Sorting of Rat SPNT in Renal Epithelium Is Independent of N-Glycosylation

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Purpose. The sodium-dependent, purine-selective nucleoside transporter, SPNT, has a unique steady-state expression pattern in renal epithelial cells. In comparison with the concentrative nucleoside transporter, CNT1, which is confined to the apical membrane, SPNT is expressed predominantly on the apical membrane but with significant expression on the basolateral membrane as well. Alternate surface expression indicates that SPNT likely has different sorting and trafficking mechanisms from CNT1. Because glycosylation has been reported to be essential for apical targeting of other transporters, and SPNT contains three unique glycosylation sites, we examined the importance of glycosylation in sorting of SPNT. Preliminary studies suggested that glycosylation affects surface expression of SPNT but not CNT1.

Methods. All three unique glycosylation sites were mutated alone and in tandem. Wild-type and mutant SPNT, tagged with green fluorescence protein, were stably transfected into MDCK. Positive clones were assayed for polarized surface expression by immunofluorescence and functional analysis.

Results. Mutation at all three sites alone or in tandem resulted in functional proteins. Removal of sites N606 and N625 resulted in proteins of reduced molecular mass. None of the unglycosylated mutants localized differently than wild-type SPNT.

Conclusion. N-linked glycosylation is not essential for polarized sorting.

KEY WORDS: concentrative nucleoside transporter; SPNT; glycosylation; sorting; MDCK.

INTRODUCTION

Nucleosides and nucleoside analogs are being used and developed therapeutically for the treatment of viral infections, neoplasms, and cardiac arrhythmias. Many of these analogs are hydrophilic molecules which require carrier-mediated transport to enter cells. This transport is achieved by two families of nucleoside transporters, concentrative and equilibrative. The concentrative nucleoside transporter (CNT) family consists of three known mammalian members (CNT1, SPNT or CNT2, and CNT3) which mediate active transport of nucleosides down the sodium gradient [Che, 1995 #106; Huang, 1994 #100; Ritzel, 2000 #28]. SPNT is a purine-selective CNT found in abundance in renal epithelium [Pennycooke, 2001 #93]. Recent work from this laboratory demonstrated that SPNT is predominantly localized to the apical membrane in renal epithelial cells (MDCK and LLC-PK_1) where it is expected to play a role in reabsorption [Mangravite, 2001 #2]. Interestingly, approximately one-fifth of the SPNT protein expressed was found on the basolateral membrane. In comparison, both CNT1 and CNT3 were completely confined to the apical membrane [Mangravite, 2001 #2]. Presence of SPNT on both membranes of renal epithelium suggests a secondary role for this transporter in addition to nucleoside salvage.

We were particularly interested in investigating the molecular determinants responsible for localization of SPNT. Sorting signals for apical membrane proteins vary widely and are poorly understood, but for some membrane proteins, glycosylation has been documented as essential [Martinez-Maza, 2001 #104; Petrecca, 1999 #105]. Analysis of SPNT as well as CNT1 and CNT3 sequences indicates that these family members are differentially glycosylated (Fig. 1). In this study, we examined the role of glycosylation as a potential sorting signal for SPNT in renal epithelium.

MATERIALS AND METHODS

Site-Directed Mutagenesis

The Stratagene QuikChange Site-Directed Mutagenesis kit was used to construct mutant cDNA following the manufacturer's protocol using wild-type rSPNT in eGFP-C1 (Clontech, Palo Alto, CA, USA) as template. The N603T mutants were altered by an A to C mutation at base pair 2079 (GenBank accession number U25055), the N606T by an A to C alteration at position 2088, and the N625T by an A to C at position 2145. All sequences were confirmed by direct DNA sequencing. Single-mutant constructs were used as template to construct double and triple mutants (N603T/N606T, N606T/N625T, or N603T/N606T/N625T).

Construction of Stably Transfected MDCK

MDCK strain II were maintained in minimal essential medium Eagle's with Earle's balanced salt solution supplemented with 5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected with mutant SPNT-GFP cDNA using the calcium phosphate method as described previously [Mangravite, 2001 #2]. Cells were selected by growth in media containing 0.7 mg/ml Geneticin (Gibco, Grand Island, NY, USA) and maintained in this media for the remainder of the experiments. Positive clones were further selected by western blot, functional uptake, and fluorescence microscopy. For all experiments, cells were permeablized by growth for 4–7 days on polycarbonate Transwell filters (Corning Costar, Corning, NY, USA).

