The UT-A1 Urea Transporter Interacts with Snapin, a SNARE-associated Protein*

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The UT-A1 urea transporter mediates rapid transepithelial urea transport across the inner medullary collecting duct and plays a major role in the urinary concentrating mechanism. To transport urea, UT-A1 must be present in the plasma membrane. The purpose of this study was to screen for UT-A1-interacting proteins and to study the interactions of one of the identified potential binding partners with UT-A1. Using a yeast two-hybrid screen of a human kidney cDNA library with the UT-A1 intracellular loop (residues 409–594) as bait, we identified snapin, a ubiquitously expressed SNARE-associated protein, as a novel UT-A1 binding partner. Deletion analysis indicated that the C-terminal coiled-coil domain (H2) of snapin is required for UT-A1 interaction. Snapin binds to the intracellular loop of UT-A1 but not to the N- or C-terminal fragments. Glutathione S-transferase pulldown experiments and co-immunoprecipitation studies verified that snapin interacts with native UT-A1, SNAP23, and syntaxin-4 (t-SNARE partners), indicating that UT-A1 participates with the SNARE machinery in rat kidney inner medulla. Confocal microscopic analysis of immunofluorescent UT-A1 and snapin showed co-localization in both the cytoplasm and in the plasma membrane. When we co-injected UT-A1 with snapin cRNA in Xenopus oocytes, urea influx was significantly increased. In the absence of snapin, the influx was decreased when UT-A1 was combined with t-SNARE components syntaxin-4 and SNAP23. We conclude that UT-A1 may be linked to the SNARE machinery via snapin and that this interaction may be functionally and physiologically important for urea transport.

In mammals, urea transporters play an integral role in the urinary-concentrating mechanism and hence in mediating transepithelial urea movement across the cell membrane of the terminal inner medullary collecting duct (IMCD)² in kidney nephrons. In the past few years, significant knowledge has been gained about the physiological role and regulation of urea transporters, which have now been cloned from many species (1). At least three subfamilies have been reported, two of them are mammalian-specific (renal tubular-type UT-A and erythrocyte vascular-type UT-B), and the third is a fish-specific (renal proximal tubular-type UT-C) urea transporter (1–3). The UT-A transporter is encoded by the Slc14A2 gene and includes six protein and nine cDNA isoforms (1). UT-A1 is the largest protein isoform and is expressed in the apical plasma membrane of the IMCD (3). Arginine vasopressin (also known as antidiuretic hormone) is the key hormonal regulator of UT-A1 transport activity in vivo. Vasopressin increases urea transport by stimulating UT-A1 accumulation in the plasma membrane and by increasing UT-A1 phosphorylation (4).

UT-A1 accumulation in the plasma membrane is a key step for the regulation of urea transport, but the mechanisms by which UT-A1 is delivered to the plasma membrane are less clearly defined. Several SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor)-mediated accessory proteins have been implicated in the vesicular trafficking of several proteins (5–9), but whether they are involved in UT-A1 trafficking has not been studied. SNARE proteins are expressed in collecting duct principal cells, including those in the IMCD, and localize with aquaporin-2-bearing vesicles (10). In particular, syntaxin 4 is present in the apical plasma membrane of collecting duct principal cells (11). SNAP23 has also been found in collecting duct principal cells both in the apical plasma membrane and in aquaporin-2-bearing vesicles (12). SNAP23 interacts with syntaxin 4 and VAMP2 (13) in SNARE complexes. Thus, several SNARE proteins are present in the same kidney cells as those that express UT-A1 and UT-A3.

Membrane trafficking or fusion is dependent on the SNARE family of proteins, which are implicated as the conserved core protein machinery for all intracellular membrane fusion events from yeast to humans (14). This fusion machinery consists of integral membrane proteins on the vesicle (v-SNAREs) and on the target (t-SNAREs) membranes that are thought to form a stable core complex in the process of vesicle docking. The SNARE; VAMP2, vesicle-associated membrane protein 2; SNAP25, 25kDa synaptosome-associated protein; PKA, protein kinase A; MDCK, MadinDarby canine kidney cell; Ad, adenovirus; CMV, cytomegalovirus; GFP, green fluorescent protein.

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‡2 The abbreviations used are: IMCD, inner medullary collecting duct; GST, glutathione-S-transferase; PBS, fetal bovine serum; FSK, forskolin; PBS, phosphate-buffered saline; pfu, plaque-forming units; SNARE, soluble NSF attachment protein receptors; v-SNARE, vesicle SNARE; t-SNARE, target SNARE; VAMP2, vesicle-associated membrane protein 2; SNAP25, 25kDa synaptosome-associated protein; PKA, protein kinase A; MDCK, Madin-Darby canine kidney cell; Ad, adenovirus; CMV, cytomegalovirus; GFP, green fluorescent protein.
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mammalian v-SNARE family consists of nine members, with vesicle-associated membrane protein 2 (VAMP2) being the dominant isoform in the brain (14, 15). The t-SNARE machinery has more complex composition, with two or three components being required in different membrane-trafficking pathways (16, 17). The syntaxin family of t-SNAREs consists of at least 12 gene products in animal cells that are localized to compartments throughout the secretory and endocytic pathways, indicating that these proteins mediate various membrane-trafficking pathways (16). The three additional t-SNAREs, SNAP25 (25 kDa synaptosome-associated protein) (18), SNAP23 (13), and SNAP29 (19), interact with syntaxin isoforms through combinatorial principles that are not yet fully understood. The SNARE complexes generally consist of four coiled-coil domains contributed by SNAREs residing in the t-SNAREs and v-SNAREs. Recent experiments have shown that only matching combinations of v- and t-SNAREs lead to successful fusion (20, 21), indicating that SNAREs not only mechanistically accomplish membrane fusion but also contribute to the specificity of this process.

