Cysteine Residues in the Transmembrane (TM) 9 to TM11 Region of the Human Equilibrative Nucleoside Transporter Subtype 1 Play an Important Role in Inhibitor Binding and Translocation Function

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ABSTRACT

Inhibitor and substrate interactions with equilibrative nucleoside transporter 1 (ENT1; SLC29A1) are known to be affected by cysteine-modifying reagents. A previous study from our laboratory established Cys222 in transmembrane (TM) 6 as the residue responsible for methyl methanethiosulfonate (a membrane-permeable sulfhydryl modifier)-mediated enhancement of the binding of the ENT1 inhibitor nitrobenzylmercaptopurine riboside (NBMPR) in intact cells. However, the capacity of charged sulfhydryl reagents to inhibit the binding of NBMPR in broken cell preparations (allowing cytoplasmic access) was not affected by mutation of any of the cysteines (Cys87, 193, 213, or 222) in the N-terminal half of the protein. We thus hypothesized that the inhibitory effects of the modifiers were due to the one or more of the six cysteine residues in the C-terminal half of ENT1, particularly one or both of those in the fifth intracellular loop (Cys414 and Cys416). Each of the cysteines were mutated to serine or alanine and expressed in nucleoside transport-deficient PK15 cells and probed with a series of methanethio-sulfonate sulfhydryl-modifying reagents. Transporter function was assessed by the site-specific binding of [³H]NBMPR and the cellular uptake of [³H]2-chloroadenosine. These studies established that Cys378 is an extracellular-located residue modified by [2-(trimethylammonium)ethyl] methane-thiosulfonate (MTSET) to inhibit the binding of NBMPR to intact cells. Mutation of Cys414 led to an enhancement of the ability of MTSET to inhibit NBMPR binding, and this enhancement was eliminated by the comutation of Cys378, indicating that disruption of the fifth intracellular loop modifies the conformation of TM10 and its extracellular extension. Mutation of Cys416 led to the loss of the ability of the charged sulfhydryl reagents to inhibit NBMPR binding in isolated membranes and also led to the loss of transport function. This finding further supports an allosteric interaction between the fifth intracellular loop and the extracellular NBMPR binding domain and implicates this region in the translocation function of human ENT1.

Introduction

Human equilibrative nucleoside transporter 1 (hENT1) is a ubiquitous integral membrane protein that mediates the movement of hydrophilic nucleosides across cell membranes. It functions by carrier-mediated facilitative diffusion and is sensitive to inhibition by nanomolar concentrations of 4-nitrobenzylmercaptopurine riboside (NBMPR) (Sundaram et al., 2001a). Inhibition of hENT1 has been shown to enhance the cardioprotective and neuroprotective actions of adenosine (Cunha, 2001; King et al., 2006; Löffler et al., 2007). hENT1 is also used by anticancer nucleoside analogs for access into tumor cells and is thus an important chemotherapeutic target (Pastor-Anglada et al., 2005; Zhang et al., 2007).

ABBREVIATIONS: hENT, human equilibrative nucleoside transporter; NBMPR, nitrobenzylmercaptopurine riboside; TM, transmembrane; ENT, equilibrative nucleoside transporter; NEM, *N*-ethylmaleimide; pCMBS, *p*-chloromercuribenzene sulfonate; MTS, methanethiosulfonate; MMTS, methyl methanethiosulfonate; MTSET, [2-(trimethylammonium)ethyl] methane-thiosulfonate; MTSES, (2-sulfonatoethyl)-methanethiosulfonate; PBS, phosphate-buffered saline; NBTGR, *S*-(4-nitrobenzyl)-6-thioguanosine riboside; 2-Br, 2-bromohexadecanoic acid; PK15-NTD, nucleoside transport-deficient pig kidney epithelial cells derived from the PK15 cell line; FTH-SAENTA, 5′-*S*-{2-(1-[(fluorescein-5-yl)thioureido]-hexanamido)-ethyl}-6-*N*-(4-nitrobenzyl)-5′-thioadenosine; BGS, bovine growth serum; WT, wild-type; NMG, *N*-methylglucamine; DMSO, dimethyl sulfoxide; TBS, Tris-buffered saline; PDVF, polyvinylidene difluoride; IL, intracellular loop.

