Localization and topology of a urate transporter/channel, a galectin, in epithelium-derived cells

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We recently cloned a cDNA that encodes a unique 322-amino acid protein and demonstrated that the 36-kDa recombinant protein that was prepared from this cDNA functions as a highly selective 10-pS voltage-sensitive urate channel in lipid bilayers (37, 38). On the basis of its function in the bilayer system, this protein has been designated urate transporter/channel (UAT). In view of the fact that the mRNA encoding UAT is present in multiple tissues (38), we have suggested that this channel is likely to perform an important housekeeping function. Insofar as the electrochemical gradient is favorable, the presence of UAT in plasma membranes would permit efflux of the urate that is produced intracellularly via metabolic degradation of the purines adenine and guanine (39). Because urate is formed in all cells that possess xanthine oxidase (4, 39, 63) and intracellular retention of urate would likely result in crystallization because of its limited solubility (64), this housekeeping function of UAT is believed to assume considerable importance in the maintenance of urate homeostasis. Moreover, subsequent to efflux from systemic cells into the extracellular compartment, urate must be eliminated from the body. The latter represents a second critical step that is required to preserve urate homeostasis. Because the kidneys serve as the major route for eliminating urate from the body, the renal handling of urate has been extensively examined (3). Two modalities of renal urate transport have been described, a sodium-independent electrogenic transporter (1, 2, 35, 52) and an electroneutral urate/anion exchanger (9, 24, 25, 31–33, 52).

Two lines of evidence led us to propose that UAT is the molecular representation of the renal electrogenic urate transporter (37, 38). First, the activity of UAT is blocked by pyrazinonic acid (37), a well-known inhibitor of renal urate transport in many species (3) and, more specifically, an inhibitor of electrogenic urate transport in rat and rabbit renal cortical membrane vesicles (1, 2, 35). Second, both UAT and the renal electrogenic urate transporter share a number of features with the hepatic peroxisomal enzyme uricase (1, 2, 35–38). One such shared characteristic is that oxonate, a specific inhibitor of the enzymatic activity of uricase (17), is a potent blocker of both UAT channel activity in the lipid bilayer system (37) and electrogenic urate transport in renal cortical membrane vesicles of rat and rabbit kidney (1, 2, 35). Another important similarity is that a polyclonal antibody to affinity-purified pig liver uricase (36) effectively blocks UAT channel activity (37, 38) and quite specifically blocks electrogenic, but not electroneutral, urate transport in membrane vesicles derived from rat renal cortex (36). It is of note that this same antibody identified the UAT cDNA clone from a rat renal expression library (38), that the recombinant UAT protein prepared from this cDNA is immunoreactive to anti-uricase (38), and that anti-uricase is reactive in rat renal proximal tubules, the nephron site of urate transport (36). Of note, we re-
recently identified (37) a local block of homology to uricase within UAT. Because this domain is homologous to the domain in uricase that contains the specific amino acid (glutamine) that X-ray crystallography has identified as being critically important in the formation of the substrate-uricase complex (15), it seems likely that this domain is responsible, at least in part, for the functional and immunologic similarities that have been observed among UAT, the renal electrogenic urate transporter, and uricase (1, 2, 35–38).

On the basis of computer analysis of the amino acid sequence of UAT and electrophysiological data obtained with recombinant UAT in the lipid bilayer system, we recently proposed (37) a topological model for UAT in which the protein has intracellular amino and carboxy termini and 4 α-helices that are long enough to represent transmembrane domains. This model incorporates the fact that local blocks of homology to membrane-spanning domains within several known integral membrane proteins, including bacterial rhodopsin (50), cytochrome-c oxidase (58) and urate/xanthine permease (22), as well as the B and E loops of aquaporin-1 (30), were detected within UAT (37). However, the amino and carboxy termini of UAT have a high degree of homology to the galectins, a family of cytoplasmic or secreted β-galactoside binding proteins (5, 8, 14, 19–21, 26, 41, 48). Indeed, complete β-galactoside binding site sequences, the signature sequence for galectins, reside within both the amino and carboxy termini of UAT. Thus, by definition, UAT is a galectin. It is important to note that the galectins are not considered to be membrane proteins that subserve a transport function. Instead, they are presumed to be soluble cytoplasmic or secreted proteins that play a role in a variety of functions including cell migration, cell adhesion, cell proliferation, immune function, and neoplasia (5, 8, 14, 19–21, 26, 29, 41, 48).

At the time UAT was originally described, a BLAST search revealed that the amino and carboxy termini of UAT were linked by a block of 61 amino acids that was unique (38). It was hypothesized that this unique linker region was probably responsible for the strikingly different function of UAT relative to the other galectins. However, subsequent to our publication (38), galectin 9 was reported in rats (60, 61), mice (60, 61), and humans (44, 45, 59). It is of note that the rat, mouse, and human galectin 9 nucleotide sequences are, respectively, 99%, 89%, and 73% identical to that of UAT (6). Again, in striking contrast to the proposed function of UAT as an integral membrane transport protein (37, 38), it has been suggested that the galectin 9s, like other members of this family, are soluble secreted proteins (44, 59–61). It is important to note that unlike the homology between UAT and galectins 1–8, the extremely high degree of homology between UAT and the galectin 9s is not confined to the amino and carboxy termini of these proteins. Rather, the above-described linker region of UAT that was presumed to be essential to its transport function is 98%, 87%, and 77% homologous to the linker regions of rat, mouse, and human galectin 9, respectively (6). Consequently, it has been extremely difficult to reconcile the disparate functions assigned to these highly homologous proteins. Indeed, despite the demonstration of urate channel activity when recombinant UAT is fused with synthetic lipid bilayers (37, 38), the prevailing data relative to the galectins (29) clearly favor the presumption that UAT is a soluble cytoplasmic or secreted protein in living cells.

