Expression of syntaxins in rat kidney

Mandon, Béatrice, Søren Nielsen, Bellamkonda K. Kishore, and Mark A. Knepper. Expression of syntaxins in rat kidney. Am. J. Physiol. 273 (Renal Physiol. 42): F718–F730, 1997.—Previously, we demonstrated that a putative vesicle-targeting protein, syntaxin-4, is expressed in renal collecting duct principal cells and is localized to the apical plasma membrane, suggesting a role in targeting aquaporin-2-containing vesicles to the apical plasma membrane. To investigate whether other syntaxin isoforms are present in the renal collecting duct, we determined the intrarenal localization of syntaxin-2 and -3. Reverse transcription-polymerase chain reaction (RT-PCR) experiments using total RNA extracted from kidney and various organs revealed that both syntaxin-2 and -3 are expressed in kidney cortex and medulla. RT-PCR experiments using microdissected tubules and vascular structures from the kidney revealed that syntaxin-3 mRNA, but not syntaxin-2, is expressed in collecting duct cells. Syntaxin-3 mRNA was also relatively abundant in the thick ascending limb of Henle's loop and in vasa recta. Syntaxin-2 mRNA was found chiefly in glomeruli. To investigate the localization of syntaxin-3 protein, a peptide-derived polyclonal antibody was raised in rabbits. In immunoblotting experiments, this antibody labeled a 37-kDa protein in inner medulla that was most abundant in plasma membrane-enriched subcellular fractions. Immunoperoxidase labeling of thin cryosections combined with immunogold electron microscopy showed that, in contrast to the labeling seen for syntaxin-4, syntaxin-3 labeling in medullary collecting duct was predominantly in the basolateral plasma membrane of intercalated cells. These results suggest the possibility that syntaxin-3 may be involved in selective targeting of acid-base transporters and/or in basolateral membrane remodeling in response to systemic acid-base perturbations.

Collecting duct; vesicle-targeting receptors; water channel; aquaporin; vasopressin

TRAFFICKING OF TRANSPORTERS to and from the plasma membrane is responsible for regulation of two important transport processes in the collecting duct of the renal medulla, water absorption and proton secretion (6). These processes are functions of different cell types. Collecting duct water absorption occurs via principal cells. The vasopressin-regulated water channel (aquaporin-2) is responsible for water transport across the apical plasma membrane (17, 32), and aquaporin-3 and -4 provide a pathway for water transport across the basolateral plasma membrane (13, 16, 41). Vasopressin, acting through increases in intracellular adenosine 3',5'-cyclic monophosphate, regulates the osmotic water permeability of the principal cells by increasing the number of aquaporin-2 water channels in the apical plasma membrane, a consequence of vasopressin-stimulated docking and fusion of aquaporin-2-containing intracellular vesicles with the apical plasma membrane (28, 31, 39, 46).

Proton secretion in the medullary collecting duct is carried out by the type A intercalated cells present in the outer medullary collecting duct and the initial part of the inner medullary collecting duct (44). A multisubunit H+-adenosinetriphosphatase (H+-ATPase) is responsible for translocation of protons from the cell to the lumen (20), whereas basolateral bicarbonate exit from the type A intercalated cells is mediated by the AE-1 subtype chloride-bicarbonate exchanger (originally referred to as "band-3") (2). Systemic acidosis has been demonstrated to trigger a redistribution of the H+-ATPase from intracellular vesicles to the apical plasma membrane, presumably by regulated trafficking (4). A similar redistribution of AE-1 has been proposed to occur in the basolateral plasma membrane of the type A intercalated cells in rabbits (42).

The molecular mechanisms responsible for regulated trafficking in principal cells and type A intercalated cells are unknown. Recently, it has been proposed that specificity of docking and fusion in vesicular trafficking is mediated by specialized membrane proteins present in vesicles and in the target membrane, which act as vesicle-targeting receptors or "SNAREs" (40). This model arose largely from studies of the molecular mechanisms by which synaptic vesicles dock and fuse with plasma membrane (3, 22, 23). Three such proteins have been identified in translocating vesicles, namely, vesicle-associated membrane polypeptides VAMP1 (synaptobrevin-1), VAMP2 (synaptobrevin-2), and cellubrevin. These have been termed "v-SNAREs." VAMP2 has been demonstrated to be present in intracytoplasmic vesicles in collecting duct principal cells (24, 25, 33). This localization raises the possibility that VAMP2 may function as a vesicle-targeting receptor for vasopressin-regulated exocytosis of aquaporin-2-containing vesicles. Similarly, several proteins have been identified that have been proposed to function as vesicle-targeting receptors in target membrane, namely, the syntaxins and synapse-associated protein-25 (SNAP-25) (3, 22, 23). These have been called "t-SNAREs." We have recently demonstrated that one of several known syntaxins, syntaxin-4, is expressed in collecting duct principal cells and is localized to apical plasma membrane (27). Thus both VAMP2 and syntaxin-4 are in appropriate membrane domains of collecting duct cells to be involved in targeting of aquaporin-2-containing vesicles to the apical plasma membrane.

