SNAP-23 in rat kidney: colocalization with aquaporin-2 in collecting duct vesicles

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SNAP-23 in rat kidney: colocalization with aquaporin-2 in collecting duct vesicles. Am. J. Physiol. 275 (Renal Physiol. 44): F752–F760, 1998.—Vesicle targeting proteins ("SNAREs") have been proposed to direct vasopressin-induced trafficking of aquaporin-2 water channels in kidney collecting ducts. A newly identified SNARE protein, SNAP-23, is proposed to mediate vesicle targeting to the plasma membrane in diverse tissues. The current studies were done to determine whether SNAP-23 is expressed in collecting ducts with an intracellular distribution compatible with a role in aquaporin-2 trafficking. RT-PCR demonstrated SNAP-23 mRNA in microdissected collecting ducts and other tubular segments including the proximal tubule and thick ascending limb. Immunoblotting using a polyclonal antibody raised against a COOH-terminal peptide revealed a solitary band at an apparent molecular mass of 30 kDa in renal medullary membrane fractions and inner medullary collecting duct suspensions. Differential centrifugation revealed that SNAP-23 is present in membrane fractions including the low-density fraction enriched in intracellular vesicles. Immunocytochemistry revealed SNAP-23 labeling at both the apical and the cytoplasm of collecting duct principal cells. Immunoblotting of intracellular vesicles immunosolated using an aquaporin-2 antibody revealed the presence of both SNAP-23 and synaptobrevin-2 (VAMP-2) in aquaporin-2-bearing vesicles. We conclude that SNAP-23 is strongly expressed in collecting duct principal cells, consistent with a role in vasopressin-regulated trafficking of aquaporin-2. However, localization of SNAP-23 in both intracytoplasmic vesicles and plasma membranes suggests a function different from that originally proposed for SNAP-25 in synaptic vesicle targeting.

syntaxin-4; VAMP-2; synaptobrevin-2; exocytosis

The regulation of renal water excretion is mediated by the peptide hormone vasopressin (the antidiuretic hormone), which acts through the intracellular messenger cAMP to increase the water permeability of the renal collecting duct, thus accelerating the reabsorption of water from the tubule lumen back to the bloodstream. The molecular target for this regulatory process is aquaporin-2, the "vasopressin-regulated water channel." Aquaporin-2 is a member of a large family of epithelial water channels that function to accelerate water permeation across plasma membranes at sites where rapid water transport is necessary for specialized physiological functions (14, 23). The short-term action of vasopressin to increase collecting duct water permeability is a result of fusion of aquaporin-2-bearing intracellular vesicles with the apical plasma membrane of the collecting duct principal cells (24). Aquaporin-2 is an integral membrane protein present in the lipid bilayer of both intracellular vesicles and the apical plasma membrane. Thus, in contrast to many other examples of regulated exocytosis, the "cargo" is an integral membrane protein rather than a soluble protein carried in the lumen of the exocytic vesicle, and the destination of the cargo is the plasma membrane rather than the extracellular space.

How vasopressin regulates this exocytic process is presently unknown. Aquaporin-2 exocytosis involves several steps, each of which could be a target for regulation, namely, transport of aquaporin-2 vesicles to the subapical domain of the principal cells, docking of the vesicles, and vesicle fusion. We and others (9, 10, 13, 15, 17, 18, 26) have postulated that the docking step could be mediated by a complex interaction between integral membrane proteins, the so-called "SNAREs," present in the vesicle (v-SNAREs) and the target membrane (t-SNAREs) as has been proposed for targeting of synaptic vesicles to the active zone of the presynaptic plasma membrane in the central nervous system (2, 12, 29). In the synapse, the core complex is made up of a v-SNARE (VAMP-2, also called synaptobrevin-2) and two t-SNAREs (syntaxin-1 and SNAP-25) (30). This 7S core complex binds an N-ethylmaleimide-sensitive factor (NSF) via an intervening soluble NSF attachment protein (α-SNAP) to form a larger 205 complex, the formation of which is thought to be vital to the eventual vesicle fusion process (30).