The present studies were conducted to determine whether UAT is, in fact, an integral membrane protein in living epithelial cells. Experimental evidence has been obtained that UAT is transcribed, translated, and inserted into plasma membranes of both renal and nonrenal epithelium-derived cells transfected with the cDNA of UAT. Consistent with the recently proposed molecular model of UAT (37), these studies have also demonstrated that UAT is a transmembrane protein whose amino and carboxy termini are both cytoplasmic. These findings, together with data previously obtained in the lipid bilayer system (37, 38) represent both a unique function and a previously undescribed subcellular localization for a galectin. Of note, Madin-Darby canine kidney (MDCK) cells were found to contain a protein immunoreactive to anti-uricase, the presumed canine homolog of UAT. Moreover, expression of a urate transporter with characteristics that suggest the presence of endogenous urate channels has been observed in all cells assessed to date. However, transfection of these cells with UAT did not modify urate uptake. Because evidence has been obtained that UAT multimerizes, we propose that the number of UAT channels in the membrane, like other transporters and channels (10, 11, 18, 27, 28, 42, 46, 49, 55, 57, 62), is regulated and that monomers of protein expressed from transfected UAT substitute for monomers of endogenous channels. In this context, the maintenance of uptake at control levels in transfected cells requires that the protein expressed from transfected UAT is functional.

**MATERIALS AND METHODS**

**Preparation of Constructs**

Restriction sites were incorporated at the amino and carboxy termini of the previously cloned full-length coding sequence of UAT (38) by PCR. Two different PCR products were prepared. In one, designated BamHI-UAT-HindIII, PCR was performed with a sense primer that incorporates the sequence for the BamHI restriction site at the 5’ end of nucleotides 1–21 of UAT and an antisense primer that includes the sequence for the HindIII at the 3’ end of nucleotides 945–966 of UAT (Table 1). The second PCR product, designated BamHI-UAT-KpnI, was amplified with the same sense primer and an antisense primer that contains the sequence for KpnI at the 3’ end of nucleotides 945–966 of UAT (Table 1). This antisense primer omits the stop codon for UAT (Table 1). These and all other primers were obtained from Genosys Biotechnologies (The Woodlands, TX). For both constructs PCR was performed using an initial denaturation at 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min with AmpliTaq DNA polymerase (PE Biosystems, Foster City, CA). Samples were maintained at 4°C after a
final cycle that prolonged the extension time at 72°C for 7 min. PCR products were electrophoresed on 1% agarose gels, purified using a gel extraction kit (Qiagen, Valencia, CA), and subcloned into pBluescript SK(−) (Stratagene, La Jolla, CA).

Minipreps of DNA were made using a plasmid miniprep kit (Qiagen) and evaluated by restriction digestion. All restriction enzymes were obtained from New England Biolabs (Beverly, MA). DNA was sequenced by automated sequence analysis with an Applied Biosystem sequencer (ABI 373A) using dye terminator chemistry to verify that the sequence of the PCR product was identical to the coding sequence of UAT. Thereafter, the BamHI-UAT-HindIII PCR product was subcloned into pcDNA 3.1(−) (Invitrogen, Carlsbad, CA). Maxipreps of the BamHI-UAT-HindIII construct in pcDNA and the BamHI-UAT-KpnI construct in pBluescript were made using a plasmid maxiprep kit (Qiagen).

A second series of constructs was prepared with the enhanced green fluorescent protein (EGFP) nucleotide sequence alone or incorporated at the amino or carboxy terminus of UAT. The coding sequence of EGFP was isolated from pEGFP-N1 (Clontech Laboratories, Palo Alto, CA) by restriction digestion with ApaI and NotI and then subcloned into pcDNA 3.1(−) (Invitrogen, Carlsbad, CA). Maxipreps of the BamHI-UAT-HindIII construct in pcDNA and the BamHI-UAT-KpnI construct in pBluescript were made using a plasmid maxiprep kit (Qiagen).

A third set of constructs was prepared with the nucleotide sequence of the FLAG epitope (Scientific Imaging Systems, Eastman Kodak, New Haven, CT) appended to the amino terminus of UAT via a triple ligation between the restriction enzyme-digested XhoI-EGFP-BamHI and BamHI-UAT-HindIII inserts and XhoI- and HindIII-digested pcDNA 3.1(−) vector. EGFP was added to the carboxy terminus of UAT by a triple ligation between the restriction enzyme-digested BamHI-UAT-KpnI and KpnI-EGFP-HindIII inserts and BamHI- and HindIII-digested pcDNA 3.1(−) vector.

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Cell Cultures

The following cell lines were purchased from American Type Culture Collection (Manassas, VA): LLC-PK1 cells, derived from porcine kidney tubules; MDCK cells, from Madin-Darby dog kidney; mIMCD cells, from mouse inner medullary collecting ducts; HEK 293 cells, a human embryonic renal cell derivative; HeLa cells, from a human adenocarcinoma of the cervix; A6, derived from adult *Xenopus laevis* kidney; and Cos-7 cells, from green monkey kidney. Cultures were grown in Falcon tissue culture flasks (Becton Dickinson, Franklin Lakes, NJ). All cells except A6 were grown in the presence of Dulbecco’s modified essential medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Life Technologies, Rockville, MD). A6 cells were cultured in medium NCTC-109 containing 15% deionized water supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Life Technologies). Culture medium was changed three times per week, and cells were passaged as necessary after treatment with cell dissociation solution (Sigma-Aldrich, St. Louis, MO). A6 cells were maintained at 27°C, whereas all other cultures were maintained at 37°C. All cells were grown in humidified incubators with 5% CO₂-95% air. To localize the cellular expression of UAT by indirect fluorescence and confocal microscopy, cells were plated on no. 1/2 22-mm square, acid-washed coverslips (Corning Laboratory and Equipment, Corning, NY) in Falcon six-well culture plates (Becton Dickinson) or 0.4-µm pore, 24-mm Transwell-clear inserts (Costar, Corning Laboratory and Equipment). When immunocytochemistry was performed before microscopy, coverslips were coated with 0.02% rat tail collagen type I (Becton Dickinson) at least 24 h before cell plating.