In the current study, we have investigated whether two other syntaxin isoforms (syntaxin-2 and -3) may be expressed in the renal collecting duct, with a view toward determining whether these syntaxins could...
play a role either in water channel trafficking in principal cells or in acid-base transporter trafficking in type A intercalated cells. The results demonstrate that syntaxin-3, but not syntaxin-2, is expressed in the renal collecting duct. Immunocytochemical localization in the medullary collecting duct indicates that, in contrast to the apical location of syntaxin-4 in collecting duct principal cells, syntaxin-3 is present predominantly in the basolateral domain of intercalated cells of the medullary collecting duct. Immunoelectron microscopy using the immunogold technique demonstrated syntaxin-3 labeling of the basolateral plasma membranes of type A intercalated cells. This localization raises the possibility that syntaxin-3 may be involved in selective targeting of acid-base transporters and/or in basolateral membrane remodeling of intercalated cells in response to systemic acid-base perturbations.

METHODS

Reverse Transcription-Polymerase Chain Reaction Amplification of Syntaxin mRNAs

Reverse transcription-polymerase chain reaction (RT-PCR) experiments were carried out as described previously (27) to localize syntaxin mRNAs in 1) total RNA samples extracted from various tissues and 2) microdissected renal tubule segments and vascular elements.

RT-PCR using total RNA samples. Total RNA samples were prepared from several tissues (heart ventricles, lung, liver, brain cortex, total kidney) and from the major regions of the kidney (inner medulla, outer medulla, and cortex) using a method adapted from that of Chomczynski and Sacchi (10). Tissue samples from decapitated Sprague-Dawley rats were homogenized in RNAzol (4 M guanidium thiocyanate, 25 mM disodium citrate, pH 7.0; Molecular Research Center, Cincinnati, OH) containing 3.6 µl/ml β-mercaptoethanol. RNA was extracted using chloroform, purified by isopropanol precipitation, and washed with 70% ethanol. The RNA pellets were resuspended in RNA dilution buffer and stored at −80°C until used for RT-PCR. The RNA dilution buffer contained 10 mM tris(hydroxymethyl)aminomethane (Tris) (pH 7.5), 0.1 mM EDTA, 2 mM dithiothreitol (DTT), and 40 U/ml ribonuclease inhibitor (Boehringer-Mannheim, Indianapolis, IN).

Sequence-specific primers for syntaxin-2 and -3 were designed with the aid of computer program OLIGO 5.0 (National Biosciences, Plymouth, MN) based on rat cDNA sequences reported by Bennett et al. (5). The chief criteria used were specificity, Tm close to 60°C, and lack of predicted secondary structure. For syntaxin-2, the sense primer sequence corresponded to bp 41–61 (5′ GGC AGC ATG GAG ACA ATG CTC 3′) and the antisense primer sequence to bp 834–856 (5′ AGC CAA TGA TTA GAG CCA GGA CA 3′). (Base pair numbering is relative to the ATG codon believed to be the site of translation initiation.) For syntaxin-3, the sense primer sequence corresponded to bp 38–60 (5′ TGA CGC AGG ATG ACA CGG AC 3′) and the antisense primer sequence to bp 880–902 (5′ AGA TTT CAG TGG AGC AGC CCC TT 3′). RT-PCR reactions were performed according to the method of Elalouf et al. (14). Briefly, each reaction used 2 µg of total RNA. RT was carried out with the antisense primer in a 50 µl reaction volume, using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD) for 45 min at 41°C. After completion of RT, the temperature was raised to 96°C for 30 s to inactivate the enzyme and denature the RNA-DNA hybrids and then lowered to 80°C. PCR was initiated by adding 50 µl of a mix containing the PCR buffer, Taq polymerase (Perkin-Elmer, Norwalk, CT), and the corresponding sense primer. The Mg²⁺ concentration used was either 2.5 or 3.5 mM as designated in the Figs. 2–4. The samples were overlaid with mineral oil and processed for 31 cycles (96°C for 30 s; 59–61°C, depending on the primer, for 30 s; 74°C for 1 min). At the end of the last cycle, the elongation time at 74°C was extended to 10 min. Ten microliters of each PCR product was electrophoresed on 1.5% agarose gels, stained with ethidium bromide, destained, and photographed.