The membrane fusion event is regulated by several accessory proteins that bind to individual SNAREs and/or channel and transporter proteins. One such protein is snapin, a SNARE-associated protein (15 kDa) that was previously characterized as a binding partner of SNAP25 and implicated in the regulation of membrane fusion events (22, 23). Although it was originally described as a brain-specific, integral membrane protein (23), it was subsequently found to be ubiquitously expressed. It is able to associate with membranes and interact with SNAP23 (22), a ubiquitous paralog of SNAP25. Snapin interacts with the assembled SNARE complex as well as isolated SNAP25 through its C-terminal coiled-coil domain. The role of snapin as a modulatory protein in exocytosis may be controlled by second messenger pathways. It was suggested that cAMP-dependent protein kinase A (PKA) phosphorylation of snapin at Ser-50 enhances its interaction with SNAP25 (24). In addition, snapin was shown to have a broad tissue distribution and to be expressed in both cytoplasmic and peripheral membrane-associated fractions (7, 22). Biochemical evidence indicated that snapin also interacts with SNAP23, suggesting that snapin might have a general role in SNARE-mediated fusion events (22). The protein has attracted wide interest because it increases binding of synaptotagmin to SNAREs, which is further enhanced upon phosphorylation of snapin (24).

In this study, we identified snapin as a UT-A1-binding protein using yeast two-hybrid genetic screening of a human kidney cDNA library. We then analyzed the interaction between UT-A1 and snapin in vitro and in vivo in rat kidney inner medulla and in UT-A1-MDCK cells. GST pulldown experiments demonstrate that snapin interacts with native UT-A1 and t-SNARE partners (SNAP23 and syntaxin-4) indicating UT-A1 participation in a SNARE complex in the IMCD. We also demonstrate a functional link between snapin and UT-A1 with t-SNARE in cRNA-injected Xenopus oocytes. We conclude that UT-A1 may be linked to the SNARE-mediated fusion machinery indirectly, most likely by interacting with snapin, and that this interaction may be important functionally for urea transport.

EXPERIMENTAL PROCEDURES

Antibodies—We used our rabbit polyclonal antibody to the C terminus of the rat renal urea transporter UT-A1 (25). Rabbit anti-snapin antibody was purchased from Synaptic Systems (Göttingen, Germany). Rabbit polyclonal anti-syntaxin 3, mouse monoclonal anti-actin, anti-GST and anti-His6, antibodies, and nonspecific rabbit serum (as control) were from Sigma-Aldrich. Mouse monoclonal anti-syntaxin 4, anti-Myc, and rabbit polyclonal anti-HA antibodies were purchased from BD Biosciences (Franklin Lakes, NJ). Rabbit polyclonal anti-SNAP-23 and VAMP2 were the kind gifts of Dr. Mark Knepper (Laboratory of Kidney and Electrolyte metabolism, NHLBI, National Institutes of Health (NIH)).

Plasmid Construction—Three intracellular fragments of human UT-A1, 1) N-terminal fragment, N-UT (residues 1–126), 2) long loop, L-UT (residues 409–594), and 3) C-terminal fragment, C-UT (residues 873–920), were amplified by PCR and cloned into pGBK17 bait vector (BD Biosciences Clontech, Palo Alto, CA) for yeast two-hybrid assay. The full-length human snapin cDNA was isolated from a human kidney cDNA library (BD Biosciences Clontech). The coding region (411 bp) of human snapin was amplified by PCR using sequence-specific primer pairs and subcloned into the multiple cloning sites of oocyte expression vector pGHI9 and various other expression vectors (e.g. pGEX-4T-snapin, pRSET-A-snapin, pEGFEP-snapin, pDsRed-Monomer-snapin, pShuttle-CMV-snapin, and pAdEasy-snapin). Full-length syntaxin 4 and SNAP-23 were cloned into the oocyte expression vector pGHI9. Snapin deletion variants and SNAP-23 and SNAP-29 were constructed in the pGEX vectors, and snapin-S50D and -S50A mutants, syntaxin 4, SNAP-23, and VAMP2 were amplified and subcloned into the pGEX-4T-2 vector (kindly provided by Dr. Z. H. Sheng, NIH). The insert sequences and reading frames were verified by sequencing.

Yeast Two-hybrid Experiments and cDNA Library Screen—Yeast two-hybrid genetic screening was performed using the MATCHMAKER GAL4-based two-hybrid system (BD Biosciences Clontech) as described by the manufacturer. The loop-bait plasmid, pGBK17-L-UT, was sequentially transformed into yeast Saccharomyces cerevisiae strain AH109, and the resulting yeast transformant was allowed to mate with S. cerevisiae strain Y187 pretransformed with the human kidney cDNA library fused to the pACT2 vector containing GAL4 activation domain. The resulting diploid cells were selected on SD plates lacking tryptophan (-Trp), leucine (-Leu), histidine (-His), and adenine (-Ade) in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Glycosynth) selection and incubated for 1 week at 30 °C. Yeast two-hybrid assays aimed at testing specific interaction pairs were carried out by double transformation of haploid S. cerevisiae strain AH109. The positive blue phenotype colonies were picked up, and the prey plasmids containing library cDNA inserts were rescued and confirmed by retransformation into fresh yeast cells with our various constructed baits or control baits. The prey plasmids were subjected to DNA sequencing and analyzed by BLAST search.

In Vitro Protein Expression and Binding Assays—The various bait genes in pGBK17 were tagged with c-Myc and contained...
T7 promoters. The target-gene, snapin, in pACT2, was obtained from cDNA library screening and was tagged with HA. [35S]Methionine-labeled recombinant fusion proteins were generated by using the TnT®-T7-coupled rabbit reticulocyte lysate system (Promega, Madison, WI). To generate [35S]Met-labeled bait and target proteins, the reactions were carried out separately in 50-μl volumes. 1 μg of plasmid DNA (each) and 10 μCi/μl [35S]-labeled methionine (Amersham Biosciences) were added to the TnT reaction mix, incubated at 30 °C in a water bath for 90 min, and terminated by placing on ice. For binding assays, in vitro translated [35S]Met-labeled baits and library proteins (10 μl each) were combined with gentle mixing and incubated at room temperature for 1 h. The recombinant protein complexes were next incubated with anti-Myc antibody or anti-HA antibody, respectively, for an additional 1 h, followed by addition of 4 μl of protein-A-Sepharose beads (BD Biosciences) to the reaction tube and incubation at 4 °C for an additional 2 h with gentle agitation. The immunoprecipitated protein complexes were extensively washed, and the proteins were resolved on 4–15% SDS-polyacrylamide gels. After protein fixation, the gels were incubated with NAMP100 (Amersham Biosciences, UK) for 30 min to enhance the signal of [35S]-labeled proteins. Gels were dried and autoradiographed. For the interaction between HA-snapin and native UT-A1 proteins, recombinant in vitro translated [35S]-labeled HA-snapin (4 μg) was first immobilized on protein-A beads (4 μl) as described above and then incubated with the rat kidney IMCD tissue lysate, solubilized in binding buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 50 mM KCl, 1 mM EDTA, 0.5% Triton X-1, 1% protease inhibitor mixture from Sigma), for 4 h at 4 °C. The snapin-bound protein complexes were extensively washed with binding buffer and resolved by 4–15% SDS-PAGE gels. The proteins were transferred to polyvinylidene difluoride membranes and analyzed by Western blot analysis using anti-UT-A1 antibody (1:6000 dilution) and anti-snapin (1:5000 dilution), respectively. The amount of membrane-bound [35S]-labeled snapin was detected by autoradiography.