Transfections

Transient transfections were performed 24 h after 5 × 10⁶ cells were plated on coverslips in six-well culture plates; 0.6 µg of DNA was added per well in the presence of Effectene (Qiagen) according to the supplier’s protocol. For stable transfections, cells were plated into Falcon T25 tissue culture flasks. When the cells reached ~80% confluence, transfections were carried out: 5.3 µg of DNA was added to each flask in the presence of Effectene, as recommended by the supplier. Forty-eight hours after transfection Geneticin (Life Technologies) was added to and subsequently maintained in the culture medium to select stable populations of transfected cells. For LLC-PK1 cells a concentration of 1.5 mg/ml Geneticin was used, and for MDCK cells a concentration of 1 mg/ml was used. In addition, clonal cell lines were established by isolating individual cells from the stably transfected LLC-PK1 cell population by flow cytometry (FACStar PLUS, Becton Dickinson). Single cells were sorted into individual wells of a 96-well culture plate and allowed to grow in the presence of conditioned medium. Clones were subsequently passed into 24-well culture plates and then into T25 tissue culture flasks. Once passed into tissue culture flasks, the cells were maintained in Geneticin-containing medium.

Indirect Fluorescence and Confocal Microscopy

Preparation of cells for microscopy. Cells that were plated at a density of 5 × 10⁶ cells per coverslip or Transwell and transiently or previously stably transfected were fixed for 5 min in 4% paraformaldehyde (Electron Microscopy Science, Ft. Washington, PA) in phosphate-buffered saline (PBS) (Life Technologies). Transiently transfected cells were fixed 48 h after transfection, whereas stable populations of cells were selected in Geneticin for at least 2 wk before plating and fixation. In some studies, plasma membranes were counterstained with a lipid soluble dye, CM-DiI or DiQ (Molecular Probes, Eugene, OR), according to the supplier’s protocol. After fixation, coverslips or Transwell inserts were mounted onto slides with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and sealed with clear nail polish. Immunochemistry. Cells that were plated at a density of 5 × 10⁶ cells per coverslip and transiently or previously stably transfected were fixed for 2 min in 2% paraformaldehyde in PBS. Transiently transfected cells were fixed 48 h after transfection, whereas stable populations of cells were selected in Geneticin for at least 2 wk before plating and fixation. After fixation, cells were washed three times (5 min/wash) in 0.3 M glycine and then permeabilized in 0.1% Triton X-100 in PBS for 5 min. In some studies, the expression of UAT was simultaneously compared in permeabilized and nonpermeabilized cells. To examine expression in nonpermeabilized cells, fixed cells were placed in PBS without Triton X-100 for 5 min. Thereafter, permeabilized and nonpermeabilized cells were handled identically. After three washes in PBS (5 min/wash), nonspecific binding sites were blocked with 5% bovine serum albumin and 10% normal goat serum (Sigma-Aldrich) in PBS for 1 h. Cells were then incubated with 10 µg/ml anti-FLAG (Sigma-Aldrich) and 1% normal goat serum in PBS for 30 min. M2 anti-FLAG was used when cells had been transfected with UAT constructs with a carboxy terminal FLAG epitope; M5 anti-FLAG was used for cells transfected with constructs containing amino terminal FLAG epitope tags. After three washes in PBS (5 min/wash), cells were incubated in a 1:200 dilution of FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min and then washed three times in PBS (5 min/wash). Finally, cells were placed in 0.1% Triton X-100 in PBS for 5 min, washed three times in PBS (5 min/wash), and then mounted on slides with Vectashield mounting medium in preparation for microscopy.

Microscopy. Fluorescence microscopy was performed with a Zeiss Axioscop (Carl Zeiss, Thornwood, NY). Brightfield and fluorescence images were captured through the appropriate filter cubes and phase rings and imported directly into Photoshop (Adobe Systems, San Jose, CA). In studies in which permeabilized and nonpermeabilized cells were compared, fluorescent images were obtained using identical exposure times. Additionally, in these studies all fields imaged were of equal size. Confocal microscopy was performed with a LEICA TCS-SP confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany) equipped with argon, krypton, and HeNe lasers. Red images (CM-DiI or DiQ) were obtained after excitation at 596 nm, and green images (EGFP or FITC) were obtained after excitation at 492 nm. When cells were labeled with two fluorophores, the red and green channels were imaged simultaneously. Confocal images were captured in the GlowOvUn look-up table and were pseudo-colored appropriately as either red or green before being saved as TCS Export TIFF image files. Images were then imported into Photoshop for analysis and processing. Dual-channel overlays of red and green confocal images were produced in Photoshop through the construction of an RGB three-channel image consisting of an empty blue channel and the red and green confocal images as the red and green channels, respectively.
Cell Surface Biotinylation

Biotinylation of cell surface proteins was performed according to the one-step recovery method described by Gotardi et al. (23). All solutions were prepared identically to those reported; the biotinylation solution that was selected contained 150 mM NaCl and was buffered to pH 9.0. Stably transfected LLC-PK1 or MDCK cells (3 × 10⁶) were plated on 0.4-μm pore, 24-mm polycarbonate Transwell supports (Costar), 10 or 6 days before biotinylation, respectively. In brief, monolayers were washed with serum-free DMEM and PBS plus calcium and magnesium and then consecutively incubated two times in a solution containing 1.5 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Pierce, Rockford, IL). The biotin-containing solution was added to both apical and basolateral chambers of the Transwells. After the biotinylation reaction was quenched with a glycine-containing buffer, filters were rinsed with PBS plus calcium and magnesium and then excised from the Transwell housing. The monolayers were solubilized in 1 ml of lysis buffer, cells were scraped into the lysis buffer, and the lysate was cleared by centrifugation. Nine hundred microliters of supernatant was removed and incubated with one hundred microliters of Immunopure imobilized streptavidin protein complexes (Pierce) overnight with end-over-end rotation. Thereafter the beads were washed three times in lysis buffer, twice with the high-salt wash solution, and once with the low-salt wash solution. The beads were then heated in 100 μl of elution solution and used for Western blots.

Aliquots of protein samples eluted from the streptavidin beads were electrophoretically resolved on a 10% polyacrylamide gel and then electrophoretically transferred to a nitrocellulose filter (Bio-Rad Laboratories, Hercules, CA). Immuno-oblots were blocked in 5.0% milk in PBS, pH 7.4, for 1 h at room temperature and then incubated for 1 h in monoclonal mouse anti-GFP IgG (Clontech) diluted 1:500 in the milk-PBS solution. After six washes at room temperature in 1.0% milk-PBS solution, the blots were incubated at room temperature with peroxidase-labeled goat anti-mouse IgG (KPL, Gaithersburg, MD) diluted 1:6,000 in 5.0% milk-PBS, washed as above, and then visualized by enhanced chemiluminescence (ECL) and exposure to Hyperfilm ECL (Amersham International, Little Chalfont, UK).