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ABBREVIATIONS: CNT, concentrative nucleoside transporter; SPNT, sodium-dependent purine selective nucleoside transporter; MDCK, Madin Darby canine kidney cells; LLC-PK_1, porcine kidney cells; GFP, green fluorescence protein; NBMPR, nitrobenzylthioribofuranosylpurine.

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                          *  *                 *
rCNT3    ----AINCHHVLE---SSKLVSNTTEVASCCQGLFNSTVARGPNDVLPGG-----NFSLY
rCNT1    PRGVEVDCVSLLN----QTVSSSSFEVYLCCRQVFQSTSS---------------EFSQV
rSPNT    PRGAETDCVSFLN----TNFTNRTYETYVCCRELFQSTLLNGTNMPSFSGPWQDKESSLR
hSPNT1   PRGAEADCVSFPN----TSFTNRTYETYMCCRGLFQSTSLNGTNPPSFSGPWEDKEFSAM
hCNT1    PRGAEVDCMSLLN----TTLSSSSFEIYQCCREAFQSVNP---------------EFSPE
hCNT3    ----DINCHHVLENAFNSTFPGNTTKVIACCQSLLSSTVAXGPGEVIPGG-----NHSLY

rCNT3    TLKSCCNLLKPPTLNCGWIPNIP
rCNT1    ALDNCCRFYN--HTVCT------
rSPNT    NLAKCCDLYT--STVCA------
hSPNT1   ALTNCCGFYN--NTVCA------
hCNT1    ALDNCCRFYN--HTICAQ-----
hCNT3    SLKGCCTLLNPSTFNCNGISNTF
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Fig. 1. Multiple alignments of predicted extracellular C-terminal tail of CNTs. The glycosylation sites in all sequences are highlighted. Asterisks mark glycosylation sites in rSPNT.

Western Blot

Samples were lysed by agitation in SDS buffer (2% SDS in PBS with protease inhibitor), and centrifuged at 14,000 RPM for 20 min. Supernatant was removed, assayed for protein content using the DC protein assay (BioRad, Hercules, CA, USA), and combined with loading buffer (15 mM TRIS pH 6.8, 1% SDS, 25 mM EDTA, 65 mM DTT, 0.025% bromophenol blue, 6% glycerol). Five micrograms of protein were loaded per sample on a 10% BioRad ready gel and separated by electrophoresis. Protein was transferred to PVDF membrane (BioRad), blocked in 5% milk, incubated first in mouse anti-GFP primary antibody (1:1000, Roche, Palo Alto, CA, USA), then in goat anti-mouse IgG-HRP conjugated secondary antibody (1:3000, BioRad), and signal was detected by the chemiluminescence (ECL) method.

Confocal Microscopy

Cells were fixed with 4% paraformaldehyde, permeablized with 0.025% (w/v) saponin, stained with Texas-red conjugated phalloidin (Molecular Probes, Eugene, OR, USA) for visualization of actin, and mounted on slides in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Cells were visualized using a BioRad MRC-1024 laser scanning confocal microscope.

Functional Localization and Statistical Analysis

Functional localization was performed as described previously [Mangravite, 2001 #2]. Briefly, cells were exposed to either Na^+ buffer (128 mM NaCl, 4.73 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, 5 mM HEPES-Tris, pH 7.4) or Na^+-free buffer (in which choline chloride is substituted for NaCl) containing 0.1 μM ^{3}H-inosine (specific activity = 25.4 Ci/mMol), 10 μM inosine and 10 μM NBMPR on either the apical or the basolateral membrane for 2 min. Reaction was terminated by washing samples three times in ice cold Na^+-free buffer. Samples were air dried and lysed by shaking for 30 min in 300 μl 10% SDS. Samples were counted on a Beckman Scintillation Counter (Anaheim, CA, USA). All experiments were performed in duplicate on four occasions. For each experiment, the ratio of apical uptake to basolateral uptake (A:B) was calculated. These were averaged and statistically analyzed by analysis of variance (ANOVA), using the 'Primer to Biostatistics' program supplied by Stanton Glantz (UCSF, San Francisco, CA, USA) ($p < 0.05$ considered statistically significant).