On the basis of antibody analyses and topology modeling, the conventional model of hENT1 combines a cytoplasmic N terminus and extracellular C terminus with 11 transmembrane (TM) domains (Sundaram et al., 1998, 2001a,b) (Fig. 1). Studies using ENT subtype chimeras and site-directed mutagenesis have identified the TM3 to TM6 region to be critical to transporter function and to contribute to interactions with hENT1 inhibitors and substrates. In particular, Gly154 and Ser160 in TM4 have an impact on NBMPR sensitivity, whereas Gly179 and Gly184 in TM5 are indicated in the affinity of nucleosides and proper membrane targeting, respectively (SenGupta et al., 2002; SenGupta and Unadkat, 2004; Endres and Unadkat, 2005).

At present, a complete structural model of hENT1 is lacking because of the difficulty in isolating hENT1 in its native membrane configuration. Alternative techniques for advancing knowledge of ENT1 structure-function have thus been used, including cysteine mutagenesis in conjunction with cysteine-selective sulfhydryl-modifying reagents (Valdés et al., 2004; Riegelhaupt et al., 2010; Park et al., 2011; Yao et al., 2011). The importance of cysteines in hENT1 function has been shown in multiple studies using N-ethylmaleimide (NEM) and p-chloromercuribenzene sulfonate (pCMBS) as sulfhydryl modifiers. In mouse Ehrlich ascites tumor cells and human erythrocytes (Vyas et al., 2002), it was found that inhibitor binding to ENT1 in intact cells was sensitive to NEM, which is membrane-permeable, whereas only the broken cell preparations were sensitive to pCMBS, a negatively charged membrane-impermeable reagent; these data are suggestive of intracellular cysteines being involved in ENT1-ligand interactions. In intact HeLa cells, both pCMBS and NEM were shown to alter the B_{max} and K_d of NBMPR binding (Dahlig-Harley et al., 1981), although NEM was much more potent in that regard, suggesting a relatively hydrophobic environment of the target cysteine.

We have recently described modification of hENT1 ligand binding and transport function using a series of methanethiosulfonate (MTS) reagents (Park et al., 2011). The membrane-permeable methyl methanethiosulfonate (MMTS) enhanced [^{3}H]NBMPR binding in a pH-dependent manner and decreased [^{3}H]2-chloroadenosine uptake, whereas the positively charged, membrane-impermeable [2-(trimethylammonium) ethyl] methane-thiosulfonate (MTSET) inhibited [^{3}H]NBMPR binding and enhanced [^{3}H]2-chloroadenosine uptake by hENT1. The negatively charged, membrane-impermeable (2-sulfonatoethyl)-methanethiosulfonate (MTSES), like pCMBS (also negatively charged), was found to inhibit NBMPR binding only in broken cell preparations, again implicating an intracellular cysteine. In that study, Cys222 was identified as the critical residue for MMTS enhancement of NBMPR binding, but the extracellular cysteine responsible for MTSET sensitivity was not identified. Likewise, the location of the MTSES-sensitive intracellular cysteine remained undefined.