Urate Uptake Studies

Endogenous urate uptake was examined in various non-transfected immortalized cell lines. In addition, uptake was assessed in pools of MDCK cells that were stably transfected with UAT or EGFP (as a control). In all studies, 4 × 10⁵ cells were plated into 35-mm tissue culture dishes (Corning) at least 4 days before analysis of urate uptake. Cells and all solutions were maintained on a warming plate at 37°C during each study. Tissue culture medium was initially removed by aspiration and replaced with 2 ml of serum-free DMEM. After 30 min of preincubation the serum-free medium was removed and replaced with 0.45 ml of either PBS containing 0.9 mM CaCl₂ and 0.49 mM MgCl₂, a 100 mM KCl solution (in mM: 100 KCl, 40 NaCl, 0.9 CaCl₂, 0.49 MgCl₂, 1.5 KH₂PO₄, and 8.1 Na₂HPO₄), or a 100 mM K-gluconate solution (in mM: 100 K-gluconate, 40 Na-gluconate, 0.9 CaOH₂, 0.49 MgSO₄, 1.5 KH₂PO₄, and 8.1 Na₂HPO₄). When utilized, 2 mM N⁶-cyclpentadienyladenosine (CPA, Sigma), or 3 mM 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS, Sigma) was dissolved into the 100 mM K-gluconate uptake medium immediately before use. Two minutes after the cells were exposed to uptake medium, the medium was pulsed with 0.05 ml of [8-¹⁴C]urate (sp act 50 mCi/mmol; American Radio Chemical, St. Louis, MO) to provide a final urate concentration of 20 μM. Thereafter, uptake was stopped at timed intervals by rapidly aspirating the medium and immediately rinsing the cells five times in ice-cold PBS containing 1 mM adenosine (Sigma). Cells were subsequently lysed for 15 min in 1 ml of 0.2% SDS diluted in deionized water. The amount of [8-¹⁴C]urate accumulated by the cells was determined by the addition of 0.5 ml of lysate to 10 ml of biodegradable counting scintillant scintillation fluid (Amersham). Radioactivity was measured in a Packard Tri-Carp spectrometer (model 4430; Packard Instrument). The protein concentration of a 0.05-ml aliquot of lysate was measured by a fluorometric assay (7) using bovine serum albumin as standards. Uptake, expressed as picomoles per milligram of protein, was calculated from the counts per minute (cpm) of the cell lysates, the cpm of the media, and the protein concentration of the lysates. In all studies uptake was determined as the mean of the uptake in triplicate dishes of cells.

Western Blot of MDCK Cells

Nontransfected MDCK cells were grown past confluence in T25 flasks. Cells were detached with cell dissociation solution (Sigma) and were pelleted by centrifugation for 4 min at 1,800 rpm. The pellet was washed twice in PBS and then lysed in PBS with 1% Triton X-100, 5 mM EDTA, and complete protease inhibitor cocktail (Roche). After clarification by centrifugation, lysate supernatant was boiled for 3 min at 95°C in 1× Laemmli sample buffer (final concentration) containing 2-mercaptoethanol. Western blots were performed on the lysates as described for Cell Surface Biotinylation studies except that polyclonal rabbit anti-pig uricase (36) diluted 1:2,500 was used as primary antibody and horse-radish peroxidase (HRP)-conjugated goat anti-rabbit IgG (KPL) diluted 1:6,000 was used as secondary antibody.

Coimmunoprecipitation Studies

To assess the possibility that the protein expressed from transfected UAT multimerizes, Cos-7 and MDCK cells were cotransfected with the chimeric cDNA constructs UAT-myc and UAT-EGFP or UAT-myc and hUAT-EGFP (the human homolog of UAT-EGFP; Ref. 40). As a control, cells were cotransfected with UAT-myc and EGFP without UAT. Cells were plated into T75 flasks and cotransfected using Lipofectamine 2000 (Life Technologies) according to the supplier’s protocol when the cells reached ~80% confluence. Forty-eight hours after transfection, cells were dissociated, pelleted, and lysed as described for Western blot of MDCK cells. All subsequent steps were performed at 4°C. Lysates were rotated for 30 min and then centrifuged for 30 min at 12,000 rpm. Supernatants were transferred to tubes containing 50 μl of protein G agarose (Roche Molecular Biochemicals, Indianapolis, IN), rotated gently for 1 h to preclar, and then centrifuged for 30 s at 13,000 rpm. The cleared supernatants were transferred to tubes containing 25 μl of fresh protein G beads. Thereafter, 5 μg of monoclonal anti-myc (Roche) or 5 μg of monoclonal anti-FLAG M5 (Sigma) was added and the tubes were rotated overnight. Anti-FLAG M5 (Sigma) served as a negative control. After the beads were washed five times in PBS, the immunoprecipitated proteins were eluted by boiling for 5 min at 95°C in 25 μl of 2× Laemmli sample buffer containing 2-mercaptoethanol. Western blots were performed on the eluates using techniques identical to those described for cell surface biotinylation studies except that polyclonal anti-GFP (Clontech) diluted 1:1,000 was used as primary antibody and HRP-conjugated anti-mouse IgG (KPL) diluted 1:6,000 was used as secondary antibody.
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RESULTS

Subcellular Localization Studies of UAT

Confocal microscopy of immortalized cell lines transiently expressing UAT. Transient transfection of LLC-PK1 cells with chimeric cDNA constructs in which EGFP was fused to either the amino (Fig. 1A) or the carboxy (Fig. 1D) terminus of UAT resulted in expression of a green fluorescent protein that was localized predominantly at the periphery of cells. In these same cells, a similar peripheral pattern of distribution of the red-fluorescing lipophilic dye DiQ (Fig. 1, B and E) was evident. Dual-channel overlays of the green and red fluorescent images yielded a yellow color (Fig. 1, C and F) indicating colocalization of UAT and DiQ. Because DiQ is lipophilic and therefore a probe of cell membranes, this finding suggests that the peripheral, circumferential pattern of distribution of the UAT-EGFP chimeric proteins is consequent to the presence of UAT in plasma membranes. It is of note that the pattern of fluorescence of these chimeric EGFP constructs is distinctly different from that observed after LLC-PK1 cells are transfected with the cDNA for EGFP. In the absence of fusion to UAT, EGFP is not targeted to plasma membranes (not depicted). Rather, as previously described (51), EGFP is observed diffusely in both the cytoplasm and the nucleus.