RT-PCR in microdissected renal tubules and vascular elements. Renal tubule segments were dissected as described (45), and RT-PCR was performed as described previously (11, 30) and summarized briefly here. The rats were killed by decapitation. Quickly, the left kidney was perfused with ice-cold dissection solution (27). This was followed by perfusion with collagenase-hyaluronidase solution. Then the kidney was removed, sliced, and incubated in the collagenase-hyaluronidase solution at 37°C with continuous oxygenation for 10–40 min. The different renal tubular segments were microdissected free hand using Dumont no. 5 forceps in dissection solution containing vanadyl ribonuclease complex (VRC), 0.5 µg/ml 2′-deoxycytidine deaminase (Tris) (pH 7.5), 0.1 mM EDTA, 2 mM dieththerol (DTT), and 40 U/ml ribonuclease (RNase) inhibitor (Boehringer-Mannheim, 0.5 µl 1 M DTT). Finally, the samples were frozen on dry ice and thawed before RT-PCR was initiated. To monitor for RNA or cDNA contamination of reagents, blanks were included in each assay with 2 µl of wash solution in lieu of tubules. Primers specific for aquaporin-2 water channel (27) were also used in some reactions with collecting ducts to provide a positive control for the assay system. The RT was initiated by adding 11.3 µl of a mix containing 50 U of avian myeloblastosis virus reverse transcriptase (Boehringer-Mannheim), 50 U RNase inhibitor, 20 nmol of each deoxynucleotide, and 1.6 µg of poly (DT)15 in Boehringer-Mannheim incubation buffer. The RT was carried out for 7 min at 37°C followed by inactivation of the enzyme for 2 min at 95°C. The tubes were then placed on ice and 80 µl of a mix containing 200 µmol of each deoxynucleotide, 50 pmol of sense and antisense primers, and 2.5 U DNA polymerase (Amplitaq, Perkin-Elmer) in Perkin-Elmer reaction buffer. The samples were overlaid with mineral oil and processed for 31 cycles [94°C, 1 min (3 min for the initial cycle); 59–61°C, depending on the primers, 1 min; 72°C, 1 min]. The elongation period in the last cycle was extended to 7 min. Ninety microliters of the PCR product were precipitated by adding 10.220.33.6 on April 14, 2017 http://ajprenal.physiology.org/ Downloaded from
containing the ethanol-precipitated PCR product was resuspended in 20 µl of TE (pH 7.5). Ten microliters were kept as the uncut fraction; 10 µl were used for restriction analysis with the appropriate enzyme (BamHI for syntaxin-2, BstXI for syntaxin-3) in the appropriate buffer, bringing the final volume to 50 µl. The digestion were carried out at 37°C for BamHI I or 45°C for BstXI I, each for 90 min. Ten microliters of the digested fraction and 10 µl of the uncut fraction were electrophoresed on 3% agarose gel (2% agarose, 1% NuSieve), stained with ethidium bromide, destained, and photographed.

**Immunoblotting Studies**

Polyclonal antibodies. To obtain a polyclonal antibody that specifically recognizes syntaxin-3, a 23-amino acid peptide corresponding to the amino terminus of the rat syntaxin-3 (with an added carboxy-terminal cysteine) was produced by solid-phase peptide synthesis (sequence: KDRLEQLKAK-QLTDQDDTDEVE) corresponding to amino acids 2–23 of the published sequence for syntaxin-3 (5). The peptide was purified by high-performance liquid chromatography (HPLC) and was conjugated to maleimide-activated keyhole limpet hemocyanin via covalent linkage to the carboxy-terminal cysteine. Two rabbits (L343 and L344) were immunized with this conjugate using a combination of Freund’s complete and incomplete adjuvants. Both rabbits developed enzyme-linked immunosorbent assay titers of 1:32,000. The antisera were affinity purified using a column on which 2 mg of the same synthetic peptide was immobilized via covalent linkage to agarose beads (SulfoLink Immobilization Kit no. 2; Pierce, Rockford, IL). Immunoblots with the two antibodies labeled identical bands. However, antibody L344 produced slightly lower background signals than L343. An immunoglobulin G (IgG) fraction of the L344 preimmune serum was purified on a protein A affinity column (Pierce) for use in control experiments. In addition, preadsorption controls were carried out by incubating the affinity-purified anti-syntaxin-3 antibody (L344, 0.5 µg/ml IgG) with 2 mg of the immunizing peptide linked to SulfoLink beads, whereas the nonadsorbed control anti-syntaxin-3 antibody (0.5 µg/ml IgG) was preincubated with cysteine-blocked SulfoLink beads.

In addition to polyclonal antibody L344, two other polyclonal anti-syntaxin-3 antibodies were used to confirm the immunocytochemical labeling observed. The first was a polyclonal antibody raised against the bacterially expressed syntaxin-3 holoprotein (26), kindly supplied by Dr. Mark Bennett (University of California, Berkeley, CA). The second was a site-directed antibody (L165) raised to a 21-amino acid peptide, CRLEQLKAKQPTQDDTDEVE, corresponding to amino acids 4–23 of the published sequence for syntaxin-3 (5). An amino-terminal cysteine was added to facilitate conjugation reactions as described above. Procedures for raising this antibody were the same as for L343 and L344 (above), except that the peptide was not HPLC purified prior to use for immunization of rabbits. This antibody was affinity purified on the same column used to purify L343 and L344. Polyclonal antibodies to syntaxin-4 (L279) (27) and aquaporin-2 (L127) (12, 32) were previously characterized.

Preparation of membrane fractions from rat tissues. Membrane fractions were prepared as described previously (27). Briefly, Sprague-Dawley rats were killed by decapitation, and tissues (whole kidney and brain cortex) were rapidly removed. Approximately 1 g of each tissue was homogenized using a tissue homogenizer (Omni 1000 fitted with a microsaw-tooth generator) in ice-cold isolation solution (250 mM sucrose, 10 mM triethanolamine) with added protease inhibitors, leupeptin (1 µg/ml; Bachem, Torrance, CA) and phenylmethylsulfonyl fluoride (0.1 mg/ml; U.S. Biochemical, Toledo, OH). The homogenates were initially spun at low speed (800 g) for 10 min to pellet incompletely homogenized fragments and nuclei. The supernatants were then spun at 17,000 g for 20 min in a Sorvall RC2-B centrifuge with an SS-34 rotor to obtain membrane fractions enriched in plasma membrane (28). The pellets were resuspended in isolation solution with protease inhibitors, and the total protein concentration was measured using the Pierce BCA Protein Assay Reagent Kit.