Cell Culture and Transfection—The UT-A1-MDCK cells, which express UT-A1 by functional assay and Western blot analysis, were generated as described previously (26). The UT-A1-MDCK and human embryonic kidney HEK-293 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO2. UT-A1-MDCK cells were transfected with pEGFP-N3-snapin, pDsRed-Monomer-N1-snapin, pcDNA3-snapin, or pQE32-His6-snapin using Lipofectamine® 2000 (Invitrogen) as described by the manufacturer.

Generation of Recombinant Adenovirus (Ad-snapin) and Infection in UT-A1-MDCK Cells—Recombinant adenoviruses were constructed using the Ad-Easy-1 system through multiple rounds of subcloning of PCR products or of restriction endonuclease fragments. Full-length snapin was first cloned into the pShuttle-CMV vector, and the resultant plasmid was linearized by digesting with restriction endonuclease Pmel, and subsequently cotransformed into Escherichia coli BJ5183 cells with an adenoviral backbone plasmid pAdEasy-1. Recombinants were selected for kanamycin resistance, and recombination was confirmed by multiple restriction endonuclease analyses. Finally, the recombinant adenoviral vectors were linearized with PacI and used to infect HEK-AD293 cells (Stratagene). Recombinant adenoviruses typically are generated within 7–10 days. Following large scale infection of human embryonic kidney HEK-AD293 cells cultures, Ad-snapin was purified with two consecutive CsCl ultracentrifugation steps and dialysis, then titrated using Adeno-X™ rapid titer kit (Clontech). Usually, titers of ~0.6 × 10^9 plaque-forming units (pfu)/ml were obtained. UT-A1-MDCK cells were grown to 90% confluence in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum as above. For infection, cells were incubated with serum-free medium containing various concentrations of 10–60 pfu/cell for 2–6 h, washed twice with phosphate-buffered saline (PBS), and further cultured in fresh medium containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, in 5% CO2/95% air at 37 °C for 18–20 h to allow transgene expression of recombinant protein. Ad-GFP was used as a control adenovirus.

Co-immunoprecipitation and Western Blotting—Adenovirus-infected snapin overexpressing UT-A1-MDCK cells were washed with cold PBS containing 1 mM EDTA and then scraped into 5 ml of lysis buffer (50 mM Tris, pH 7.4, 50 mM NaCl, 0.5% Triton X-100, and 1% protease inhibitor mixture). Cells were homogenized with a glass Teflon homogenizer (10 strokes at 900 rpm) and lysed by passing the extract through a 25-gauge needle five times. The extract was centrifuged at 12,000 × g for 30 min at 4 °C to remove insoluble material, and the supernatants were solubilized in binding buffer (50 mM Tris, pH 7.4, 50 mM NaCl, 0.1% bovine serum albumin, and 0.1% Triton X-100) and centrifuged at 20,000 × g for 30 min at 4 °C. Finally, the resultant supernatant was used to immunoprecipitate with indicated various antibodies (anti-snapin, anti-UT-A1, or rabbit IgG) at 4 °C for 2 h with continuous mixing. Protein-A beads were added, and incubation was continued for an additional 2 h. Subsequently, the antibody-protein complexes were immobilized on protein-A beads and washed five times with binding buffer. Immunoprecipitated proteins were resuspended in SDS-PAGE loading buffer (60 mM Tris, 2% SDS, 10% glycerol, 5 mM EDTA, 2% β-mercaptoethanol, 0.01% bromphenol blue, pH 6.8), heated at 85 °C for 5 min, and separated on a 4–15% SDS-PAGE polyacrylamide gel. Following transfer to polyvinylidene difluoride membranes, bound proteins were identified by immunoblotting. Experiments were performed in duplicate or triplicate.

Preparation of GST Fusion Proteins and Pulldown Assays—The full-length open reading frame encoding snapin was cloned into GST fusion vector pGEX-4T-2 and then expressed in E. coli BL21-Codon plus-(DE3) (Stratagene) cells, followed by induction with 1 mM isopropyl β-D-thiogalactopyranoside, essentially as described previously (2). Bacterial pellets were sonicated and lysed in binding buffer (20 mM HEPES, pH 7.4, 140 mM KCl, 20 mM NaCl, 0.5% Triton X-100). GST-snapin fusion proteins were immobilized on glutathione-Sepharose (Amersham Biosciences) and washed twice with binding buffer. Quantity and quality of GST-fusion protein was checked by Bradford protein assay or by SDS-PAGE analysis using Coomassie Brilliant Blue staining. Rat kidney inner medulla tissue
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(300 μg) was homogenized in the lysis/binding buffer containing 1% Triton X-100 with a glass Teflon homogenizer (12 strokes at 900 rpm), sheared by passing the extract through a 25-gauge needle (5 times), and centrifuged at 12,000 × g for 30 min. The supernatants were resuspended in the same binding buffer containing 0.5% Triton X-100 under glyceration overnight and centrifuged at 30,000 × g for 30 min at 4 °C. The resultant supernatant was incubated with the immobilized GST-snapin resin. After extensive washing, the bound protein complexes were resolved on a 4–15% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The blots were probed with the indicated antibodies.