Fusion of the cDNA of EGFP to the cDNAs of several integral membrane proteins does not appear to interfere with expression, targeting, insertion into plasma membranes, or function of these various proteins (13, 43, 47). Despite these observations, it was considered essential to determine whether the cellular localization of UAT was similarly unaffected by the production of UAT-EGFP chimeric proteins. Thus the cellular localization of UAT was evaluated after transfection of the same cell line with either UAT-EGFP or UAT-FLAG chimeric constructs. The latter was used because translation of this construct would result in production of a protein with a 30-fold smaller appendage, the eight-amino acid FLAG epitope. This requirement seemed particularly relevant because UAT and EGFP are relatively similar in size, approximating 36 and 27 kDa, respectively. As demonstrated in Fig. 2, independent of whether FLAG is fused to the amino (Fig. 2A) or carboxy (Fig. 2B) terminus of UAT, the resultant fusion protein is targeted to the plasma membrane. Thus the cellular pattern of distribution of the UAT-FLAG chimeric proteins is identical to that observed when UAT-EGFP fusion proteins are expressed in LLC-PK1 cells (Fig. 1, A and D). On the basis of the concordance of findings with the EGFP and FLAG fusion proteins we have concluded that transfection with chimeric cDNA constructs of EGFP or FLAG that are linked to the amino or carboxy terminus of UAT, like fusion with other membrane proteins (13, 43, 47), does not influence cellular targeting of UAT and, therefore, can be utilized as a valid tool to examine the localization of UAT in living cells.
To assess the possibility that targeting of UAT to plasma membranes is a phenomenon that is restricted to LLC-PK1 cells, the cellular localization of UAT was evaluated in six additional epithelium-derived cell lines. As depicted in Fig. 3, transient transfection of Cos-7 (Fig. 3A), mIMCD (Fig. 3B), HEK 293 (Fig. 3C), and Xenopus A6 (Fig. 3D) cells with the cDNA construct in which EGFP is fused to the carboxy terminus of UAT also results in translation of a chimeric UAT protein that is targeted to the plasma membrane. Similarly, transient transfection of HeLa (Fig. 3E) and MDCK (Fig. 3F) cells with the cDNA construct in which the FLAG epitope is linked to the carboxy terminus of UAT results in plasma membrane localization of the expressed protein. These findings indicate that protein expressed from the rat cDNA for UAT localizes to plasma membranes in a variety of immortalized cells that are driven via a cytomegalovirus promoter to transiently overexpress UAT.

Confocal microscopy of immortalized cell lines stably expressing UAT. Electrogenic urate uniport has been reported in both brush border and basolateral membrane vesicles derived from the rat kidney cortex (1, 2). To determine whether UAT protein is similarly targeted in living cells, the localization of UAT-EGFP chimeric proteins has been assessed in two immortalized renal epithelial cell lines that polarize in culture, LLC-PK1 and MDCK (53). As depicted in Fig. 4, the localization of UAT in plasma membranes is not a phenomenon that is limited to transiently transfected cells (Figs. 1–3). In a stable clone of LLC-PK1 cells (Fig. 4, A and B) and a stable pool of MDCK cells (Fig. 4, C–F), the chimeric UAT-EGFP proteins and the lipophilic fluorophore CM-DiI colocalize to the plasma membrane. As noted above, evidence of colocalization is provided by the yellow color of the dual-channel overlay of the green- and red-fluorescing images. In addition to revealing that UAT is expressed and targeted to the cell membrane after chromosomal integration of the UAT cDNA, an examination of sagittal as well as horizontal images of monolayers of these stably transfected cells permitted a determination of whether UAT is selectively targeted to apical or basolateral membranes. As demonstrated in the horizontal images (Fig. 4, C, D, and F), it is evident that UAT is targeted to lateral cell membranes. Sagittal images of these same cells (Fig. 4, A, C, and E) reveal that UAT is not selectively targeted to lateral cell membranes. At the stage of cell differentiation achieved in these monolayers, at least 5 days after cell plating, UAT is clearly targeted to both apical and basolateral plasma membranes. This same distribution of the chimeric UAT-EGFP protein was observed in MDCK cells grown on Transwells (Fig. 4, C and D) or coverslips (Fig. 4, E and F).

Topology Studies Utilizing FLAG Epitope Tag: Amino and Carboxy Terminal Epitope Tags

Our topological model (37) predicts that UAT has intracellular amino and carboxy termini and four transmembrane domains. To test the validity of this model, studies were conducted to determine whether the amino and carboxy termini of UAT are located on the extracellular or the intracellular side of the plasma membrane. As depicted in Fig. 5, despite the presence of numerous cells (Fig. 5A) amino terminus FLAG-tagged UAT was not detected in nonpermeabilized cells (Fig. 5B). In contrast, in the presence of a comparable number of cells (Fig. 5C) this chimeric protein was clearly evident in paired cells that were permeabilized before application of the same antibody (Fig. 5D). Identical results were obtained in cells transfected with the cDNA construct in which the FLAG epitope was ap-
epend to the carboxy terminus of UAT: the FLAG epitope was not detectable in nonpermeabilized cells (Fig. 5, E and F) but was readily visualized in paired cells permeabilized before exposure to anti-FLAG (Fig. 5, G and H). Although the fluorescence microscopy images suggest that both of the FLAG-tagged UAT constructs are located in the plasma membrane (Fig. 5, D and H), confirmation was sought by confocal microscopy. As in the case of cells transiently transfected with the FLAG-tagged UAT (Fig. 2), the confocal images confirm that the fusion proteins expressed in permeabilized LLC-PK1 cells stably transfected with the amino (Fig. 5I) and carboxy (Fig. 5J) FLAG-tagged UAT constructs also reside in plasma membranes. Insofar as these studies have demonstrated that the FLAG epitopes can only be detected when cell membranes are permeabilized (Fig. 5, B, D, F, and H), both the amino and carboxy termini of UAT must reside on the cytoplasmic side of the plasma membrane.