Preparation of inner medullary membranes. Inner medullary tissues were removed from kidneys of six Sprague-Dawley rats and were minced finely with a razor blade. The minced tissue was suspended in isolation solution with protease inhibitors and was homogenized finely as above. The homogenate was subjected to differential centrifugation as follows. An initial spin was carried out at 800 g to remove nuclei and incompletely homogenized cellular debris. The supernatant was then spun at 17,000 g for 20 min to yield a membrane fraction enriched in plasma membranes (13, 28). The 17,000 g supernatant was subjected to further high-speed centrifugation (200,000 g for 60 min in a Beckman L8-M ultracentrifuge with a Ti 100.5 rotor) to obtain a low-density microsome fraction enriched in intracellular vesicles (13, 28). The total protein concentration of all fractions were measured using the Pierce BCA Protein Assay Reagent Kit.

Electrophoresis and immunoblotting of membranes. The membrane fractions were solubilized at 60°C for 15 min in Laemml buffer, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on minigels of 12% polyacrylamide (Novex, San Diego, CA). The proteins were transferred from the gels electrophoretically to nitrocellulose membranes using a Bio-Rad transfer apparatus (Bio-Rad, Hercules, CA). After being blocked with 5% nonfat dry milk in wash buffer (150 mM NaCl, 50 mM NaH2PO4, 0.05% Tween 20, pH 7.5), the nitrocellulose membranes were probed with the affinity-purified polyclonal antibody to syntaxin-3 (L344) at an IgG concentration of 0.5 µg/ml in antibody dilution buffer solution (wash buffer containing 0.02% sodium azide and 0.1% BSA, pH 7.5). The secondary antibody was donkey anti-rabbit IgG conjugated to horseradish peroxidase (Pierce no. 31458) used at a concentration of 0.16 µg/ml in the milk block buffer described above. Sites of antigen-antibody reaction were visualized using luminol-based enhanced chemiluminescence (LumiGlo; Kirkegaard and Perry Laboratories, Gaithersburg, MD) before exposure to light-sensitive imaging film (Kodak no. 165–1579 Scientific Imaging Film).

**Immunocytochemistry**

Tissue preparation. Male Wistar rats, weighing 250–300 g, were obtained from Møllegaard Breeding Centre. The rats had free access to water and standard rat chow. Rats were anesthetized with intraperitoneal pentobarbital sodium, and kidneys were fixed by retrograde perfusion via the aorta with ice-cold fixative (4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4). The kidneys were then fixed by retrograde perfusion via the aorta with ice-cold fixative (4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4). The kidneys were then removed and processed for immunocytochemistry. Tissue blocks were prepared from inner medulla. The blocks were postfixed for 2 h in 4% paraformaldehyde and infiltrated with 2.3 M sucrose-2% paraformaldehyde for 30 min. The blocks were then mounted on holders and rapidly frozen in liquid nitrogen.

Sectioning and immunolabeling. Thin (0.85 µm) cryosections, cut on a Reichert Ultracut FCS cryoultramicrotome, were incubated with the affinity-purified antibodies against syntaxin-3 (L343, L344, L165, or the antibody against bacterially expressed syntaxin-3 described above). Additional sections were labeled with the affinity-purified antibody against syntaxin-4 or aquaporin-2 described above. The labeling was.
visualized using horseradish peroxidase-conjugated secondary antibodies (Dako P448, 1:100; Dako, Glostrup, Denmark). Controls using preimmune serum, nonimmune IgG, or omission of primary or secondary antibody revealed no labeling.

Immunoelectron Microscopy

Frozen tissue blocks were prepared for freeze substitution and embedding at low temperature in Lowicryl HM20 in a Reichert AFS (Reichert, Vienna, Austria) as previously described (34, 35). Briefly, the samples were sequentially equilibrated over 3 days in 0.5% uranyl acetate dissolved in anhydrous methanol at temperatures gradually increasing from −80 to −75°C and then rinsed in pure methanol for 24 h at −75 to −45°C. At −45°C, the samples were infiltrated in Lowicryl HM20 and methanol at 1:1, 2:1, and finally, pure HM20 before ultraviolet polymerization for 2 days at 45°C and 2 days at 0°C. Immunolabeling was performed essentially as described before (34, 36). Ultrathin sections (70–80 nm) were collected on 400 mesh, noncoated nickel grids. Sections were treated with a saturated solution of NaOH in absolute ethanol (for 2–3 s), rinsed, and preincubated in 0.1% sodium borohydride and 50 mM glycine in Tris-buffered saline containing 0.1% Triton X-100 (TBST) for 10 min at room temperature followed by blocking in 0.1% skimmed milk in TBST for 10 min. Sections were then incubated overnight at 4°C in affinity-purified anti-syntaxin-3 (L165) in TBST containing 0.1% skimmed milk. The labeling was visualized with goat anti-rabbit IgG conjugated to 10-nm colloidal gold particles (GAR.EM10, 1:50; BioCell Research Laboratories, Cardiff, UK). Sections were stained 10 min with uranyl acetate and 5 s in lead citrate and examined in a Philips CM100 electron microscope. The following controls confirmed specificity of immunolabeling: 1) incubation with nonimmune rabbit IgG and secondary antibody.