In the collecting duct principal cell, we have demonstrated that a v-SNARE, synaptobrevin-2 (also called "VAMP-2") is present in aquaporin-2 vesicles (26) and that the t-SNARE syntaxin-4 is present in the apical plasma membrane (18). However, no homolog of the third component of the 7S complex, SNAP-25, had been identified in epithelia including the renal collecting duct. Recently, however, cDNAs for a novel SNAP-25 homolog called SNAP-23 were cloned from human B lymphocytes (28) and mouse adipocytes (34). These cDNAs encode orthologous 211-amino-acid proteins. Human SNAP-23 is 59% identical to SNAP-25 at the amino acid level and was found to bind syntaxins 1–4.
and synaptobrevin-1 and -2 in vitro (28). Northern blot analysis revealed the SNAP-23 mRNA is broadly expressed among tissues including kidney (28). Based on these properties, we hypothesize that SNAP-23 may be the third SNARE protein involved in regulated trafficking of aquaporin-2 in the collecting duct principal cell. To localize SNAP-23 in kidney, we carried out RT-PCR studies in microdissected renal tubules and prepared peptide-directed antibodies for immunoblotting and immunocytochemistry in the kidney.

METHODS

Antibodies. To prepare a polyclonal antibody against SNAP-23, a 20-amino acid peptide corresponding to the carboxy-terminal sequence of the human SNAP-23 sequence was synthesized and used for immunization of rabbits. A search of the available protein sequence data bases with the BLAST algorithm revealed that the immunizing peptide sequence is distinct from that of all other known eukaryotic proteins including SNAP-25. The peptide was purified by high-performance liquid chromatography and conjugated to maleimide-activated keyhole limpet hemocyanin through covalent linkage to the amino-terminal cysteine before immunization using a combination of Freund's complete and incomplete adjuvants. For rabbit L.394, ELISA titers were >1:32,000 before exsanguination. This antiserum was affinity purified using a column on which 2 mg of the same synthetic peptide was immobilized through covalent linkage to activated agarose beads (Immunobilization kit no. 2; Pierce, Rockford, IL). An IgG fraction of the preimmune serum was purified using a protein A affinity column (Pierce) for a negative control in immunoblots and immunocytochemistry.

Antibodies recognizing aquaporin-2 (L127) (5), aquaporin-1 (L266) (32), VAMP-2 (L220) (18), and syntaxin-4 (L279) (18) have been previously characterized. They were affinity purified as described above. A mouse monoclonal anti-SNAP-25 antibody was purchased from Sternberger Monoclonals, Baltimore, MD (catalog no. SMI-81, lot 3).

Differential centrifugation. Subcellular fractions were prepared by differential centrifugation as described before (20). Male Sprague-Dawley rats weighing between 200 and 300 g were used. Several tissues (cerebral cortex, lung, heart, liver, spleen, pancreas, kidney, skeletal muscle) were quickly re-suspended with bicarbonate-buffered isotonic solution, each sample was homogenized and centrifuged at 4,000 g for 10 min to completely eliminate noncovalently bound IgG. The supernatants from this high-speed centrifugation were re-suspended in isolation solution, the protein concentration was measured spectrophotometrically with the Pierce bicinchoninic acid protein assay reagent kit. These fractions were solubilized at 60°C for 15 min in Laemmli sample buffer prior to immunoblotting (see below).

Inner medullary collecting duct (IMCD) and non-IMCD tubule suspensions. IMCD and non-IMCD tubule suspensions were prepared from rat whole inner medulla suspensions as described by Chou et al. (4). Inner medullas were dissected from rat kidneys and digested at 37°C with collagenase B (3 mg/ml; Boehringer-Mannheim, Indianapolis, IN) and hyaluronidase (600 U/ml; Worthington Biochemicals, Freehold, NJ) in bicarbonate-buffered isotonic solution (118 mM NaCl, 5 mM KCl, 25 mM NaHCO3, 4 mM Na2HPO4, 2 mM CaCl2, 1.2 mM MgSO4, 5 mM CH3COONa, and 5.5 mM glucose) containing 0.5% (wt/vol) BSA (ICN Biomedicals, Aurora, OH) under continuous supplement of 95% air-5% CO2 until IMCDs were free from adherent thin limbs. DNase I (Boehringer-Mannheim) at a final concentration of 0.001% (wt/vol) was added to the digesting solution to reduce aggregation of separated tubule segments. After incubation for another 15 min in this solution, one-fourth of the suspension was removed and kept on ice ("whole inner medulla" sample). The remaining suspension was separated into IMCD and non-IMCD containing fractions by low-speed centrifugation (50 g) (Sorvall RT600B). The pellet was resuspended in bicarbonate-buffered isotonic solution, and the 50 g centrifugation was repeated three times. The final pellet ("IMCD" sample) contained mostly IMCD fragments, whereas the pooled supernatant contained thin limbs and vascular elements ("non-IMCD" sample). Subsequently, all three samples were pelleted by centrifugation at 4,000 g for 20 min (Tomy, MTX-150). After resuspension with bicarbonate-buffered isotonic solution, each sample was homogenized and centrifuged at 200,000 g for 1 h. After measurement of protein content, all samples were solubilized in Laemmli sample buffer.