**Cell Surface Biotinylation Studies**

To determine whether UAT actually spans the plasma membrane, cell surface biotinylation studies were performed utilizing Sulfo-NHS-SS-Biotin, a hydrophilic, membrane impermeant biotinylation reagent that only binds to primary amine groups of lysine residues of membrane proteins that have exposed extracellular domains (23, 54). LLC-PK1 and MDCK cells stably transfected with chimeric constructs of EGFP at either the amino or the carboxy terminus of UAT or with EGFP without UAT (as a control) were grown on Transwell supports for 6–10 days and then biotinylated. In the method used, biotinylation is reversible because the S-S bond is cleaved in the process of eluting proteins from the streptavidin-conjugated beads. Because biotin remains attached to the streptavidin beads, eluted proteins migrate at approximately their endogenous, nonbiotinylated size during SDS-PAGE; however, the NHS groups remain bound to primary amines and partially restrict the protein’s mobility during electrophoresis. As depicted in Fig. 6, lanes containing eluates from both LLC-PK1 and MDCK cells transfected with the UAT chimeric constructs contained bands at ~70–75 kDa independent of whether EGFP was linked to the amino or the carboxy terminus of UAT. Bands were not detected in eluates of cells transfected with the intracellular soluble protein EGFP without UAT. This finding is important because EGFP contains numerous lysines (twenty) that would be reactive had they been accessible to the Sulfo-NHS-SS-Biotin (23, 54). These findings therefore require that UAT contains at least one extracellular domain that binds biotin.

**Urate Uptake Studies**

To evaluate the presence and nature of urate transport in various cells, 20 μM radiolabeled urate uptake was assessed in confluent, nontransfected cultured
cells in 35-mm dishes. At 1 min of incubation in the presence of PBS, urate uptake was significantly above background in each cell line evaluated (LLC-PK1, HeLa, A6, Chinese hamster ovary (CHO), human umbilical vein endothelial cells (HUVEC), Cos-7, Leydig, mMCD, MDCK, immortalized mouse podocytes, and mesangial cells; not depicted). A comparison of uptake at 1 min and/or 10 min in Cos-7, MDCK, CHO, HUVEC, podocytes, and mesangial cells incubated in PBS or in 100 mM K-glucuronate revealed that in each cell line urate uptake was increased approximately twofold when the membrane potential was depolarized (high-K media; Fig. 7A). To determine whether urate uptake could be further augmented, uptake was also evaluated in MDCK cells stably transfected with either UAT or EGFP. A time-dependent curvilinear increase in urate uptake was observed, and, at each time point, uptake was inhibited ~75% by coincubation with the adenosine analog (Fig. 7C). Residual uptake was not inhibited by 3 mM DIDS, suggesting that urate/anion exchange is not responsible for the adenosine-insensitive uptake in these cells (not depicted).

Western Blot of MDCK Cells

The demonstration of electrogenic, adenosine-sensitive urate transport in MDCK cells (Fig. 7) is consistent with the presence of a canine UAT homolog in MDCK cells. To determine whether this putative homolog shares other characteristics of UAT, Western blots were performed on lysates from nontransfected MDCK cells probed with anti-uricase, an antibody that is immunoreactive to both UAT and hUAT (38, 40). As depicted in Fig. 8, MDCK cells clearly express a protein that both is immunoreactive to anti-uricase and migrates at 36 kDa, the approximate size of both UAT and hUAT.

Coimmunoprecipitation Studies

The failure to modify urate uptake in cells transfected with UAT could be consequent to nonfunction of the expressed protein. Alternatively, because virtually all channels are multimeric, the failure to modify transport might simply be the result of substitution of monomeric subunits that derive from the endogenous membrane potential-sensitive transporter with those expressed from the transfected UAT. The latter possibility was assessed with the technique of coimmunoprecipitation. As depicted in Fig. 9 (lanes 3 and 4), eluates prepared from lysates of Cos-7 cells cotransfected with UAT-myc and UAT-EGFP and immunoprecipitated with anti-myc contained a band immunoreactive to anti-GFP. This band migrated at the approximate size of UAT-EGFP (63 kDa). Additionally, as depicted in Fig. 9 (lanes 5 and 6), coimmunoprecipitation was observed in cells cotransfected with UAT-myc and hUAT-EGFP. In contrast, in cells cotransfected with EGFP and UAT-myc, EGFP did not

Fig. 7. [8-14C]urate uptake studies in cultured cell lines. A: endogenous urate uptake in paired studies in nontransfected cells incubated for 1 and/or 10 min in the presence of PBS or KCl. B: 1-min urate uptake in paired studies (means ± SE; n = 3) performed on pools of MDCK cells stably transfected with either UAT or EGFP in the presence of PBS or 100 mM K-glucuronate. C: urate uptake in paired studies (means ± SE; n = 4) performed on pools of MDCK cells stably transfected with UAT in the presence or absence of 2 mM N6-cyclopentyladenosine (CPA) at 1, 5, and 15 min of incubation. Where not shown, the SE bars are incorporated within the symbols.

Fig. 8. Western blot of MDCK cell lysate probed with polyclonal anti-pig liver uricase. The immunoreactive band at 36 kDa is presumed to represent the canine homolog of UAT.
coimmunoprecipitate (Fig. 9, lanes 1 and 2). Moreover, EGFP did not bind nonspecifically to the protein G beads or to an irrelevant antibody, anti-FLAG M5 (not depicted). Similar results were obtained in transiently cotransfected MDCK cells (data not shown). Because UAT-EGFP was detected subsequent to immunoprecipitation of UAT-myc with anti-myc, these data indicate that UAT-EGFP and UAT-myc must multimerize when coexpressed in cultured cells. Additionally, because hUAT-EGFP was similarly coimmunoprecipitated with UAT-myc it is evident that the human and rat homologs of UAT are capable of heteromultimerization. In each sample, an additional band migrating at ~32 kDa was detected. Because this band was evident independent of the constructs transfected, it is presumed that this band represents an endogenous protein that is precipitated nonspecifically and is reactive to either the primary or the secondary antibody used to probe the Western blots. A faint band migrating at ~50 kDa (Fig. 9, lanes 3-6) was also often detected. The nature of this 50-kDa protein is unknown, but it may represent UAT/hUAT-EGFP that was degraded at some point after expression.