Functional Analysis in X. laevis Oocytes—Functional characterization of UT-A1 with SNARE proteins was determined by measuring urea uptake in Xenopus laevis oocytes using the urea uptake procedure described previously (2). Briefly, the full-length coding region of UT-A1, snapin, syntaxin-4, and SNAP23 were subcloned into oocyte expression vector pGH19. After linearization, cRNAs were synthesized by T7 polymerase using mMessage mMACHINE T7 Ultra kit (Ambion, Austin, TX) and injected into collagenase-treated oocytes (2 ng/oocyte). Stage V–VI oocytes were used for cRNA injection, obtained from two to three animals for each experimental series. Two nanograms of each cRNA in 23 nl of water were injected into each oocyte. Control oocytes were injected with a similar volume (23 nl) of water. Injected oocytes were maintained in OR3 medium for 2–3 days at 18 °C. Four days later, healthy oocytes were selected for urea influx measurements. For the urea uptake assay, oocytes were preincubated with 1 ml of uptake solution (200 mM mannitol, 2 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM HEPES buffer, 5 mM Tris-HCl, pH 7.4) for 1 h. Cells were then moved to incubate at different time points with 2 μCi of [14C]urea/ml and 1 mM cold urea in uptake solution. Cells were washed four times with ice-cold uptake solution containing 1 mM cold urea. Each individual cell was dissolved in 10% SDS, followed by scintillation counting. Results shown in the bar graphs represent means ± S.D. for 12–18 oocytes in each group. Statistical significance was determined by unpaired t-tests, and differences were judged significant when p < 0.05.

Confocal Immunofluorescence Microscopy—A 0.41-kb BamHI–EcoRI fragment containing the complete coding region of the snapin cDNA was subcloned into pDsRed Monomer N1 and named pDsRed-snapin. UT-A1–MDCK cells (0.7–0.9 × 106) were plated on 35-mm plates and transiently cotransfected with 1.5 μg of pDsRed-snapin or pDsRed Monomer N1 vector using the Lipofectamine™2000 reagent. Cells were grown on Transwell filters to confluence. Before immunostaining, cells were rinsed twice with PBS, fixed with 4% formalin in PBS for 10–15 min at 4 °C, washed with PBS three times, permeabilized in PBS containing 0.1% Triton X-100 for 10 min, and blocked with 1% bovine serum albumin in PBS (blocking buffer) for 30 min at 25 °C (27). Cells were then incubated with primary antibody, anti-UT-A1 (C-terminal), diluted 1:4000 in blocking buffer for 1 h at room temperature. Cells were washed with blocking buffer several times to remove unbound antibody and then incubated with secondary antibody, goat anti-rabbit IgG-fluorescein isothiocyanate (Sigma), diluted 1:250 in blocking buffer for an additional 1 h. To remove unbound secondary antibody, cells were washed three more times with blocking buffer and one time with deionized water. The cells were mounted between slide and coverslip using Vectashield mounting media and sealed with nail polish. Fluorescence was detected using a Zeiss Axioplan fluorescence microscope equipped with a confocal laser-scanner unit CSU10 (Yokogawa Electronic, Tokyo, Japan). Images were obtained with a high resolution digital charge-coupled device camera (C4742-95, Hamamatsu Photonics) and processed by using a Zeiss LSM5 image software browser (LSM 510 META; Carl Zeiss microimaging, Inc., Thornwood, N.Y.). The brightness and contrast of the final images were adjusted with Photoshop (Adobe Systems).

Animal Preparation and Tissue Collection—All animal protocols were approved by the Emory University Institutional Animal Care and Use Committee. Male Sprague-Dawley rats weighing 250–350 g were kept in cages with autoclaved bedding and received free access to water and a standard diet (Diet 5001, Purina). Immediately after the rats were killed, their kidneys were dissected to separate the cortex, outer medulla, and inner medulla. These tissues were placed into an ice-cold isolation buffer (10 mM triethanolamine, 250 mM sucrose (pH 7.6), 1 μg/ml leupeptin, and 2 mg/ml phenylmethylsulfonyl fluoride), homogenized, SDS was added to a final concentration of 1%, and samples were sheared with a 25-gauge needle. Total protein concentration in each sample was measured by a modified Lowry assay (DC Protein Assay Kit, Bio-Rad).

Plasma Membrane Isolation—Plasma membrane isolation of UT-A1–MDCK cells was performed as described previously (27) with some modification. Ad-snapin-infected UT-A1–MDCK confluent cells (in 6-well plates) were treated with 10 μM forskolin (FSK, Sigma) for 20 min and then frozen at −80 °C for 1 h. The cell pellets were collected and resuspended in ice-cold homogenization buffer (250 mM sucrose, 2 mM EDTA, 10 mM Tris, pH 7.4) containing protease inhibitor mixture (Sigma) at 4 °C. Cells were disrupted via Dounce homogenization, and the cell mixture was centrifuged for 15 min at 700 × g to remove intact cells and debris. Plasma membranes were separated by loading supernatant on a five-step sucrose gradient (2.0, 1.6, 1.4, 1.2, and 0.8 M sucrose) and ultracentrifuged in an SW-28 rotor at 20,000 × g for 4 h at 4 °C. After centrifugation, plasma membranes were collected from the 1.2/0.8 M density interface and diluted (1:3) to 25 ml in homogenization buffer for further ultracentrifugation with an SW-50.1 rotor at 25,000 × g for 6 h. The pellets were resuspended in homogenization buffer containing protease inhibitor mixture and stored at 4 °C for short-term or at −20 °C for long term storage (>1 month). Total protein concentration in each sample was measured as above and adjusted to ∼1 μg/μl with the same homogenization buffer as for Western blot analysis.