RESULTS

Syntaxin-2 and Syntaxin-3 mRNAs are Expressed in Kidney

To determine whether syntaxin-2 and -3 are expressed in the kidney at an mRNA level, we carried out RT-PCR amplifications using total RNA extracted from the major regions of kidney (cortex, outer medulla, and inner medulla) and compared the products to those obtained with total RNA extracted from heart, liver, lung, and cerebral cortex (Figs. 1 and 2). The results confirm that syntaxin-2 and syntaxin-3 are broadly expressed among nonneural tissues (5). Syntaxin-2 mRNA appears to be relatively abundant in heart, lung, and kidney (Fig. 1), whereas syntaxin-3 mRNA is comparatively abundant in lung and kidney (Fig. 2). Furthermore, mRNAs for both syntaxin-2 and -3 are relatively abundant in all three regions of the kidney (Figs. 1 and 2). In all cases, the amplification products were single bands corresponding to the predicted size of the target sequence.

Because mRNAs for both syntaxin-2 and -3 were found in the kidney, further RT-PCR experiments were carried out to determine whether they are expressed in collecting ducts at high levels relative to other renal structures (Figs. 3 and 4). To do this, microdissected collecting ducts, nephron segments and vascular structures from collagenase-digested rat kidney were trans-
limbs, distal convoluted tubules, connecting tubules, and cortical collecting ducts appear to display lower levels of syntaxin-3 mRNA. Little or no syntaxin-3 mRNA was found in proximal tubules.

To confirm the identity of the PCR products, they were digested with restriction endonucleases chosen to cleave the intended product at a single site. With each set of PCR products and restriction enzymes, appropriate-size restriction products were obtained (Fig. 5).

When the syntaxin-3 RT-PCR product from microdissected tubules was cloned and sequenced, the determined sequence was found to be 99% identical to the reported sequence for rat syntaxin-3 (5) (861 of 865 bases identical), thus confirming that the RT-PCR amplified syntaxin-3 mRNA and not some other similar mRNA.

Syntaxin-3 protein is expressed in renal collecting ducts. Based on the finding that syntaxin-3 is expressed in collecting ducts at an mRNA level, we carried out further studies using peptide-directed polyclonal antibodies to determine whether syntaxin-3 protein is detectable in collecting duct cells. Polyclonal antibodies were raised in rabbits against a 23-amino acid synthetic peptide corresponding to the carboxy terminus of syntaxin-3, a region that differs from that of the other known syntaxins (5). Figure 6 shows an immunoblot run with a crude membrane preparation from whole kidney (left) and cerebral cortex (right) and probed with affinity-purified anti-syntaxin-3 antibody. The antibody labeled a distinct 37-kDa band in both kidney and brain, consistent with the expected size of the syntaxin-3 protein. In addition, the antibody labeled a weaker 67-kDa band in both brain and kidney. This band was seen with all three peptide-directed antibodies. In contrast to crude membrane fractions, neither band was visible on immunoblots using whole homogenates of renal inner medulla (not shown), suggesting that the syntaxin-3 protein is of relatively low abundance.

Figure 7 shows results of a competition experiment in which the syntaxin-3 antibody was preadsorbed with 2 mg of the immunizing peptide and used to probe an immunoblot run using a crude membrane fraction from cerebral cortex. Preadsoption ablated both the 37- and 67-kDa bands, supporting the view that the 37-kDa band represents syntaxin-3 and that the 67-kDa band may contain syntaxin-3 (e.g., in an oligomeric complex). However, we cannot rule out that the 67-kDa band represents another protein that possesses the same epitope.

Figure 8 shows syntaxin-3 immunoblots displaying the results of differential centrifugation of a homogenate from rat inner medulla. The low-speed fraction (17,000 g pellet), which we have shown is enriched in collecting duct plasma membranes (13, 28), contained only the 37-kDa band, whereas the high-speed fraction (200,000 g pellet), which is enriched in intracellular vesicles (13, 28), showed exclusively the 67-kDa band. In addition, the 67-kDa band was detectable in the 200,000 g supernatant. Both the 37- and 67-kDa bands were absent when an identical blot was probed with preimmune IgG from the same rabbit. These findings show that, in inner medulla, the 37- and 67-kDa proteins are present in different cellular compartments.

Immunocytochemical localization. Figure 9 shows results from immunoperoxidase labeling of thin cryosections from the outer part of rat kidney inner medulla. All three peptide-derived syntaxin-3 antibodies labeled predominantly a minority cell type in the collecting duct (Fig. 9, a and b, arrowheads) that was identified as the type A intercalated cell (see below). Thin limbs and vascular structures were unlabeled. Within the intercalated cells, strong labeling was observed in the basolateral plasma membrane domains (arrows), and weaker
labeling of cytoplasmic components was seen (Fig. 9a). The apical region was unlabeled. Labeling with a polyclonal antibody raised against a bacterially expressed syntaxin-3 holoprotein (kindly provided by Dr. Mark Bennett) revealed the same labeling pattern (not shown). Controls using nonimmune IgG (Fig. 9d) gave no labeling. As illustrated in Fig. 9c, the antibody to aquaporin-2 strongly labels the majority cell type in the collecting duct, namely, the principal cells (32). Labeling of nearby thin sections with anti-syntaxin-3 (Fig. 9b) and anti-aquaporin-2 (Fig. 9c) demonstrated that the two antibodies labeled different cells, confirming that the syntaxin-3 labeling is in the intercalated cells. Immunocytochemistry using the anti-syntaxin-4 antibody revealed predominant labeling of the apical plasma membrane domain of collecting duct principal cells (Fig. 9e) consistent with previous observations (27). Intercalated cells were unlabeled with the syntaxin-4 antibody (not shown). Thus syntaxin-3 and syntaxin-4 were localized in different plasma membrane domains in different cells types of the initial part of the inner medullary collecting duct. A similar localization was found in the outer medullary collecting duct (not shown).