Immunosolubilization of aquaporin-2 containing intracellular vesicles. Aquaporin-2-containing intracellular vesicles were immunosolubilized from the HS membrane fraction of inner medulla (200,000 g pellet from 17,000 g supernatant as described above under Differential centrifugation) which has been demonstrated to be virtually devoid of plasma membrane elements from collecting duct cells. This technique used the aquaporin-2 antibody covalently coupled to magnetic beads (Dynabeads M-280 sheep anti-rabbit IgG; Dynal, Lake Success, NY) following the manufacturer's instructions. A quantity of 5.1 µg of affinity-purified aquaporin-2 antibody or the IgG fraction of preimmune serum was incubated with 1.7 mg magnetic beads in 100 µl of wash solution (PBS, pH 7.4, 0.1% BSA with 0.02% azide) overnight at 4°C with gentle mixing. After washing four times in the same solution with bidirectional mixing for 30 min each, beads were resuspended in 1 ml of cross-linking buffer solution (0.2 M triethanolamine, pH 8.2) and were washed two more times with the same solution. Dimethylpimelimidate (DMP), 20 mM, in 10 ml of cross-linking buffer solution was added to and incubated with the beads for 45 min at room temperature with bidirectional mixing. After incubation in 10 ml of cross-linking buffer solution without DMP for a further 2 h, beads were resuspended and incubated in 10 ml of 1% Nonidet P-40 (NP-40) containing the cross-linking buffer solution for another 10 min to completely eliminate noncovalently bound IgG. The high-speed membrane fraction (200,000 g pellet from 17,000 g supernatant as described above) from inner medulla was resuspended in wash buffer. After the beads were washed in 1 ml of wash buffer three times, 166 µl of the high-speed
fraction was incubated with beads for 5 h at 4°C with gentle mixing. After a wash in 1 ml of wash buffer three times, vesicles were eluted with 100 µl of Laemmli sample buffer and were incubated at 60°C for 10 min to solubilize membrane proteins.

Electrophoresis and immunoblotting. Samples prewarmed to 37°C for 20 min were loaded on precast 12% SDS-PAGE minigels (Novex, San Diego, CA) and electrophoresed using an X-Cell II minicell (Novex). The proteins on the gel were transferred electrophoretically to nitrocellulose membranes using a Bio-Rad Mini Trans-Blot cell (Bio-Rad, Hercules, CA). After blocking with 5% (wt/vol) nonfat dry milk in blot-wash buffer (150 mM NaCl, 50 mM NaH2PO4, 0.05% (vol/vol) Tween-20, pH 7.5) for 30 min at room temperature, the membranes were incubated with either the anti-SNAP-23 antibody, the anti-aquaporin-1 antibody, or the anti-aquaporin-2 antibody in antibody dilution buffer solution (blot-wash buffer with 0.1% BSA and 0.02% NaN3) overnight at 4°C. After a wash with blot-wash buffer, the nitrocellulose membranes were incubated with 0.16 µg/ml of donkey anti-rabbit IgG conjugated to horseradish peroxidase (no. 31458; Pierce, Rockford, IL) in blot-wash buffer containing 5% (wt/vol) nonfat dry milk at room temperature for 1 h. For the immunoblots incubated with the mouse monoclonal antibody to SNAP-25, 0.16 µg/ml of rabbit anti-mouse IgG conjugated to horseradish peroxidase (Pierce no. 31450) was used. Sites of antibody-antigen reaction were visualized by chemiluminescence using SuperSignal Substrate (Pierce) and exposure to light-sensitive imaging film (Kodak no. 165–1579 Scientific Imaging Film).