Materials

Cell culture media and supplements were purchased from the Cell Culture Facility (UCSF, San Francisco, CA, USA). ECL Detection was illuminated using Western Lightning (Perkin Elmer, Boston, MA, USA). Complete Mini Protease Inhibitor Cocktail was purchased from Roche and radiolabeled inosine from Morevak Biochemicals (Brea, CA, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA).

RESULTS

SPNT but Not CNT1 Localization Is Sensitive to Tunicamycin

Preliminary experiments indicated that administration of tunicamycin for 24 h caused a small decrease in molecular mass of SPNT and CNT1 characteristic of deglycosylation. When viewed by confocal microscopy, this shift was associ-

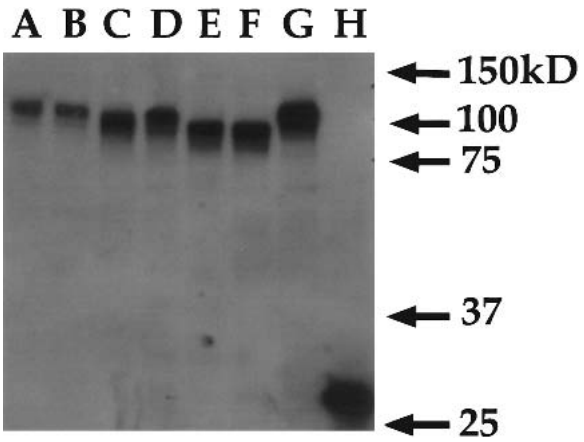

Fig. 2. Western blot analysis of SPNT glycosylation mutants. MDCK stably transfected with SPNT mutants N603T (A), N606T (B), N625T (C), N603T/N606T (D), N606T/N625T (E), N603T/N606T/N625T (F), wild type SPNT (G), or GFP (H) were polarized by growth for seven days on permeablized support and prepared for western blot as described in Materials and Methods. Five micrograms of protein per lane were loaded on a 10% SDS-PAGE gel and protein was separated by electrophoresis, transferred to PVDF and probed with GFP antibody. Weight of molecular standards is indicated by arrows to right of gel.