Cell Surface Biotinylation—After FSK stimulation as described above, cells were placed on ice to minimize protein trafficking and endocytosis and washed twice with ice-cold phosphate-buffered saline supplemented with 1 mM MgCl2 and 1 mM CaCl2 (PBS-CM, pH 8.0). UT-A1–MDCK cell surface protein biotinylation was done as described previously (27) with some modification. Briefly, cells were labeled twice with freshly prepared 0.5 mg/ml EZ-Link™sulfo-N-hydroxysuccinimide.
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RESULTS

Snapin Specifically Interacts with the Intracellular Loop Domain of UT-A1—The UT-A1 protein has three long intracellular domains, based upon topology (Kyte-Doolittle algorithm): the N terminus (residues 1–126), a long loop region (residues 409–594), and the C terminus (residues 873–920), which we have designated as N-UT, L-UT, and C-UT, respectively (Fig. 1A). To assess the physiological role of the UT-A1-encoded gene product, we screened a human kidney cDNA library for potential intracellular interaction partners using a yeast two-hybrid assay, with L-UT as the bait. Approximately 10^6 independent clones were screened, and 12 potential L-UT-interacting candidate clones were identified, including snapin. The strongest induction of the α-galactosidase reporter was observed for two clones; both contained full-length cDNAs encoding human snapin (Fig. 1B, panels a and b). As shown in Fig. 1B (panel c), mating with various baits of UT-A1 and snapin led to expression of the reporter gene, confirming the interaction between snapin and L-UT in yeast. Specificity of the UT-A1-snapin association was shown by transformations of control bait and prey plasmids. This interaction was also verified by co-transforming the identified pACT2-snapin construct with the L-UT bait into yeast Y187, allowing to mate with strain Y187 pretransformed human kidney cDNA library (Fig. 2A, middle panel). The yeast strain AH109 was co-transformed with pACT2-snapin, and various baits in pGBKT7. Growth of yeast cells containing different protein constructs (panel d) on selective SD/-Leu/-Trp/-His/-Ade assay plate was analyzed (panel e). Positive control clone was used with p53 and SV40 T-antigen. The negative control utilized yeast expression vector pGBK7 and pACT2 or snapin.

As shown in Fig. 2A, we demonstrated that recombinant L-UT bait detects snapin-binding yeast colonies (lane 2) on a LacZ assay plate (panel a) and exhibited blue color upon Gal testing (panel b) assay plate. The yeast strain AH109 was co-transformed with prey plasmid pACT2-snapin, and various baits in pGBK7. Growth of yeast cells containing different protein constructs (panel d) on selective SD/-Leu/-Trp/-His/-Ade assay plate was analyzed (panel c). Positive control clone was used with p53 and SV40 T-antigen. The negative control utilized yeast expression vector pGBK7 and pACT2 or snapin.

Identification of snapin as a UT-A1 interacting protein. A, hypothetical structural model and schematic representation of three intracellular domains (N-UT, L-UT and C-UT) of human UT-A1 protein. B, these intracellular domains were fused to Myc-tagged pGBK7 vector and used as baits to transform into yeast strain AH109, and the resulting yeast transformant were allowed to mate with strain Y187 pretransformed human kidney cDNA library in pACT2. Upon mating, only L-UT bait detects snapin-binding yeast colonies (1 and 2) on a LacZ assay plate (panel a) and exhibited blue color upon Gal testing (panel b) assay plate. The yeast strain AH109 was co-transformed with prey plasmid pACT2-snapin, and various baits in pGBK7. Growth of yeast cells containing different protein constructs (panel d) on selective SD/-Leu/-Trp/-His/-Ade assay plate was analyzed (panel e). Positive control clone was used with p53 and SV40 T-antigen. The negative control utilized yeast expression vector pGBK7 and pACT2 or snapin.

To confirm the selective interaction between UT-A1 and snapin, we performed in vitro binding analyses of recombinant HA-tagged snapin and the various Myc-tagged baits of UT-A1. We demonstrated in vitro binding of recombinant Myc-L-UT and HA-snapin by co-immunoprecipitation using Myc or HA antibody (Fig. 2A) respectively. Snapin could be co-immunoprecipitated only with L-UT, whereas in the case of N-UT and C-UT, no snapin was detected in the immunoprecipitated com-

disulfide-biotin (Pierce) in borate buffer (pH 8.0) for 30 min at 4 °C with gentle shaking. Cells were washed with PBS-CM and incubated with quenching buffer (100 mm lysine and 25 mm Tris, pH 8.0, in PBS-CM). After washing four times with ice-cold PBS-CM, the cells were homogenized in 500 μl of lysis buffer (1% Triton X-100, 20 mm HEPES, pH 7.4, 1 mM EDTA, 150 mM NaCl, and protease inhibitors). The homogenates were centrifuged at 14,000 g for 20 min to remove cell debris. Total protein concentration in each sample was measured and adjusted to ~1 μg/μl with the same lysis buffer. 50 μl of supernatant was incubated with 80 μl of ImmunoPure-immobilized streptavidin-agarose beads (Pierce) overnight at 4 °C with gentle shaking. The beads were washed five times with ice-cold lysis buffer, and biotin-labeled proteins were resuspended in 50 μl of Laemmli sample buffer, boiled, and analyzed by Western blot.

FIGURE 1. Identification of snapin as a UT-A1 interacting protein. A, hypothetical structural model and schematic representation of three intracellular domains (N-UT, L-UT and C-UT) of human UT-A1 protein. B, these intracellular domains were fused to Myc-tagged pGBK7 vector and used as baits to transform into yeast strain AH109, and the resulting yeast transformant were allowed to mate with strain Y187 pretransformed human kidney cDNA library in pACT2. Upon mating, only L-UT bait detects snapin-binding yeast colonies (1 and 2) on a LacZ assay plate (panel a) and exhibited blue color upon Gal testing (panel b) assay plate. The yeast strain AH109 was co-transformed with prey plasmid pACT2-snapin, and various baits in pGBK7. Growth of yeast cells containing different protein constructs (panel d) on selective SD/-Leu/-Trp/-His/-Ade assay plate was analyzed (panel e). Positive control clone was used with p53 and SV40 T-antigen. The negative control utilized yeast expression vector pGBK7 and pACT2 or snapin.