RT-PCR amplification of SNAP-23 mRNA in isolated renal tubule segments. Renal tubule segments were microdissected as previously described (36) and as recounted briefly in the following. The rats were killed by decapitation. The left kidney was perfused through the aorta with 100 mg of collagenase B (Boehringer-Mannheim) and 250,000 U hyaluronidase (Worthington Biochemicals) in 50 ml of dissection solution (135 mM NaCl, 5 mM KCl, 0.1 mM Na2HPO4, 0.3 mM NaH2CO3, 0.12 mM Na2SO4, 2.5 mM CaCl2, 1.2 mM MgSO4, 5 mM HEPES, and 5.5 mM glucose, at pH 7.4) with added 0.1% BSA (ICN Biomedicals). Then, the kidney was removed, sliced, and incubated in digestion solution (4 mg of collagenase B and 10,000 U of hyaluronidase in 2 ml dissection solution) at 37°C with continuous oxygenation for 10–80 min. The different segments were microdissected freehand using Dumont no. 5 forceps in dissection solution containing 1:40 vanadyl ribonucleoside complex (VRC, Life Science), a ribonuclease inhibitor. After a wash in dissection solution with 0.1% BSA, one segment in 2 µl of dissection solution with 0.1% BSA was transferred into a PCR tube containing 6.7 µl of Triton X-100 solution [mix of 96 µl 2% Triton X-100, 3.5 µl RNase placental inhibitor (Boehringer-Mannheim), and 0.5 µl 1 M DTT] to monitor RNA or cDNA contamination, blanks were included in each RT-PCR experiment in which 2 µl of the wash solution was transferred without a tubular segment. The samples were immediately frozen on dry ice and stored at −80°C until use.

RT-PCR was carried out as described previously (7). After each sample was thawed, 11.3 µl of master mix [4 µl of 5X incubation buffer, 50 U RNase inhibitor, 20 nmol of each deoxynucleotide, 1.6 µg of poly(dT)15, 10 U of AMV reverse transcriptase (Boehringer-Mannheim), and 1.28 µl of 0.1 M DTT] was added to each PCR tube. To be sure that products are not the result of amplification of genomic DNA, we added diethyl pyrocarbonate-treated water to some tubes instead of diethyl pyrocarbonate-treated water to all tubes instead of water. The PCR primers were designed from the mouse SNAP-23 cDNA sequence (34) (GenBank accession no. U73143). The sequences of the sense and antisense primer were 5’-CAAC-TAAATCGATAGAAAGG 3’ (bp 151–173) and 5’-CCACCTTGAGTCAGGTT 3’ (443–460 bp), respectively. The expected size of the PCR product is 327 bp.

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Expression of SNAP-23 mRNA along the renal tubule. Previous Northern blotting studies have demonstrated that SNAP-23 mRNA is expressed in kidney (28). To localize SNAP-23 mRNA among intrarenal structures in the rat, RT-PCR was carried out in microdissected tubules, glomeruli, and vasa recta using primers based on the reported cDNA sequence for the mouse (34). These experiments demonstrated that SNAP-23 mRNA is broadly expressed among kidney structures. Figure 1 typifies the results. The signal was strongest in the glomerulus, vasa recta, cortical thick ascending limb (CTAL), connecting tubule (CNT) and collecting ducts from cortex, outer medulla, and inner medulla (CCD, OMCD, and IMCD). Substantial signals were also seen in the proximal convoluted tubule (PCT), type I and II thin descending limbs (TDL-I and TDL-II), medullary thick ascending limb (MTAL), and distal convoluted tubule (DCT). No signal was seen in a
“blank” control in which the reaction was run without added tissue and in an RT-negative control in which the reverse transcription step was omitted. These results therefore demonstrated the presence of SNAP-23 mRNA in the segments that express aquaporin-2, i.e., the collecting ducts, but the expression is not limited to these segments.