Cys414 and Cys416 are the only cysteine residues predicted to be located in an intracellular aqueous accessible region (loop between TM10 and TM11). Thus, we hypothesized that it was one of these two cysteines that was the target for the charged MTS reagent effects on ENT1 function. Further evidence for a role of the C-terminal end of ENT1 being involved in ligand binding comes from studies on a truncated splice variant of mouse ENT1 missing the last three transmembrane domains (mENT1Δ11). This variant lost the ability to be photolabeled with NBMPR and was also found to be less sensitive to NEM, relative to its full-length counterpart (Robillard et al., 2008). Although this result suggested that the region was not essential for transporter function, it did imply that NEM sensitivity of NBMPR binding may be due to modification of cysteines encompassed by TM9 to TM11. In addition, Visser et al. (2005, 2007) have shown
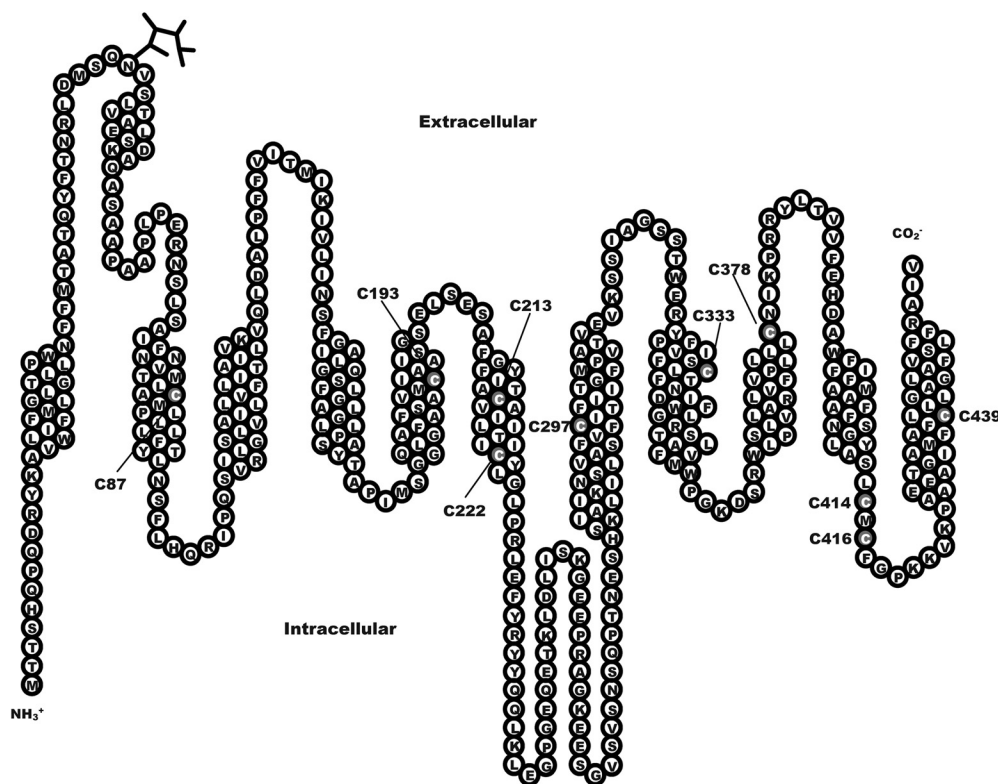

Fig. 1. Predicted transmembrane topology of hENT1 with locations of the 10 cysteine residues indicated. The cysteines targeted in the present study were Cys297, Cys333, Cys378, Cys414, Cys416, and Cys439 in the C-terminal half of the protein.

that Leu442 in TM11 of hENT1 (one helix turn above Cys439) (Fig. 1) is involved in transporter-permeant selectivity and may contribute to the interaction of the inhibitor dipyridamole with hENT1.

In the present work, we have examined the roles of the six cysteines found within the C-terminal half of hENT1 with regard to transporter function and the binding of NBMPR. With the use of site-directed mutagenesis, combined with differential sensitivity to a series of MTS reagents, we have described residues Cys378, Cys414, and Cys416 as having important but divergent roles in hENT1 function and ligand binding.

Materials and Methods

Materials. [^{3}H]NBMPR (5.5–20.1 Ci/mmol), [^{3}H]2-chloroadenosine (9.1 Ci/mmol), and [^{3}H]water (1 mCi/g) were obtained from Moravek Biochemicals (Brea, CA). MMTS, MTSET, and MTSES were acquired from Toronto Research Chemicals, Inc. (North York, ON, Canada). Culture-grade phosphate-buffered saline (PBS), modified Eagle's medium, G418 (Geneticin), penicillin-streptomycin, trypsin-EDTA, sodium pyruvate, nonessential amino acids, Lipofectamine 2000, and Lipofectamine LTX were purchased from Invitrogen (Burlington, ON, Canada). Oligonucleotide primers were obtained from Sigma-Genosys (Oakville, ON, Canada). NBMPR, 2-chloroadenosine, dipyridamole, and S-(4-nitrobenzyl)-6-thioguanosine (NBTGR) and p3XFLAG-CMV10 vector were purchased from Sigma-Aldrich (Oakville, ON, Canada). 2-Bromohexadecanoic acid (2-Br) was purchased from Sigma-Aldrich. The primary mouse monoclonal anti-FLAG antibody and secondary goat anti-mouse antibody were purchased from Sigma-Aldrich. The primary mouse monoclonal anti-Na$^+$/K$^+$-ATPase antibody was purchased from Abnova (Cambridge, MA). Cell lysis buffer (10×) and LumiGLO chemiluminescent substrate were purchased from Cell Signaling Technology (Danvers, MA). The mammalian protease inhibitor cocktail was from Calbiochem (San Diego, CA). The Bradford colorimetric protein assay was purchased from Thermo Fisher Scientific (Waltham, MA), and the Pierce Cell Surface Protein Isolation Kit was purchased from Thermo Fisher Scientific. Pig kidney nucleoside transporter-deficient (PK15-NTD) cells were generously provided by Dr. Ming Tse (The Johns Hopkins University, Baltimore, MD). 5′-S-{2-(1-[(Fluorescein-5-yl)thioureido]-hexanamido)ethyl}-6-N-(4-nitrobenzyl)-5′-thioadenosine (FTH-SAENTA) was generously donated by Dr. John K. Buolamwini (The University of Tennessee Health Science Center, Memphis, TN).

