. 11). In addition, we found RalA, an exocyst-associated small GTPase, in the immunoisolated membranes, further supporting the conclusion that some subset of AQP2 intracellular vesicles contains the exocyst complex. This complex, which is associated with trafficking to the lateral plasma membrane (31), is also present in recycling endosomes and TGN (16). Polishchuk et al. (8) propose that some apically targeted proteins move to the apical plasma membrane by transcytosis after exocyst-associated basolateral targeting and that the transcytosis is initiated by internalization via caveolae. It appears possible that this pathway could be involved in AQP2 trafficking. Conceivably this indirect targeting model may provide an explanation to the finding in IMCD (2, 32) and earlier parts of the collecting duct system (33) that a substantial fraction of total cellular AQP2 is “mistargeted” to the basolateral plasma membrane.

Ubiquitin was readily detectable in AQP2-immunoisolated vesicles both by mass spectrometry (Table I) and immunoblotting (Fig. 10). Ubiquitin was present throughout most of the molecular weight range investigated, suggesting that ubiquitinated proteins were present in most fractions. Monoubiquitination is recognized as a signal that targets cargo proteins from the plasma membrane to the endosomal pathway, whereas polyubiquitination targets proteins to the proteasome (34).

Another class of proteins that was relatively well represented in immunoisolated AQP2 vesicles are the myosins (Table I). We identified non-muscle myosins IIA and IIB as well as the unconventional myosins 1C, VI, and IXB in association with AQP2-immunoisolated vesicles. Non-muscle myosin II is
involved with organization of actin microtubules in cells and with cell shape changes. A role of non-muscle myosin II has been proposed in vesicle budding at the TGN along with heterotrimeric G-proteins (also identified in this study) (35–37). Recent studies have implicated non-muscle myosin II in AQP2 trafficking (38).

LC-MS/MS analysis also identified a number of heterotrimeric G-protein subunits including G_{i1}, G_{i2}, G_{i3}, G_{i4}, G_{i6}, G_{i8}, and G_{i3} (Table I). Previous studies have implicated heterotrimeric G-proteins in vesicle fusion events in pancreatic zymogen granules (39) and GLUT4 vesicles (40). Additionally, Donaldson et al. (41) showed that heterotrimeric G-proteins may be involved in the regulation of β-COP and ARF binding to Golgi membranes. Although the presence of heterotrimeric G-proteins in the collecting duct cytoplasm has been reported previously (42), a role for heterotrimeric G-proteins in vasopressin-induced AQP2 trafficking, beyond their roles in G-protein-coupled receptor signaling, has undergone limited investigation. Valenti et al. (43) found that G_{ai} may play a role in vasopressin-induced insertion of AQP2 into the apical membrane of CD8 RC.SV3 rabbit cortical collecting duct cells. However, the nature of this involvement remains unclear.

CONCLUSIONS

This study provides the first step toward establishment of the feasibility of a new approach to the investigation of membrane trafficking in cells involving a combination of "immuno-dissection" of individual membrane compartments and large scale protein identification by LC-MS/MS. Immunolocalization using an antibody to AQP2 yielded a heterogeneous mixture of membrane compartments, which includes both endosomes and the RER. A logical next step would be to use antibodies to marker proteins associated with specific membrane compartments to selectively identify their respective proteomes.

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