Expression of SNAP-23 protein in the kidney. To localize SNAP-23 protein in the kidney, we raised a rabbit polyclonal antibody to a 20-amino-acid peptide corresponding to the carboxy terminus of human SNAP-23 (see METHODS). Immunoblotting was carried out with the affinity-purified L394 antibody (Fig. 2). The SNAP-23 antibody labeled a single band in membrane fractions from kidney cortex, outer medulla, and inner medulla (Fig. 2, left). As seen previously with SNAP-25 (30), the apparent molecular mass, 30 kDa, is greater than the predicted mass based on the open-reading frame from the cDNA sequence. No band was seen in a membrane fraction from cerebral cortex, supporting the view that this antibody does not cross-react with SNAP-25, a relatively abundant protein in the central nervous system. Preadsorption of the affinity-purified anti-SNAP-23 antibody with an excess of the immunizing peptide completely ablated labeling of the 30-kDa band (Fig. 2, right).

Expression of SNAP-23 protein in the IMCD. To determine whether SNAP-23 protein is expressed in the IMCD, we carried out immunoblotting using tubule suspensions from the rat renal medulla after a low-speed centrifugation procedure that divides the suspensions into IMCD-enriched and non-IMCD fractions (4) (Fig. 3). As seen in Fig. 3, middle and bottom, aquaporin-2 (a collecting duct marker) was enriched in the IMCD fraction and aquaporin-1 (a descending limb/vasa recta marker) was enriched in the non-IMCD fraction relative to whole inner medulla. Figure 3, top, illustrates that SNAP-23 protein is more abundant in the IMCD-enriched fraction (middle lane) than in the whole inner medulla sample (as seen with aquaporin-2), supporting the conclusion from the RT-PCR studies that SNAP-23 is expressed in the IMCD. If SNAP-23 were not expressed in the IMCD, then the IMCD fraction would be expected to be relatively depleted in SNAP-23 as seen for aquaporin-1 (Fig. 3, bottom).

Subcellular distribution of SNAP-23 protein. To determine the subcellular distribution of SNAP-23 protein in rat medulla, immunoblotting was carried out using samples prepared by differential centrifugation as described in METHODS. As shown in Fig. 4, SNAP-23 was present in the samples prepared from 17,000 g and 200,000 g membrane pellets but was absent in the...
cytosolic fraction (200,000 g supernatant). In previous studies of the rat inner medulla (8, 20), we have demonstrated using appropriate markers that plasma membranes are abundant in the 17,000 g fraction but are virtually absent in the 200,000 g fraction. Thus the differential centrifugation results demonstrate that SNAP-23 is not limited to the plasma membrane, at least in the inner medulla where the differential centrifugation technique has been validated.

Immunocytochemical localization of SNAP-23 protein in kidney. To establish further the distribution of SNAP-23 in the kidney, immunocytochemistry was carried out in thin cryosections of rat kidney with the affinity-purified SNAP-23 antibody using a horseradish peroxidase-conjugated secondary antibody (Fig. 5). Figure 5A shows a renal cortical section showing strong labeling of the PCTs with a predominant apical distribution (arrows). Labeling was absent in the cortex when an IgG fraction of the preimmune serum was substituted for the primary antibody (Fig. 5B). Figure 5C shows a renal outer medullary section demonstrating weak but significant labeling of the thick ascending limbs not seen when the preimmune IgG was substituted (Fig. 5D). Finally, as was deduced from the immunoblotting studies, there was labeling of the IMCD cells with the SNAP-23 antibody (Fig. 5E), including labeling of the apical aspect of the cells (arrows) and the cytoplasmic domains, i.e., in intracytoplasmic vesicles. As shown in Fig. 5F, IMCD labeling was absent when an equal concentration of preimmune IgG was substituted.

Presence of SNAP-23 in aquaporin-2-bearing intracellular vesicles. To investigate whether SNAP-23 is present in aquaporin-2-bearing intracellular vesicles, membranes from renal inner medulla were immunoisolated from a low-density membrane fraction (200,000 g pellet from 17,000 g supernatant; see METHODS) using the aquaporin-2 antibody covalently coupled to magnetic beads. Figure 6 shows immunoblots of the immunoisolated proteins probed with the aquaporin-2, VAMP-2, SNAP-23, and syntaxin-4 antibodies. As expected, aquaporin-2 was enriched in the sample from aquaporin-2 immunoisolated vesicles relative to material isolated from beads coated with an equal amount of affinity-purified preimmune IgG (Fig. 6). As previously reported (26), VAMP-2 was also present in aquaporin-2-bearing intracellular vesicles (Fig. 6). In addition, SNAP-23 was also enriched in these vesicles (Fig. 6). On the other hand, syntaxin-4, another t-SNARE in collecting duct, was not detectable in the aquaporin-2 vesicles (Fig. 6). These results demonstrate that aquaporin-2-bearing intracellular vesicles possess SNAP-23 as well as VAMP-2, but not syntaxin-4.