Site-Directed Mutagenesis. Cys297, Cys333, Cys378, Cys414, Cys416, and Cys439 were mutated to serine onto the hENT1-p3XFLAG template using the QuikChange mutagenesis kit (Agilent Technologies, Santa Clara, CA) as instructed by the manufacturer. Cys416 and Cys439 were also mutated to alanine for some aspects of the study. All cysteine mutants were transformed into a XL1-Blue strain of *Escherichia coli*, amplified, purified by the Qiagen Miniprep Kit (QIAGEN, Valencia, CA), and sequenced at the London Regional Genomics Centre (London, ON, Canada).

Stable Cell Line Generation. Cysteine mutants were expressed in PK15-NTD cells as described previously (Park et al., 2011). In brief, cells were cultured in modified Eagle's medium supplemented with 10% BGS, 100 units of penicillin, 100 μg/ml streptomycin, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate at 37°C in 5% CO$_2$ and plated (90% confluent) onto 12-well plates. Cells were transfected with plasmid DNA using Lipofectamine 2000 at a 1:3 ratio (4.8 μl of Lipofectamine 2000 and 1.6 μg of plasmid DNA). After a 24-h incubation period, cells were placed under selection for 3 weeks using G418 at a concentration of 500 μg/ml. Individual colonies surviving the selection period were isolated and grown in the above medium supplemented with a lower G418 concentration (300 μg/ml) at 37°C in 5% CO$_2$.

Transient Transfections. PK15-NTD cells were transfected with p3XFLAG (vector control), hENT1-p3XFLAG, and hENT1-cysteine mutants-p3XFLAG using Lipofectamine LTX following the manufacturer's instruction (Invitrogen). In brief, 18.75 μg of plasmid DNA was incubated with 3.75 ml of Opti-MEM medium, 18.75 μl of Plus reagent, and 46.87 μl of Lipofectamine LTX for 30 min. The DNA-Lipofectamine complex was then added slowly to near (90%) confluent cells in T75 flasks void of antibiotics, and the cells were then incubated at 37°C and 5% CO$_2$ for at least 24 h before harvesting for use in experimental procedures.

Cell Membrane Preparations. PK15-NTD cells expressing wild-type hENT1 (hENT1-WT) or hENT1-mutants were harvested with 0.05% trypsin-0.53 mM EDTA, diluted with medium containing 10% (v/v) BGS, collected by centrifugation at 6000g and washed twice with PBS and finally suspended in 5 mM sodium phosphate buffer (containing a mammalian protease inhibitor cocktail). Cells were incubated for 30 min on ice to induce cell swelling and then were sonicated using a Sonic Dismembrator model 150 for 30 s and centrifuged at 3000g for 30 min at 4°C to pellet nuclei and unbroken cells. The supernatant containing the crude cell membranes was then centrifuged for 1 h at 30,000g at 4°C. The membrane pellet was suspended in 5 mM sodium phosphate buffer (plus protease inhibitors), and protein content was determined by the Bradford colormetric assay (Bradford, 1976).