FIGURE 2. Selective interaction of snapin with loop domain of UT-A1. A, autoradiogram of coprecipitation of snapin with L-UT. [35S]Methionine-labeled L-UT and snapin were generated in a Tris reticulocyte lysis system. L-UT protein was incubated with snapin, and the protein–protein interaction complexes were immunoprecipitated with Myc antibody (M) and HA antibody (HA), separated by SDS-PAGE, and analyzed by autoradiography. Lanes: 1 and 2, antibody verification; 3 and 4, complimentary binding proof; 5 and 6, negative controls. B, in vitro translated [35S]labeled snapin was incubated with protein A bead alone (Resin) or each of the three [35S]-labeled Myc-tagged bait proteins (C-UT, N-UT, and L-UT) followed by immunoprecipitation with Myc antibody. Snapin was pulled down only in the presence of L-UT. C, Triton X-100 extracts of rat kidney IMCD tissue lysates were incubated with resin alone (lane 2) or nonspecific rabbit IgG (lane 3) or in vitro translated HA-tagged snapin (lane 3) and immunoprecipitated with HA antibody. Bound proteins were analyzed by immunoblotting with anti-UT-A1 antibody (top panel) and anti-HA antibody (middle panel). The amount of membrane bound [35S]-labeled snapin was detected by autoradiography (bottom panel). Total lysate used in the binding reaction was run in parallel (lane 1).
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To map the region of snapin involved in the binding of UT-A1, we performed binding assays of UT-A1 wild-type (wt) with intact GST-snapin or truncated snapin fusion proteins. Analysis of the cDNA sequence of snapin predicted two coiled-coil α-helical domains (H1 and H2). The truncated mutants were constructed by deleting specific domains of snapin as indicated. The PKA phosphomimetic mutant (S50D) was constructed by changing a single amino acid. The column on the right (dotted box) is a summary of the experiments in B. "*" indicating the presence and "-" indicating the absence of interaction between snapin construct and UT-A1. B, pulldown assay of UT-A1 from lysates of UT-A1-MDCK cells by GST-snapin constructs, followed by Western blotting with anti-UT-A1 antibody. Only the snapin peptides containing the C-terminal H2 domain interacted with UT-A1 similar to the wild-type protein (top part). Blots were stripped and re-probed with anti-GST antibody to ensure equivalent loading of GST proteins (bottom part).

UT-A1 interacts with C-terminal α-Helical H2 Domain of Snapin—To map the region of snapin involved in the binding of UT-A1, we performed binding assays of UT-A1 wild-type (wt) with intact GST-snapin or truncated snapin fusion proteins. Analysis of the cDNA sequence of snapin predicted two coiled-coil α-helical domains (H1, amino acids 37–65 and H2, amino acids 83–126). The C-terminal coiled-coil domain (H2) was characterized as a binding domain for SNAP25 and SNAP23 (22, 23). Hydropathy analysis indicated that the first 20 amino acid residues from the N terminus form an uncharged, mostly hydrophobic domain, potentially a signal peptide for membrane insertion (23). To further characterize the UT-A1-binding site within snapin, we generated six deletion mutants and tested them for their interaction with UT-A1 by GST-affinity chromatography (GST pulldown assay). The predicted C-terminal helical domain (H2) of snapin was sufficient to confer binding to UT-A1, whereas a predicted hydrophobic domain (HD) or the N-terminal helical domain (H1) was dispensable for the observed association (Fig. 3A).

UT-A1 to mimic PKA phosphorylation (S50D) did not affect the binding of UT-A1. Deletion analysis, therefore, indicated that UT-A1 interacts with the H2 domain when present. Deletion of the H2 domain eliminated UT-A1 binding. The truncated H2 domain resulted in no interaction, suggesting that UT-A1 interacts with H2 domain of snapin.

Snapin, a SNARE-associated Protein, Interacts with Native UT-A1—We used adenovirus-mediated snapin-overexpressing UT-A1-MDCK cells and followed the expression pattern in response to increasing doses of adenovirus (Ad-snapin). As a control, separate cultures were infected with an adenoviral construct for green fluorescent protein (Ad-GFP) (Fig. 4A). Two days after infection, UT-A1-MDCK cells were treated with arginine vasopressin or FSK for 20 or 30 min, respectively, and then subjected to membrane biotinylation. Streptavidin-bound proteins were immunoblotted with anti-UT-A1 antibody.

FIGURE 3. Mapping of UT-A1 interaction sites on snapin. A, domain map of snapin indicating the positions of the N-terminal hydrophobic domain (HD) and two predicted coiled-coil α-helical domains (H1 and H2). The truncated mutants were constructed by deleting specific domains of snapin as indicated. The PKA phosphomimetic mutant (S50D) was constructed by changing a single amino acid. The column on the right (dotted box) is a summary of the experiments in B. "*" indicating the presence and "-" indicating the absence of interaction between snapin construct and UT-A1. B, pulldown assay of UT-A1 from lysates of UT-A1-MDCK cells by GST-snapin constructs, followed by Western blotting with anti-UT-A1 antibody. Only the snapin peptides containing the C-terminal H2 domain interacted with UT-A1 similar to the wild-type protein (top part). Blots were stripped and re-probed with anti-GST antibody to ensure equivalent loading of GST proteins (bottom part).

FIGURE 4. Adenovirus-mediated snapin overexpression in UT-A1-MDCK cells. A, UT-A1-MDCK cells grown in 6-well plates were infected by Ad-snapin at the indicated recombinant virus concentrations (microliter) corresponding to ~30 pfu/cell and grown to confluence for 20 h before the assay. The adenovirus-mediated total levels of snapin expression were detected by Western blotting using anti-snapin antibody. Membrane-associated UT-A1 was detected by anti-UT-A1 antibody in the plasma membrane fraction of corresponding Ad-snapin-infected UT-A1-MDCK cells. Ad-GFP was used as a control adenovirus. B, 2 days after infection, UT-A1-MDCK cells were treated with arginine vasopressin or FSK for 20 or 30 min, respectively, and then subjected to membrane biotinylation. Streptavidin-bound proteins were immunoblotted with anti-UT-A1 antibody.
To investigate further the interaction between snapin and SNARE proteins, we performed in vitro binding assays using recombinant fusion proteins (Fig. 5). In this experiment, SNARE proteins fused to GST were immobilized on glutathione beads, incubated with the lysate of Ad-snapin expressing UT-A1-MDCK cells, and the bound proteins were analyzed by immunoblotting. Ad-GFP-mediated UT-A1-MDCK cell lysate was used as a control. An interaction between SNARE-associated proteins (synaptotagmin 1, SNAP-25, SNAP-23, and syntaxin-4) and snapin was observed (Fig. 5, top). No snapin band was detected with control (GST), GST-VAMP2, or GST-SNAP29. Blots were stripped and re-probed with anti-UT-A1 antibody to observe the interaction between snapin-coupled SNARE proteins and UT-A1 (Fig. 5, middle). A strong interaction between GST-snapin and native UT-A1 was detected, a moderate interaction between snapin-coupled SNARE proteins and UT-A1 was observed, and no background UT-A1 was observed in controls and other GST-SNARE proteins (Fig. 5, bottom). These results indicate that the UT-A1 protein associates with the SNARE machinery indirectly, most likely by interacting with snapin.