Expression of SNAP-23 and SNAP-25 in nonrenal tissues. To investigate the distribution of SNAP-23 among tissues, membrane fractions were prepared from several organs including brain, heart, skeletal muscle, lung, pancreas, liver, spleen, kidney cortex, and kidney inner medulla (Fig. 7). SNAP-23 was relatively abundant in lung, spleen, and kidney. In addition, there were weak but perceptible bands in heart, pancreas, and liver. In contrast to SNAP-23, SNAP-25 was detected only in brain (Fig. 7, right). SNAP-23 and SNAP-25 were present in both the 17,000 g and the 200,000 g membrane fractions, but absent from the cytosolic fraction (200,000 g supernatant).

DISCUSSION

Regulation of aquaporin-2 trafficking by vasopressin is central to the overall process that precisely regulates body water balance. Hence, an understanding of the molecular mechanisms involved in regulation of aquaporin-2 trafficking is crucial to the understanding of the pathophysiology of water balance disorders. Aquaporin-2 is stored in the membranes of intracellular vesicles

![Fig. 3. Immunoblots showing relative abundance of SNAP-23 in IMCD and non-IMCD elements of rat inner medulla (IM). Blots were probed with affinity-purified anti-SNAP-23 (0.125 µg/ml) (top), anti-aquaporin-2 (AQP-2, 0.077 µg/ml; middle), and anti-aquaporin-1 (AQP-1, 0.120 µg/ml; bottom). A quantity of 1 µg protein from each fraction was loaded for aquaporin-1 and aquaporin-2 immunoblots. A quantity of 10 µg protein was loaded for SNAP-23.

![Fig. 4. SNAP-23 immunoblot of fractions from differential centrifugation of kidney medulla. Fractions were prepared by a series of centrifugations as described in METHODS. A quantity of 10 µg protein from each fraction was loaded. OM; outer medulla, IM; inner medulla]
in the collecting duct principal cells (25). Upon stimulation by vasopressin, these aquaporin-2-bearing vesicles fuse with the apical plasma membrane, thus increasing the water permeability of the apical plasma membrane and concomitantly of the epithelium as a whole (24). Docking of these vesicles with the apical plasma membrane has been proposed to be mediated by binding of vesicle-targeting receptor proteins present in the

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**Fig. 5.** Immunocytochemical localization of SNAP-23 in kidney cortex and outer and inner medulla. Kidney was perfusion fixed with a 4% paraformaldehyde-containing fixative. Thin sections were prepared, and horseradish peroxidase immunocytochemistry was carried out with SNAP-23 antibody (A, C, and E) and preimmune IgG (B, D, and F) as control. Labeling with anti-SNAP-23 antibody (arrows) was present in proximal tubule (A), thick ascending limb of Henle's loop (C), and collecting duct (E). Labeling was not seen in preimmune IgG controls (B, D, and F). P, proximal tubule; T, thick ascending limb of Henle's loop; CD, collecting duct. Magnification, ×840.
vesicles (v-SNAREs) with vesicle-targeting proteins in the target membrane (t-SNAREs) (9, 10, 13, 15, 17, 18, 26). Such a process has been demonstrated to be essential for synaptic vesicle exocytosis in the central nervous system, where at least one v-SNARE (synaptobrevin or “VAMP”) forms a tight SDS-resistant complex with at least two t-SNAREs, syntaxin and SNAP-25 (30). The likelihood that such a complex may be involved in aquaporin-2 vesicle targeting to the apical plasma membrane of collecting duct principal cells drew support from the observations that the v-SNAREs VAMP-2 and cellubrevin have been identified in aquaporin-2 vesicles (9, 26) and that a t-SNARE (syntaxin-4) is present in the apical plasma membrane of principal cells (18). In this study, we demonstrate that a second putative t-SNARE, SNAP-23, is expressed in the principal cells of the renal collecting duct. Thus we have obtained evidence that collecting duct principal cells express all three components of a putative SNARE complex analogous to that demonstrated in the central nervous system, namely, VAMP-2, syntaxin-4 and SNAP-23. However, SNAP-23 was found not only in plasma membrane but also in intracellular vesicles. Indeed, SNAP-23 was relatively abundant in a membrane fraction that was immunoisolated from a low-density membrane fraction using an antibody to aquaporin-2, a finding suggesting that SNAP-23 is present in at least a subpopulation of aquaporin-2-bearing intracellular vesicles. This finding suggests that SNAP-23 may not function strictly as a t-SNARE for vesicle targeting to the plasma membrane and adds to evidence from studies in other tissues that putative t-SNARES for plasma membrane targeting can be found in intracellular membrane domains as well. The significance of these findings is discussed in greater detail below.