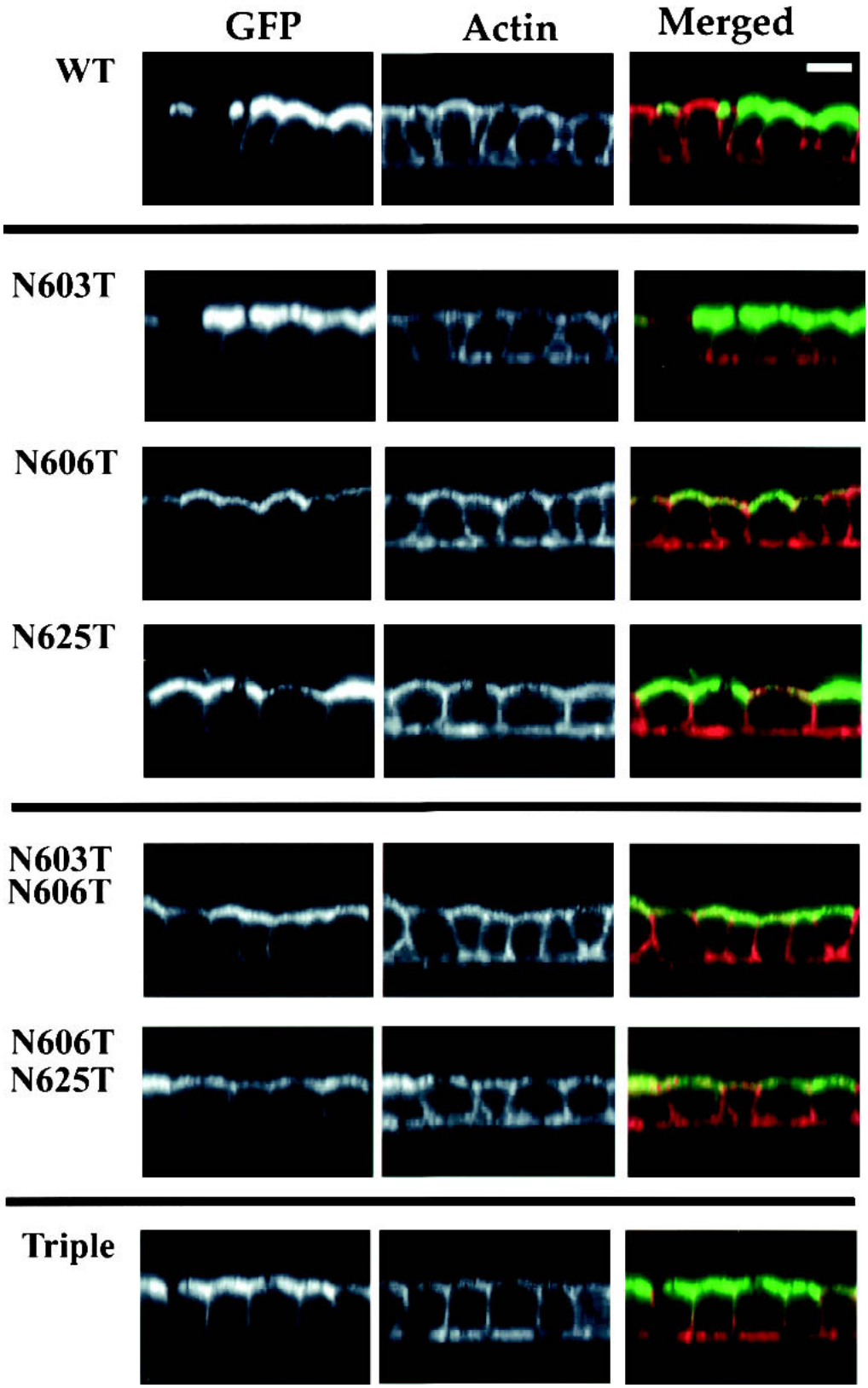

Fig. 3. Localization of SPNT glycosylation mutants in MDCK. MDCK stably transfected with wild type or mutant SPNT (as indicated to left of each row) were polarized by growth on permeable support, fixed, permeablized, stained for actin with Texas-red conjugated-X Phalloidin and visualized by laser scanning confocal microscopy. Horizontal lines separate wild type, single, double, and triple mutants. Left column: GFP. Middle column: Actin. Right column: Merged image. Vertical optical sections are shown with apical membrane on top. Bar, 10 μm.

ated with partial internalization of SPNT but not CNT1. (Data not shown.) The SPNT amino acid sequence contains five putative N-glycosylation sites, two of which (N439 and N539) are predicted to be located in transmembrane domains and are unlikely to be glycosylated. The other three sites (N603, N606, and N625) are located in the extracellular C-terminal tail [Hamilton, 2001 #103]. Alignment of the C-terminal tail of human and rat CNTs indicated that these three sites are unique to SPNT (Fig. 1). Two of these three glycosylation sites are conserved across species (N606 and N625) while the third (N603) is specific to rat and mouse SPNT.

Stable Transfections of Unglycosylated SPNT Mutants in MDCK

Using site-directed mutagenesis, the three unique glycosylation sites in SPNT-GFP were removed. We began by making single mutations (N603T, N606T, and N625T) and stably transfecting into MDCK cells. Positive clones were selected by immunoblot analysis, confocal microscopy, and functional analysis for maximal protein expression and function. In addition, two double mutants (N603T/N606T and N606T/N625T) and a triple mutant containing none of the putative glycosylation sites were constructed and transfected into MDCK. We examined the effect these mutations had on molecular mass of SPNT by immunoblot analysis (Fig. 2). Wild-type tagged SPNT had a molecular mass of approximately 105 kDa, representing ~70 kDa SPNT plus ~27 kDa GFP plus glycosylation. The N603T mutant appeared to have a similar molecular mass while the N606T mutant demonstrated a minimal mobility shift of approximately 5 kDa. The N625T mutant had a more-pronounced decrease in size (~15 kDa). Western blot analysis of the double and triple mutants indicated that the effects of glycosylation on protein size were additive. The molecular mass of these mutants was smaller than either wild-type or single mutant proteins. No degradation product was seen in any of the lanes. Interestingly, the N603T and N606T single-mutant clones appeared to contain less protein than wild-type or other mutant clones. It is unclear whether this is a consequence of transfection or whether mutation at these sites affects protein stability.