DISCUSSION

In a previous study (27), we demonstrated that the vesicle targeting protein, syntaxin-4, is expressed in the apical plasma membrane of collecting duct principal cells and concluded that this localization is consistent with a role for syntaxin-4 in the targeting of vesicles containing the water channel aquaporin-2 to...
apical plasma membrane in response to the antidiuretic hormone, vasopressin. In the present study, we sought to determine whether two other putative targeting proteins, syntaxin-2 and syntaxin-3, are expressed in the renal collecting duct. mRNA localization using RT-PCR in microdissected renal tubule segments demonstrated the presence of syntaxin-3 mRNA but not syntaxin-2 mRNA in collecting ducts. Direct sequencing of the RT-PCR product confirmed that the amplified product was derived from syntaxin-3 mRNA. The presence of syntaxin-3 protein in rat renal medulla was demonstrated by immunoblotting, and its presence in the collecting duct was demonstrated by immunocytochemistry. The localization of syntaxin-3 protein in the medullary collecting duct was found to differ from that previously determined for syntaxin-4 (27). First, syntaxin-3 was most abundant in type A intercalated cells rather than principal cells where syntaxin-4 is most abundant. Second, syntaxin-3 was located predominantly in the basolateral domain of these cells rather than the apical domain, as seen for syntaxin-4. This localization raises the possibility that syntaxin-3 may be involved in selective targeting of membrane proteins to the basolateral plasma membrane of type A intercalated cells. In the following, we discuss the physiological significance of these findings in greater detail in the context of the relevant literature.

Possible Role of Syntaxin-3 in Targeting of Acid-Base Transporters in Collecting Duct Type A Intercalated Cells

Type A intercalated cells play a vital role in the regulation of systemic acid-base balance by carrying out regulated acid secretion, a process critical to precise regulation of renal net acid excretion. Acid secretion by the type A cells is a consequence of vectorial transport across both the apical and basolateral plasma membranes. Carbonic acid formed by hydration of carbon dioxide dissociates to form protons that are secreted across the apical plasma membrane and bicarbonate, which is absorbed into the blood across the basolateral plasma membrane. Apical proton secretion is mediated by the action of a H+-ATPase localized to the apical plasma membrane (2, 7, 8). Basolateral bicarbonate transport is believed to be mediated by a chloride/bicarbonate exchanger AE-1 (2, 43), a slightly modified version of the band-3 protein of present in red blood cells. In the present study, we have demonstrated that syntaxin-3 is expressed at relatively high levels in type...
A intercalated cells with predominant localization in the basolateral plasma membrane, corresponding to the localization of AE-1 in these cells.

Whether syntaxin-3 is involved in regulation of AE-1 trafficking in type A intercalated cells is unknown. Previous studies in rabbits have demonstrated that prolonged systemic acid loading, a well-known stimulus for acid secretion by type A intercalated cells, is associated with redistribution of AE-1 from intracellular vesicles to the basolateral plasma membrane (42). This response was associated with proliferation of the basolateral plasma membrane surface area causing the type A cells to extend laterally in a stellate pattern. More recent studies have not demonstrated any apparent redistribution of AE-1 from intracellular vesicles to the basolateral plasma membrane (42). This response was associated with proliferation of the basolateral plasma membrane surface area causing the type A cells to extend laterally in a stellate pattern. More recent studies have not demonstrated any apparent redistribution of AE-1 in type A intercalated cells of the rat cortical collecting duct in response to acute systemic acid or alkali loading (38). Thus it is unclear whether regulation of AE-1-mediated transport of bicarbonate across the basolateral plasma membrane is dependent on regulated vesicular trafficking, particularly as a means of short-term adjustment of acid secretion in type A intercalated cells. Conceivably, however, syntaxin-3 plays a role in the maintenance of cell polarity in the type A intercalated cells, segregating

Fig. 5. Restriction endonuclease digestion of PCR products after amplification of target sequences for syntaxin-2 (A) and syntaxin-3 (B). Two micrograms of total RNA extracted from each renal region were loaded into individual PCR tubes. Amplifications were carried out using 3.5 mM Mg²⁺ with syntaxin-2 (A) or syntaxin-3 (B) specific primers (see METHODS). After RT-PCR was run for 31 cycles, each product was split into 2 aliquots. One aliquot was incubated with the appropriate restriction enzyme (BamHI for syntaxin-2, BstXI for syntaxin-3). Products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide as described in METHODS. For each region, left lane represents product of the restriction enzyme digestion, and right lane shows the original RT-PCR product. Predicted fragment sizes are 1) BamHI digestion of syntaxin-2 RT-PCR product, 591 and 225 bp; and 2) BstXI digestion of syntaxin-3 RT-PCR product, 618 and 247 bp. OM, outer medulla; IM, inner medulla.