DISCUSSION

The present studies have provided evidence that UAT localizes to plasma membranes in a number of cell lines that are derived from mammalian renal (LLC-PK1, Cos-7, mIMCD, HEK 293, and MDCK) and nonrenal (HeLa) epithelia of multiple species (pig, monkey, mouse, human, and dog) as well as amphibian renal epithelia (A6) (Figs. 1–6). In two of these cell lines, LLC-PK1 and MDCK, data have been acquired that document that UAT is not simply a protein that closely associates with the cytoplasmic face of the plasma membrane but rather is a protein with at least one extracellular domain (Fig. 6). Insofar as UAT has an extracellular domain and the present studies have also demonstrated that the amino and carboxy termini of UAT are both located on the intracellular side of the membrane (Fig. 5), at a minimum UAT must contain two transmembrane domains. It is of note that this preliminary topological analysis of UAT in plasma membranes of mammalian cells is consistent with the model of UAT that has recently been proposed on the basis of both computer analyses and electrophysiological data obtained with recombinant UAT in planar lipid bilayers (37).

In addition to documenting that UAT is a transmembrane protein, the present studies have demonstrated that UAT is targeted to both apical and basolateral plasma membranes in stably transfected, polarized, transporting LLC-PK1 and MDCK cells (Fig. 4). Evidence for the transport function of these cells is based on the formation of domes in cells grown on coverslips, as captured in the sagittal confocal image in Fig. 4E. It is of note that the morphological localization of UAT in these polarized cells is similar to the functional localization of the electrogenic urate transporter in brush border (apical) and basolateral membranes of rat renal cortical cell membranes (1, 2). Moreover, rabbit anti-pig liver uricase, the same antibody that identified UAT in the rat renal cDNA library (38), reacts with recombinant UAT protein (38), blocks UAT channel activity (37, 38), and inhibits electrogenic urate transport in renal membrane vesicles (36), similarly demonstrates a morphological pattern of distribution of immunoreactive protein in rat kidney that is entirely consistent with the functional localization of the electrogenic urate transporter. Light microscopy reveals reactivity in proximal tubule brush border membranes (36), whereas electron microscopy demonstrates immunogold particles in both brush border and basolateral proximal tubule cell membranes (unpublished observations). It is of interest that a protein that is immunoreactive to rabbit anti-rat liver uricase was recently described in apical and basolateral plasma membranes as well as membranes of secretory/endocytic compartments of digestive gland cells of mussels (12). This protein has not yet been cloned, and, therefore, its homology to UAT remains unknown. However, as urate oxidase enzymatic activity was not detected in any subcellular fractions of mussel digestive glands, it has been postulated that the immunoreactive protein in digestive gland plasma membranes, like that in renal proximal tubule cell membranes (36), is not uricase per se but rather may represent the urate transporter of mussel intestine (12).

It is of note that to date we have not detected a single cell line that is null for urate transport. Each has a basal level of urate uptake, and, where evaluated, uptake is stimulated by cell depolarization (Fig. 7, A and B) and blocked by extracellular adenosine (Fig. 7C), findings consistent with the presence of endogenous urate transporter/channels in plasma membranes. Moreover, although recombinant UAT prepared from an identical cDNA functions as a highly selective urate channel in lipid bilayers (37, 38) and confocal microscopy, immunocytochemistry, and cell surface biotinylation studies have clearly demonstrated that UAT protein expressed from transfected UAT cDNA resides in plasma membranes in stably transfected MDCK cells (Figs. 1–6), it has not been possible to demonstrate that the expression of UAT increases urate uptake above the basal levels detected in control cells (Fig. 7B).

Fig. 9. Western blot of eluates of cotransfected Cos-7 cells immunoprecipitated with anti-myc and probed with a polyclonal antibody to EGFP. Lanes 1 and 2, cells cotransfected with UAT-myc and EGFP; lanes 3 and 4, cells cotransfected with UAT-myc and UAT-EGFP; lanes 5 and 6, cells cotransfected with UAT-myc and human UAT-EGFP.
One possible explanation for this observation is that UAT that is expressed in the plasma membrane subsequent to transfection is nonfunctional. Of note, however, our data indicate that UAT multimerizes (Fig. 9). It therefore seems highly probable that each UAT channel (like most other channels) is multimeric. Moreover, we have documented that multimers can form between monomers of UAT derived from different species (rat and human) and that MDCK cells contain a 36-kDa protein that is immunoreactive to anti-urate (Fig. 8), presumably the dog homolog of UAT. In this context, in MDCK cells expressing both endogenous dog and transfected rat UAT, individual channels are likely composed of monomeric subunits derived from each source. If protein expressed from transfected UAT was nonfunctional, then multimerization with endogenous subunits would be expected to alter channel properties (i.e., conductance and/or open probability) and consequently modify net urate transport. Because uptake was not affected in the presence of protein expressed from transfected UAT, an alternate explanation is favored.

There is considerable precedence for regulation of channel and transporter function at the level of plasma membrane insertion and retrieval (10, 11, 28, 42, 46, 49, 55, 57, 62). Examples of proteins regulated in this manner include the epithelial sodium channel (ENaC; Ref. 57), aquaporin 2 (42), the cystic fibrosis transmembrane conductance regulator (CFTR; Ref. 10), sodium-protein exchanger type 3 (NHE3; Ref. 55), the rat outer medullary K⁺ channel (ROMK; Refs. 46, 49, 62), and the type IIa Na-Pᵢ cotransporter (28). An additional level of regulation also occurs via the phosphorylation status or other posttranslational modifications of proteins already present in the membrane. Examples of the latter include ENaC (56), the cardiac L-type calcium channel (34), the anion exchanger AE4 (27) and the chloride channel CIC-2 (18). On the basis of the well-documented regulation of these membrane proteins it is suggested that the sum total of functional multimeric UAT channels in the plasma membrane, whether derived in whole or in part from endogenous and/or transfected DNA, is also tightly regulated under basal steady-state conditions.