Treatment with MTS Reagents. Cells were harvested using 0.05% trypsin-0.53 mM EDTA, diluted with medium containing 10% (v/v) BGS, collected by centrifugation at 6000g, and washed twice with, and ultimately suspended in, PBS (137 mM NaCl, 6.3 mM Na$_2$HPO$_4$, 2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, 0.5 mM MgCl$_2 \cdot$ 6H$_2$O, and 0.9 mM CaCl $\cdot$ 2H$_2$O, pH 7.4, 22°C) or sodium-free N-methylglucamine (NMG) buffer (140 mM NMG, 5 mM KCl, 4.2 mM KHCO$_3$, 0.36 mM K$_2$HPO$_4$, 0.44 mM KH$_2$PO$_4$, 10 mM HEPES, 0.5 mM MgCl$_2$, and 1.3 mM CaCl$_2$, pH 7.25, 22°C) for [^{3}H]NBMPR binding or [^{3}H]2-chloroadenosine uptake assays, respectively. Cells were treated with 0.1% DMSO (controls) or MTS reagent (1 mM MMTS, 5 mM MTSET, and 5 mM MTSES) for 10 min at room temperature We previously showed via a concentration/time course analysis that a 10-min incubation at the MTS concentrations used in this study was sufficient to achieve a maximal effect of each of the reagents on hENT1 activity. (Park et al., 2011). In some cases, 1 mM adenosine or 10 nM NBMPR was included with the MTS reagent incubation protocol to assess the ability of these ENT1 ligands to protect against MTS reagent modification of the cysteines. After the incubation period, cells were washed (6000g for 3 min) three times with PBS or NMG to remove residual MTS reagent and then resuspended in the appropriate buffer for subsequent experimental analyses.

[^{3}H]NBMPR Binding. Cells ($\sim$75,000 cells/assay) suspended in PBS were incubated with a range of concentrations of [^{3}H]NBMPR for 45 min at room temperature ($\sim$22°C). Cells were then collected on Whatman Binder-Free Glass Microfiber Filters type 934-AH using a 24-port Brandel cell harvester, washed twice with Tris buffer (10 mM, pH 7.4, 4°C), and analyzed for ^{3}H content using standard liquid scintillation counting techniques. Specific binding was defined as total binding minus cell-associated [^{3}H]NBMPR in the presence of 10 μM NBTGR (nonspecific binding).

FTH-SAENTA Inhibition Assay. PK15-NTD cells transfected with hENT1-WT, hENT1-C416A, or hENT1-C439A were incubated with 5 nM [^{3}H]NBMPR for 40 min in the presence and absence of 100 nM FTH-SAENTA (membrane-impermeable) or 10 μM NBTGR (membrane-permeable) and then processed as described above for the [^{3}H]NBMPR binding assays. FTH-SAENTA would be expected to displace only the [^{3}H]NBMPR bound to hENT1 on the plasma membrane because the large fluorescein tag prevents access to intracellular pools (Visser et al., 2007; Paproski et al., 2008).

[^{3}H]2-Chloroadenosine Uptake. Uptake was initiated by the addition of suspended cells ($\sim$750,000 cells/assay) in NMG buffer to [^{3}H]2-chloroadenosine layered over 200 μl of silicon-mineral oil (21:4, v/v) in 1.5-ml microcentrifuge tubes. Parallel assays were conducted

in the absence (total uptake) and presence (nonmediated uptake) of 5 μM NBMPR-dipyridamole to define the transporter-mediated uptake (total minus nonmediated uptake) of substrate in each cell treatment condition. After a defined incubation time, uptake was terminated by centrifugation for 10 s ($\sim$12,000g). Aqueous substrate and oil layers were removed by aspiration, and pelleted cells were digested in 1 M sodium hydroxide overnight (12–16 h). A sample of the digest was removed and analyzed for [3]H content using standard liquid scintillation counting techniques. Uptake data are presented as picomoles per microliter of intracellular volume after correction for the amount of extracellular [3]H in the cell pellet. Total volume was determined by incubating cells with [3]H$_2$O for 3 min and processing as above. Extracellular water space was estimated by extrapolation of the linear time course of nonmediated uptake to zero time.