Fig. 6. Immunoblots of crude membrane fractions of whole kidney and brain probed with the anti-syntaxin-3 antibody (L344). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was run using 12% polyacrylamide gels (10 µg protein/lane), and separated proteins were then transferred to nitrocellulose membranes. Blots were probed with affinity-purified anti-syntaxin-3 antibody at immunoglobulin G (IgG) concentration of 0.50 µg/ml. As described in RESULTS, antibody labeled distinct 37- and 67-kDa proteins in both brain and kidney membrane preparations.

Fig. 7. Preadsorption control for anti-syntaxin-3 antibody. Ten micrograms of crude membrane fraction from cerebral cortex were loaded in each lane. SDS-PAGE was run using 12% polyacrylamide gels, and separated proteins were then transferred to nitrocellulose membranes. Blot was split in half. One-half (left) was probed with the affinity-purified anti-syntaxin-3 antibody (L344, 0.5 µg/ml IgG) preincubated with 2 mg of immunizing peptide linked to SulfoLink (Pierce) beads (see METHODS). Other was probed with the affinity-purified anti-syntaxin-3 antibody at same IgG concentration preincubated with cysteine-blocked SulfoLink beads (right). Both the 37- and 67-kDa bands were ablated by peptide preadsorption of the antibody.
rule out the possibility that syntaxin-2 protein is
occurs chiefly in the glomerulus. However, we cannot
suggest that renal expression of syntaxin-2 (5) and therefore is unlikely to play a targeting role in
the central nervous system and in neuroendocrine cells
forms. Syntaxin-1 is believed to be expressed only in
only syntaxins-1 through -4 are plasma membrane
mammalian syntaxins that have been described,
other syntaxins may be expressed at these sites. Among
basolateral domain of principal cells suggests that
distribution of syntaxins in type A and type B cells.
Further investigations will be required
involving an inversion of the polarity of the interca-
temic acid-base state through a remodeling process
cells can interconvert in response to changes in sys-
known v-SNAREs, i.e., VAMP1, VAMP2, or cellubrevin,
may be present in type A intercalated cells has not been
investigated.

Role of Syntaxins in Water Channel Trafficking

The present study confirmed the presence of syn-
taxin-4 in the apical plasma membrane of collecting
duct principal cells (27) (Fig. 9e). Furthermore, we
previously documented that the v-SNARE protein
VAMP2 is present in substantial amounts in aquaporin-
containing vesicles (33). VAMP2 has also been demon-
strated in renal medullary vesicles by other investiga-
tors (24, 25). These findings led us to the proposal that
binding of the VAMP-2 present in aquaporin-2 vesicles
and syntaxin-4 present in the apical plasma membrane
is responsible for the selective targeting of aquaporin-2
to the apical plasma membrane. The present finding
that syntaxin-3 is expressed predominantly in type A
intercalated cells, a cell type that does not express any
of the known aquaporins, suggests that syntaxin-3 is
not involved in water channel trafficking. However, we
cannot rule out the possibility that syntaxin-3 is ex-
pressed in principal cells at low levels that may not be
detectable by immunoperoxidase labeling.

Subcellular Distribution of Syntaxin-3

Immunoblotting using the anti-syntaxin-3 antibody
demonstrated that the antibody recognizes a protein
with an apparent molecular mass of 37 kDa, which is
present in a relatively high-density membrane fraction
prepared from renal inner medullary homogenates
(Fig. 8). This molecular mass is compatible with the
expected molecular mass of the syntaxins (5). Previ-
ously, we have demonstrated that most of the collecting
duct plasma membranes are found in this “low-speed
fraction” pelleted at 17,000 g, i.e., the same fraction
that contains the 37-kDa syntaxin-3 protein. Thus the
differential centrifugation results are compatible with
localization of syntaxin-3 in plasma membranes. This
was corroborated by immuneelectron microscopy (Fig.
10). It appears possible that syntaxin-3 is not limited to
the plasma membrane. Indeed, immunocytochemical
labeling of collecting ducts suggested that, in addition
to the basolateral plasma membrane of intercalated
cells, syntaxin-3 may be present in intracytoplasmic
structures (Figs. 9, a and b, and 10).

In addition to the 37-kDa band, the anti-syntaxin-3
antibody also labeled a 67-kDa band present in the

AE-1 and other basolaterally expressed proteins in the
basolateral domain as a result of targeting of constitu-
tive trafficking processes.