Glycosylation Does Not Affect Localization of SPNT Mutants

Localization of SPNT glycosylation mutants was examined by immunofluorescence and functional analysis. In all cases, experiments were repeated on more than one positive clone with similar results. Localization of single, double, and triple mutants mirrored that of wild-type SPNT-GFP. Visualization by confocal microscopy indicated that all clones localized primarily to the apical membrane with minor populations residing on the basolateral membrane (Fig. 3). For the N603T and N606T clones, basolateral signal was present but difficult to view because of low levels of protein expression.

In all cases, sodium-dependent nucleoside uptake was greater across the apical membrane than across the basolateral membrane (Fig. 4). Statistical analysis of the ratios of apical to basolateral function (A: B ratios, Fig. 4), performed using ANOVA, indicated that these ratios did not differ significantly ($p < 0.05$).

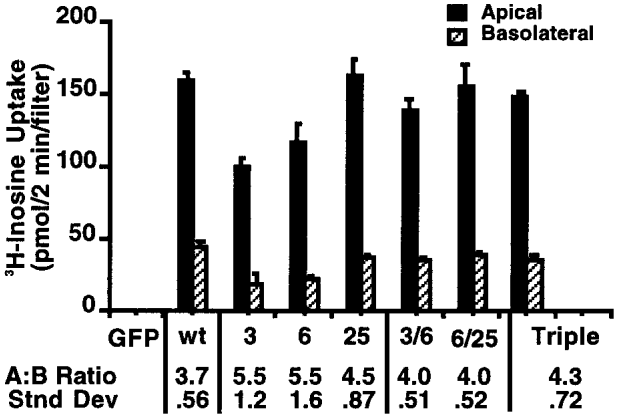

Fig. 4. Functional localization of SPNT glycosylation mutants. MDCK cells stably transfected with GFP, wild type SPNT (wt), or the glycosylation mutants N603T (3), N606T (6), N625T (25), N603T/N606T (3/6), N606T/N625T (6/25), or N603T/N606T/N625T (Triple) were polarized by growth on permeable support. ^{3}H-inosine uptake was measured across either the apical or basolateral membrane in the presence of Na^+. Data represent the average of four experiments. The averaged ratio of apical to basolateral uptake (A:B ratio) for each mutant is listed below each column.

DISCUSSION

SPNT is the most uniformly expressed of the CNTs. It is abundant throughout the gastrointestinal tract and present in liver and kidney where it is expected to play a role in nucleoside reabsorption. In addition, SPNT is widely expressed in small amounts throughout the rest of the body including tissues in which signaling via adenosine receptors is important, such as heart, testis, and lung [Pennycooke, 2001 #93; Felipe, 1998 #107]. In fact, the A_1 adenosine receptor, considered the key adenosine receptor in the kidney, exhibits a similar localization pattern to SPNT when expressed in MDCK [Saunders, 1996 #95]. Expression of SPNT is dependent on cell cycle, upregulated by exposure to PKC activating molecules and affected by growth factors including insulin and glucagon [Pastor-Anglada, 1998 #108].

Differential localization of SPNT in comparison with CNT2 or CNT3 in renal epithelial cells suggests that this protein has an alternate sorting mechanism. Recent observations from Pastor-Anglada suggest that SPNT sorts differently from CNT1 in hepatocytes as well [Pastor-Anglada, 2001 #102]. Sorting signals within membrane proteins vary but often act by selective interaction with trafficking machinery specific for movement to one type of membrane or vesicle. These signals tend to be discrete motifs within the protein and often can be identified. Within epithelial cells, proteins are not simply sent to the plasma membrane but specifically contain signals appropriate for targeting to either the apical or basolateral membrane.

While basolateral sorting signals often depend on a specific amino acid motif, apical sorting signals are less well characterized. They may involve anything from structure within a transmembrane domain, as is seen for the influenza virus hemagglutinin protein, to post-translational modifications such as glycosylation as in the case of the high affinity glycine transporter, GLYT1 [Lin, 1998 #110; Olivares, 1995 #109]. SPNT has three conserved glycosylation sites, all of which are

unique from CNT1 and CNT3. To examine the importance of N-glycosylation on sorting of SPNT, we observed the effect on localization of removing these glycosylation sites. We determined localization by both confocal microscopy and functional analysis. Our data indicated that sorting of SPNT is independent of glycosylation, though glycosylation may play a role in other aspects of SPNT biology such as protein turnover or stability. The molecular determinants responsible for sorting of SPNT remain to be identified.

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