Anti-SNAP-23 antibody recognizes SNAP-23, but not SNAP-25. The antibody used for these studies was raised in rabbits against a synthetic peptide corresponding to the terminal 20 amino acids of the SNAP-23 protein. This portion of the SNAP-23 molecule was chosen, not only because the sequence was likely to be immunogenic, but also because it lacks substantial similarity to SNAP-25 or to any other protein present in the GenBank data base (BLAST analysis). Immunoblots (Figs. 2 and 7) demonstrated that the antibody does not cross-react with any protein abundantly expressed in rat brain (a rich source of SNAP-25), supporting the conclusion that the antibody specifically recognizes SNAP-23. In immunoblots of kidney and other organs, the antibody recognizes a solitary band at an apparent molecular mass of ~30 kDa, which was ablated by preadsorption with the immunizing peptide. As seen previously for SNAP-25, the apparent molecular weight of SNAP-23 estimated by SDS-PAGE and immunoblotting is greater than the predicted molecular weight based on the open-reading frame of the cloned cDNA (35). This property could be in part due to the fact that SNAP-25 and SNAP-23 are believed to be palmitoylated (28), which would alter both the size and shape of the protein.

SNAP-23 is broadly expressed among organs. Immunoblotting experiments (Fig. 7) demonstrated that SNAP-23 is expressed in several organs other than kidney. In agreement with observations by Wong et al. (35), SNAP-23 protein is particularly abundant in lung and spleen in addition to kidney. Furthermore, weaker but unequivocal expression was seen in liver, heart, skeletal muscle, and pancreas. In contrast to SNAP-25, little or no SNAP-23 expression was detected in brain.

**Fig. 6.** Aquaporin-2, VAMP-2, SNAP-23, and syntaxin-4 immunoblots of aquaporin-2 immunosolated vesicles from rat renal inner medulla. In each case, aquaporin-2-isolated vesicle proteins are compared with proteins obtained with substitution of preimmune IgG for anti-aquaporin-2 on beads. Equal volumes of eluted material are compared in each case (see METHODS).

![Aquaporin-2, VAMP-2, SNAP-23, and syntaxin-4 immunoblots of aquaporin-2 immunosolated vesicles from rat renal inner medulla.](https://example.com/aquaporin-2-vmsnap-23-syntaxin-4-immunoblots)

**Fig. 7.** Comparison of the tissue distribution of SNAP-23 and SNAP-25. Fractions from tissues were prepared by a series of centrifugations as described in METHODS. Blots were probed with polyclonal anti-SNAP-23 (0.125 µg/ml) and monoclonal anti-SNAP-25 antibody (Sternberger, cat. no. SMI-81, lot 3, dilution 1:1,000). A quantity of 10 µg protein was loaded for all tissues except for brain (2 µg/ml) for SNAP-25. Brain sample was from cerebral cortex. CX, cortex; IM, inner medulla.
Previous studies have demonstrated that SNAP-23 is strongly expressed in adipocytes (1, 35), where it appears to be involved in insulin-mediated trafficking of the glucose carrier GLUT-4 to the plasma membrane. SNAP-23 has also been identified in human neutrophils, where it has been proposed to play a role in granule exocytosis (21). These results suggest that in contrast to SNAP-25, SNAP-23 may be involved in a broad array of trafficking events seen in many tissues.