In view of the apparent multimeric nature of UAT channels and the likelihood that the number of functional UAT channels in plasma membranes is restricted under basal conditions, we conclude that protein expressed from transfected UAT is, in fact, functional and that it substitutes for monomeric subunits of endogenous protein and consequently results in a level of urate uptake comparable to that in non-transfected cells. Clearly, a direct demonstration of transport function of exogenously expressed UAT will require the utilization of cells that are null for an endogenous urate transporter. As noted above, no such cells have been identified to date and future attempts to directly document function of UAT may require production of a cell line in which the endogenous transporter is nonfunctional. Because the apparent ubiquitous presence of these transporters is consistent with the proposed housekeeping function of allowing urate efflux subsequent to intracellular production (4, 39, 63), it seems possible that channel deletion may be poorly tolerated.

Subsequent to our report of the cloning and functional assessment of recombinant UAT (38), in which we noted a high degree of homology between UAT and the galectin family of proteins, the sequences of rat (60), mouse (60), and human (44, 45, 59) galectin 9 were identified. It is of note that 965 of the 966 nucleotides that encode UAT (GenBank accession no. U67958) and rat galectin 9 (accession no. U59462) are identical. As a consequence of this single nucleotide difference, amino acid 155, which is translated as histidine in UAT, is replaced by leucine in rat galectin 9. Whether this discrepancy reflects a naturally occurring polymorphism between the animals utilized in the different cloning strategies (38, 60), a mutation introduced by PCR during the cloning of galectin 9 (PCR was not used to clone the coding sequence of UAT), or simply a sequencing error in either UAT or galectin 9, it seems highly likely that UAT and rat galectin 9 represent the same gene product. In support of the identity of UAT and rat galectin 9 are the highly similar differential patterns of expression of UAT and rat galectin 9 in the various tissues assayed by Northern analysis of rat multitissue blots (38, 60). Although there is currently no experimental evidence for the physiological role of rat galectin 9, this protein has been presumed to be soluble and potentially secreted and not to be a membrane protein (60). Although the present studies provide no evidence to contradict this presumption, they do provide direct evidence that UAT is an integral plasma membrane protein in a large variety of immortalized epithelial cells (Figs. 1–6). Clearly, it remains to be determined whether cellular trafficking, localization, and function of UAT/rat galectin 9 are possibly tissue- and/or cell type specific.

Although UAT has the highest degree of homology to rat galectin 9, UAT also has a strikingly high degree of homology to mouse galectin 9 (accession no. U55060) as well as human galectin 9/ecalectin (accession nos. Z49107, AB006782, and AB005894), with the nucleotide sequences that encode these proteins having 89% and 73% identity to UAT, respectively (44, 45, 59, 60). Mouse galectin 9 has been detected both intracellularly and at plasma membranes in intestinal and hepatic cells, whereas its protein distribution appears to be diffuse rather than enriched at plasma membranes in renal proximal tubular epithelial cells (61). On the basis of the enrichment of mouse galectin 9 at intestinal and hepatocyte plasma membranes it has been postulated, but not documented, that mouse galectin 9, like some other members of the galectin family of proteins, is secreted by some nonclassic mechanism (8, 16). The finding that murine thymocytes, but not hepatocytes, undergo apoptosis in the presence of recombinant mouse galectin 9 suggested that locally secreted galectin 9 may play a role in thymocyte/epithelial interactions that are relevant to clonal deletion of T cells during development of the thymus.
(60, 61). However, the actual functional role of mouse galectin 9 in the many other tissues in which it is expressed (60, 61) remains to be determined. In view of the high degree of homology between mouse galectin 9 and UAT, it is suggested that the apparent membrane localization of the mouse protein in some tissues (61) may represent a protein that, like UAT, both resides and functions in the plasma membrane rather than simply being a soluble, membrane-associated protein that is either about to be or has just been secreted.

As in the case of rat and mouse galectin 9, human galectin 9 (60) and ecalectin (44) are also believed to be secreted proteins. Although human galectin 9 and ecalectin differ by only four amino acids (44, 45, 59), it has been proposed that human galectin 9 participates in cellular interactions of the immune system (59), whereas ecalectin (an apparent variant of galectin 9) has been described as a selective eosinophil chemottractant (44). In contrast to both of these suggested functions for the human homolog, our laboratory has obtained evidence (40) that recombinant human galectin 9/ecalectin, like rat UAT, functions as a highly selective urate transporter/channel in planar lipid bilayers. Furthermore, as with the rat UAT, protein expressed from the human homolog was documented to reside in plasma membranes and to contain at least two transmembrane domains in cultured cells (40).

Although functional differences have clearly been reported for UAT and mouse and human galectin 9/ecalectin, it is important to note that no two laboratories have specifically assayed the highly homologous proteins for the same functional properties. In this context, it is of interest that despite the distinct differences in the rat and human sequences, the recombinant proteins prepared from both respective cDNAs have been shown to function as highly selective urate transporter/channels when functionally assessed with the same methodology in a single laboratory (37, 38, 40). Furthermore, it seems possible that some of the functional differences between UAT and the rat, mouse and human galectin 9 sequences, either demonstrated or proposed, are likely to reflect evolutionary modifications of a single gene product whose subcellular localization and function may be significantly modified by the cell in which it is expressed. This possibility derives support from the apparent highly cell-specific apoptosis-inducing function of mouse galectin 9 (61). It is evident that the present studies have not reconciled the different functions (or subcellular localizations) ascribed to this “branch” of the galectin family. Rather, the body of data obtained provides evidence that UAT is an integral plasma membrane protein with intracellular amino and carboxy termini that multimerizes and is targeted to both apical and basolateral membranes in polarized renal epithelial cells. The consistent plasma membrane localization of UAT in multiple cells (Figs. 1–6), the documented function of the same protein in the lipid bilayer system (37, 38) in conjunction with the indirect evidence for functionality of protein expressed from transfected UAT, and the demonstration of endogenous membrane potential-sensing urate transport in multiple cell types support our proposal that UAT is a transmembrane protein that likely serves a critical housekeeping function in permitting urate efflux from systemic cells subsequent to intracellular production as well as facilitating urate excretion via renal tubular and intestinal epithelial cells.

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REFERENCES


20. Gitt MA, Massa SM, Leffler H, and Barondes SH.