Syntaxins as a Possible Determinant of Epithelial
Cell Polarity

Despite some recent progress, mechanisms by which
cell polarity in epithelial cells is maintained are incom-
pletely understood (15, 29). It appears possible that the
syntaxins, which have been proposed to be a determi-
ant in specific targeting of membrane trafficking in
neurons (3), play a role in maintenance of epithelial cell
polarity. Recently, it has been proposed that type B
type B (bicarbonate-secreting) intercalated cells and type A
cells can interconvert in response to changes in sys-
temic acid-base state through a remodeling process
involving an inversion of the polarity of the interca-
tated cells (1). Further investigations will be required
to determine what syntaxins are expressed in type B
cells and whether changes in acid-base state alter the
distribution of syntaxins in type A and type B cells.
The absence of either syntaxin-3 or syntaxin-4 in the
apical plasma domain of type A intercalated cells or the
basolateral domain of principal cells suggests that
other syntaxins may be expressed at these sites. Among
the mammalian syntaxins that have been described,
only syntaxins-1 through -4 are plasma membrane
forms. Syntaxin-1 is believed to be expressed only in
the central nervous system and in neuroendocrine cells
and therefore is unlikely to play a targeting role in
the kidney. RT-PCR experiments in the present study
suggest that renal expression of syntaxin-2 occurs chiefly in the glomerulus. However, we cannot
rule out the possibility that syntaxin-2 protein is
present in kidney epithelial cells. Indeed, it has re-
cently been reported that syntaxin-2 is endogenously
expressed in both cultured Madin-Darby canine kidney
epithelial cells (26) and in rat pancreatic acinar cells
(18). It appears likely that additional plasma mem-
brane syntaxins will be identified in the future, which
may be expressed in collecting duct cells.

The presence of syntaxin-3 in type A intercalated
cells suggests that cognate receptor proteins, i.e., synap-
totagmins (VAMPs) must be expressed in these cells to
mediate docking of vesicles to specific domains defined
by polarized syntaxin expression. Whether any of the
known v-SNAREs, i.e., VAMP1, VAMP2, or cellubrevin,
may be present in type A intercalated cells has not been
investigated.

Fig. 8. Distribution of 37- and 67-kDa bands in subcellular fractions
of inner medulla obtained by differential centrifugation of inner
medullary homogenate. Ten micrograms total protein from each
sample were electrophoresed and transferred to nitrocellulose mem-
branes as described in METHODS. Blots were probed with either
affinity-purified anti-syntaxin-3 antibody (0.50 µg/ml, A) or with
protein A-purified preimmune IgG fraction (0.50 µg/ml, B). As shown,
37-kDa protein was found only in the low-speed (plasma membrane
enriched) fraction, whereas high-speed fraction (enriched in intracell-
ular vesicles) contained only the 67-kDa protein. The 67-kDa band
was also seen in the supernatant from the high-speed (200,000 g)
centrifugation. Both bands were absent in blot probed with preim-
minute IgG, although a nonspecific band at 36 kDa appears in
supernatant lane with preimmune IgG.
low-density membrane fraction (200,000 g pellet) from renal inner medulla and in the 200,000 g supernatant. This band was ablated by preadsorption of the antibody with the immunizing peptide and was absent when blots were probed with the IgG fraction of the preimmune serum, suggesting that the labeling is specific. It is conceivable that this 67-kDa band may represent a unique syntaxin-3-like protein or, alternatively, could be an unrelated protein that shares an epitope with the amino-terminal region of syntaxin-3. However, another possibility is that the 67-kDa band represents an SDS-stable protein complex containing syntaxin-3. Indeed, the syntaxins are known to bind with high affinity to a number of proteins including VAMPs (mol mass, 14–17 kDa) (9, 40), SNAP-25 (mol mass, 25 kDa) (40), and mammalian homologs to yeast Sec1 (mol mass, 10.220.33.6 on April 14, 2017 http://ajprenal.physiology.org/)
mass, 67 kDa) (19, 21, 37). It is possible that the 67-kDa band represents a naturally occurring complex that has an important regulatory role, e.g., blockade of the ability of syntaxin-3 to bind to its cognate v-SNARE when it is retrieved from the plasma membrane by endocytosis. Additional studies are needed to address this possibility. It is interesting that in our previous study localizing syntaxin-4 in the kidney (27), we found...
Differently Among Renal Structures

Although the chief purpose of this study was to determine which syntaxins are expressed in the renal collecting duct and hence which syntaxins could play a role in regulation of water and acid-base transport in the collecting duct, a number of other structures were surveyed for expression of syntaxin mRNAs in part to provide positive controls ensuring that the RT-PCR method could successfully detect the respective mRNAs. The pattern of syntaxin expression is nonetheless of interest because it may point to other potential roles of syntaxins in renal structures other than the collecting ducts. Interestingly, syntaxin-2 mRNA was localized chiefly to the glomeruli in the renal cortex (Fig. 3). Because the glomeruli are made up of a number of cellular components, it is impossible to guess what role syntaxin-2 could play. One possibility is that it may be involved in the regulated exocytosis responsible for renin secretion by the intraglomerular portion of the afferent arteriole. Although syntaxin-2 mRNA was detectable in samples of total RNA extracted from the outer and inner medullary regions, our microdissection experiments did not identify which structures were responsible for the positive signal in the medulla. This result suggests that cell types that are not obtainable via microdissection, e.g., capillary endothelium, fibroblasts, or interstitial cells, could be responsible for the presence of syntaxin-2 mRNA in the renal medulla.

In contrast to syntaxin-2, the distribution of syntaxin-3 mRNAs was quite broad. Using RT-PCR, we demonstrated that syntaxin-3 mRNA is detectable in several nephron segments and in the collecting duct system. Greatest expression, however, was seen in inner medullary collecting ducts, medullary thick ascending limbs, and outer medullary vasa recta. Among the three syntaxins surveyed in this study and our previous one (27), the syntaxin with the broadest mRNA expression was syntaxin-4, found in substantial quantities in glomeruli, vasa recta, all loop of Henle segments, the connecting tubule, and all collecting duct segments. Further studies will be needed to determine the role of syntaxins in structures other than the collecting duct.

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