SNAP-23 is broadly expressed among structures in the kidney. Previous studies have demonstrated that SNAP-23 mRNA (28) and SNAP-23 protein (35) are relatively abundant in the kidney. Our studies using RT-PCR, immunoblotting, and immunocytochemistry strongly support that conclusion. Furthermore, all three techniques provide evidence that SNAP-23 is expressed in the collecting duct, the site at which vasopressin regulates aquaporin-2 vesicle trafficking, as noted above. Aside from the collecting duct, several other structures were found to express SNAP-23 mRNA, including the proximal tubule, the thick ascending limb of Henle's loop, the connecting tubule, the glomerulus, and vasa recta. This distribution is similar to that seen for syntaxin-4 (18) but not syntaxin-2 and -3 (19). Immunocytochemical localization showed that SNAP-23 protein is relatively abundant in proximal tubule and thick ascending limb as well as the collecting duct. The broad renal expression suggests that SNAP-23 could be involved in multiple vesicular trafficking processes in the kidney, although the specific roles played by SNAP-23 in these structures remain to be elucidated. The parallel distribution of syntaxin-4 and SNAP-23 is concordant with the observation that among the known renal syntaxins, SNAP-23 was found to bind most strongly to syntaxin-4 when tested in yeast two-hybrid assays (1). Indeed, SNAP-23 was originally cloned using the yeast two-hybrid system with syntaxin-4 as bait (28). In view of these observations, we hypothesize that SNAP-23 is a SNARE protein that is preferentially expressed in plasma membrane domains that contain syntaxin-4. This combination may be involved in vesicle docking, for example, at sites where exocytosis is triggered by factors other than a rise in intracellular calcium such as vasopressin-regulated trafficking of aquaporin-2 in the renal collecting duct (18) or insulin-regulated trafficking of GLUT-4 in adipocytes (35). These observations also raise the possibility that other SNAP-25/SNAP-23 homologs may exist that interact preferentially with syntaxin-2 and -3.

In collecting duct principal cells, SNAP-23 is not limited to the plasma membrane. The SNARE hypothesis proposes that SNARE proteins determine the specificity of vesicular trafficking to the correct target membrane domains (30). If SNAP-23, a putative t-SNARE, is a determinant of targeting of aquaporin-2 vesicles to the apical plasma membrane, then one would expect predominant expression in plasma membranes. However, differential centrifugation studies (Fig. 4), immunocytochemical localization (Fig. 5), and immunoblotting of aquaporin-2 immunosolated vesicles demonstrate that SNAP-23 is distributed to membrane components of the cytoplasm, i.e., intracisternal vesicles, in addition to the plasma membrane. The intracisternal localization in collecting duct cells contrasts with the observation that there is little or no SNAP-23 in intracellular vesicles of 3T3-L1 adipocytes (1, 3, 35), where SNAP-23 is proposed to play a role in insulin-regulated GLUT-4 vesicle trafficking to the plasma membrane. The intracellular localization of SNAP-23 seen in the present study, however, is in accord with observations indicating that the SNAP-23 homolog, SNAP-25, is present in synaptic vesicles (6, 16, 33) and in chromaffin granules (11, 31), as well as the respective plasma membranes. Furthermore, recent studies have provided evidence that SNARE complexes containing both t-SNAREs and v-SNAREs can form in synaptic vesicles where they can be activated at a presynaptic site by NSF (27). Indeed, it has shown in a homotypic fusion system that NSF is not even needed at the time of vesicle docking and fusion; it is only needed at some step prior to docking (22).

Although the intracisternal localization of SNAP-23 appears to be at odds with the SNARE hypothesis as originally proposed, it is possible that the targeting role of SNAP-23 in vesicle trafficking to the plasma membrane is dependent on the physical state of the protein, which could be altered by posttranslational modification when it is present in the plasma membrane vs. the intracellular compartment. For example, SNAP-23 could undergo phosphorylation, which could alter its binding characteristics. Conceivably, SNAP-23 may need to be phosphorylated (or dephosphorylated) at one or more of several possible phosphorylation sites to bind efficiently with VAMP-2 and syntaxin-4 to form a 7S complex and dock with the plasma membrane. Additional studies will be required to test this hypothesis.

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