**Co-immunoprecipitation of UT-A1 and Snapin Indicates in Vivo Interaction**—In the present study we found that snapin interacts with UT-A1 in vivo in UT-A1-MDCK cells. We performed co-immunoprecipitation studies using Ad-snapin-infected UT-A1-MDCK cells. UT-A1 and snapin were immunoprecipitated from Triton X-100-solubilized lysate obtained from snapin-overexpressing UT-A1-MDCK cells using anti-UT-A1 and anti-snapin antibodies, respectively. As expected, snapin was co-immunoprecipitated when anti-UT-A1 antibody was used (Fig. 6A), and UT-A1 was co-immunoprecipitated when anti-snapin antibody was used (Fig. 6B). Snapin and UT-A1 were not precipitated with nonspecific rabbit IgG and protein-A beads. Neither snapin nor UT-A1 was co-immunoprecipitated when GFP adenovirus (Ad-GFP)-infected UT-A1-MDCK cells were used (data not shown). Thus, our data suggest that snapin and UT-A1 may exist in a same core complex in UT-A1-MDCK cells.

**Co-localization of UT-A1 and Snapin to Both the Membrane and Cytosol in UT-A1-MDCK Cells**—To determine if there is a spatial interaction between UT-A1 and snapin in UT-A1-MDCK cells, full-length snapin was inserted into the pDsRed-nmonomer-N1 expression vector (pDsRed-snapin), which allows the production of a red fluorescent fusion protein. pDsRed-snapin plasmid was transiently transfected into UT-A1-MDCK cells and grown on Transwell filters. Cultured cells were then fixed and immunostained with UT-A1 antibody and fluorescein isothiocyanate-tagged secondary antibody, thus making UT-A detectable by its green fluorescence (panel a) and snapin by its red fluorescence (panel b). The merged image (yellow, panel c) indicates that the two proteins show co-localization in both the cytoplasm and in the plasma membrane (Fig. 7). The results provide evidence that snapin is associated with UT-A1 within cells. This is more clearly seen in the x-z section view, where the UT-A1 signal is predominantly localized in the apical membrane region of the cells, and snapin is localized in both the apical and basolateral membranes (Fig. 7d).

**Snapin Modulates Urea Transport Activity in Xenopus Oocytes**—The highly conserved t-SNAREs, syntaxin-4 and SNAP23, share a common motif composed of an amphipathic α-helix. In addition to t-SNAREs interacting with each other to
form a binary complex, they interact with snapin, which could create a link between UT-A1 and the vesicle. We sought to characterize urea transport activity by these binary complexes in *Xenopus* oocytes. Urea transport activity was induced by UT-A1 cRNA injection in oocytes, and this activity was increased by co-injection of UT-A1 with snapin alone or with snapin together with the t-SNARE components, syntaxin-4 and SNAP23 (Fig. 8). Interestingly, urea transport activity was markedly decreased by co-injection of UT-A1 with syntaxin-4 and SNAP23 in the absence of snapin. Thus, snapin enhances urea transport activity, but in the absence of snapin, the binary t-SNARE complexes reduced the transport activity. Furthermore, mutant snapin in which the putative PKA phosphorylation site (Ser-50) was changed to either aspartate (S50D, phospho-null) or alanine (S50A, phospho-null), did not stimulate urea flux.

**Snapin Detects UT-A1 and t-SNARE Components in Kidney Inner Medulla by Pulldown Assay**—To investigate the interaction of snapin with UT-A1 and other membrane proteins in inner medulla, the full-length coding region of snapin fused to GST (GST-snapin) was immobilized on glutathione beads, incubated with rat inner medullary tissue lysate, and analyzed by immunoblotting. We analyzed the bound and non-bound material in parallel to determine the binding efficiency. As shown in Fig. 9, snapin detected UT-A1 and t-SNARE components (Syntaxin-4 and SNAP23) in the inner medulla. This result indicates that snapin is an adapter molecule that can be linked to t-SNARE components and to UT-A1 in the IMCD. However, VAMP2 was not detected in the bound fraction. A very similar result was observed using His<sub>6</sub>-snapin in place of GST-snapin (data not shown). Collectively, these data suggest that UT-A1 recruits snapin to participate in the SNAP machinery in the IMCD.

**DISCUSSION**

The UT-A1 urea transporter is the largest protein isoform in the UT-A gene family and plays a central role in the urine-concentrating mechanism. Vasopressin-mediated trafficking of UT-A1 to the plasma membrane is a key step for the regulation of UT-A1, but the mechanism by which UT-A1 is delivered to the plasma membrane remains unclear. The major finding in the present study is the identification of snapin, a SNARE-associated protein, as a protein that interacts with the loop region of UT-A1. This interaction was initially suggested by the results of a yeast two-hybrid screen, confirmed by both in vitro and in vivo binding assays, and verified by GST pulldown assays. Based on the domain map of snapin, we investigated the UT-A1 interaction site on snapin and showed that it is located in the C-terminal H2 domain of snapin. We also identified t-SNAREs that form a complex with snapin and UT-A1, providing the first evidence for the SNAP vesicular trafficking system in the plasma membrane accumulation of UT-A1. The protein-protein interaction of UT-A1 and snapin increases urea flux in oocytes, suggesting that this interaction results in a change in function. Finally, the ability of snapin to immunoprecipitate UT-A1, SNAP23, and syntaxin-4 from rat kidney inner medullary tissue lysate suggests that these interactions may be physiologically relevant.

Correct fusion of a vesicle to its destination membrane is implicit in all membrane trafficking events. The vesicle fusion is directed by the formation of a stable SNAP core complex between the fusing vesicle v-SNARE protein and its target membrane t-SNAREs to enable fusion. It has been proposed that the specific pairing of t-SNAREs are essential for the fidelity of membrane recognition and fusion (15). The t-SNAREs...
that mediate GLUT4 exocytosis, syntaxin-4 and SNAP23, form stable and SDS-resistant complexes with VAMP2 (28). In the present study we demonstrate that immobilized GST-snapin efficiently binds to UT-A1 and t-SNARE components (syntaxin 4 and SNAP23) in the kidney inner medulla, where UT-A1 trafficking plays an important role in the urine concentrating mechanism. Hence, snapin is an adapter molecule that may link between UT-A1 and t-SNAREs for vesicle fusion. These data suggest that UT-A1 could be involved in SNARE-mediated fusion machinery indirectly, most likely by interacting with snapin. Snapin has been reported to form a ternary complex with syntaxin 4 and SNAP23 (t-SNARE) in non-neuronal cells (22). The t-SNAREs composed of syntaxins 2–4 interacting with SNAP23 are involved in distinct exocytic pathways in non-neuronal cells (13, 14). Our data indicate that snapin does not affect the binding of UT-A1 to the complex of syntaxin 4 and SNAP23. SNAREs are now generally accepted to be the major players in the final stage of the docking and fusion of vesicle-mediated transport events. Vesicles with v-SNARE combine with t-SNARE of the target membrane to form a complex and fuse to deliver bound cargo. Two prominent members of the SNARE family, syntaxin and SNAP, have been shown to interact and regulate a number of ion channels and transporters (29).

It remains to be determined whether snapin exerts modulatory effects on v-SNARE (VAMP) binding to t-SNARE (syntaxin 4/SNAP23) for UT-A1 trafficking. Among the plasma membrane-localized syntaxins in non-neuronal tissues, only syntaxin 4 interacts with VAMP2 (30), the v-SNARE implicated in insulin-stimulated trafficking of GLUT4 to the plasma membrane. The SNARE complexes consisting of syntaxin 4, SNAP23, and VAMP2 were also found in rat adipose cells (31). Recent reports demonstrate that snapin interacts with EBAG9 (estrogen-receptor-binding fragment-associated gene 9) (7), vanilloid receptor (6), and BLOC-1 (biogenesis of lysosome-related organelles complex-1) (9) and provide evidence for the role of snapin role in intracellular membrane trafficking. In support of a more general role, additional interaction partners for snapin have been identified that are neuronal specific, among them the regulator of G protein signaling 7 (32), adenyl cyclase type VI (5), and tryptophan/methionine tyrosine kinase (8), whose function is seemingly unrelated to membrane fusion.

We also demonstrated a functional coupling of UT-A1 with snapin, syntaxin 4, and SNAP23 in cRNA-injected Xenopus oocytes. The present findings of enhanced urea influx show a functional interaction of snapin with UT-A1. This interaction is predicted to govern the proper positioning of UT-A1 to the plasma membrane. In contrast, injecting oocytes with UT-A1 and t-SNAREs, but not snapin, reduced urea transport activity. This finding is similar to what has been found for the Ca\textsuperscript{2+} channel in chromaffin cells, when it encounters highly abundant t-SNAREs (syntaxin 1A/SNAP25), which have been shown to occupy a large area of the cell membrane (33). Thus, it is possible that the binary t-SNARE complex modulates UT-A1 urea transport activity by occupying a large area of plasma membrane and reducing UT-A1 accumulation in the membrane.

It is not clear from our data how important snapin phosphorylation might be for the role of snapin in enhancing urea transport. Instead of leading to a potential further enhancement of urea flux by mimicking phosphorylation, replacing the consensus phosphorylation site with the negatively charged aspartate (S50D) abolished the enhancing effect of wild-type snapin (Fig. 8), similar to the mutant S50A whose phosphorylation site had been removed. There is no ready explanation for this observation beyond the suggestion that snapin phosphorylation at Ser-50 may not be essential for coupling UT-A1 to the SNARE machinery.

The t-SNAREs could act as the principal intermediate in coupling the fusion machinery with the ion-channel or transporter (29, 34). Interestingly, the interaction of syntaxin 1A/SNAP25 in a 1:1 ratio was suggested to serve as a SNARE assembly intermediate prior to the association with synaptotubulin (35). The pre-association of UT-A1 with syntaxin-4/SNAP23 at the release sites of non-neuronal cells could be an important step for further engagement of snapin and recruitment of the vesicle to the plasma membrane. The essential role of t-SNAREs in linking snapin to the fusion machinery is a new pathway for the trafficking of UT-A1. Although the order of binding of each protein to the urea transporter is not yet determined, t-SNARE addition to a snapin-associated UT-A1 transporter could provide a distinct conformation and a functional link between the vesicle tethering apparatus, the urea transporter source, and the fusion machinery. Such a putative exocytotic unit, called an exitosome, could temporally and spatially facilitate vesicle fusion with the cell membrane (29, 36). Our functional data suggest that the t-SNARE complex enhances the association of snapin with UT-A1. This snapin-t-SNARE interaction serves to position vesicles at the release sites and is consistent with Ca\textsuperscript{2+}-independent binding of synaptotagmin to t-SNARE heterodimer (37). Overall, our study demonstrated that snapin can be found in the plasma membrane and, together with the t-SNARE components, generates a stable core complex.

In summary, in this study we demonstrated that snapin may be linked between the SNARE-mediated fusion machinery and UT-A1 by protein-protein interactions, similar to events in non-neuronal cells. We identified a functional coupling of UT-A1 with snapin and t-SNARE components (SNAP23/syntaxin 4) in cRNA-injected Xenopus oocytes. Although the binding kinetics of each SNARE-protein and snapin-UT-A1 complex is not yet determined, the role of snapin in linking t-SNAREs may be an important mechanism for UT-A1 trafficking. Our study concludes that UT-A1 urea transporter can recruit snapin to the plasma membrane where it forms a cargo complex with t-SNARE and that this docking/fusion machinery may be functionally important to the ability of UT-A1 to orient in the membrane and transport urea.

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