Cell Surface Biotinylation. The biotinylation and isolation of cell surface proteins for Western blot analysis was performed as per the manufacturer's instructions (Thermo Fisher Scientific). In brief, PK15-NTD cell monolayers transiently transfected with hENT1-WT or hENT1-C416A (or untransfected controls) were washed with ice-cold PBS and incubated for 30 min with membrane-impermeable Sulfo-NHS-SS-Biotin on a rocking platform at 4°C. The cells were then harvested and washed with Tris-buffered saline (TBS) by centrifugation at 500g for 3 min. Cell pellets were lysed, sonicated, and vortex-mixed periodically on ice for 30 min. Cell lysate was centrifuged at 10,000g for 2 min at 4°C after which the supernatant was added to a column of NeutrAvidin Agarose and incubated for 60 min at 20°C with end-over-end mixing. The column was then washed extensively with TBS, and biotinylated proteins were eluted from the column using end-over-end mixing for 60 min with SDS-polyacrylamide gel electrophoresis sample buffer containing dithiothreitol.

The isolated biotinylated protein samples were loaded into 12% polyacrylamide gels (1.5 M Tris, pH 8.8, 0.1% SDS, 30% bis-acrylamide, 0.05% ammonium persulfate, and 0.05% N,N,N',N'-tetramethylethylenediamine) and resolved in the Mini-PROTEAN Tetra Cell electrophoresis system for $\sim$1 h at 120 V. After electrophoresis, gels were transferred to polyvinylidene fluoride (PVDF) membranes using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell at 440 mA, 20 V limit for 45 min. Membranes were blocked for 1 h at room temperature with 5% skim milk-TBS-Tween buffer (TBST) (0.5 mM Tris, 13.8 mM NaCl, 2.7 mM KCl, and 0.05% Tween 20) and then incubated overnight at 4°C with primary monoclonal mouse anti-FLAG antibody (1:2500 in 5% skim milk-TBST). Membranes were washed with TBST and incubated for 1 h at room temperature with secondary goat anti-mouse horseradish peroxidase-conjugated antibody (1:25,000 in 5% skim milk-TBST), and then washed further with TBST. Membranes were incubated for 1 min with LumiGLO chemiluminescent reagent and then imaged using the Molecular ImagerVersaDoc 5000 MP System (Bio-Rad Laboratories, Hercules, CA). Na$^+$/K$^+$-ATPase was used as a plasma membrane loading control. PVDF membranes were stripped with mild stripping buffer (200 mM glycine, 1% Tween 20, and 0.1% SDS; pH 2.2), washed twice each with PBS and TBST before being blocked for 1 h with 5% skim milk. The PVDF membranes were then incubated overnight at 4°C with primary mouse antibody to Na$^+$/K$^+$-ATPase (1:2500 in 5% skim milk-TBST). After three washes with TBST, membranes were incubated for 1 h at room temperature with secondary goat anti-mouse horseradish peroxidase-conjugated antibody (1:25,000 in 5% skim milk-TBST). After a final three washes, the PVDF membranes were visualized as described above.

Data Analysis and Statistics. Data are presented as means ± S.E.M. fitted to rectangular (one-site binding/uptake) hyperbolic curves using GraphPad Prism 5 software. Statistical analyses were performed using one-way analysis of variance (Dunnett's post-test) or Student's t test (paired or unpaired), as appropriate, with $P < 0.05$ considered significant.

Results

We showed previously that a 10-min incubation of intact cells with 1 mM MMTS enhanced the B_{max} of [^{3}H]NBMPR binding and decreased the V_{max} of [^{3}H]2-chloroadenosine uptake, whereas 5 mM MTSET caused a small but significant decrease in the B_{max} of [^{3}H]NBMPR binding and increased the V_{max} of [^{3}H]2-chloroadenosine uptake (Park et al., 2011). It was also determined that treatment with MMTS, MTSET, or MTSES decreased [^{3}H]NBMPR binding to hENT1 in broken cell preparations. Similar observations were made for the effects of these MTS reagents on hENT1-WT when tested intermittently throughout the conduct of the present work (Fig. 2, A and B).

Mutation of Cys297. hENT1-C297S-transfected cells bound [^{3}H]NBMPR with a K_d of 0.30 ± 0.04 nM and transported [^{3}H]2-chloroadenosine with a K_m of 61 ± 13 μM, which were not significantly different from the affinities obtained for hENT1-WT (Table 1). The B_{max} of [^{3}H]NBMPR binding and the V_{max} of [^{3}H]2-chloroadenosine uptake by hENT1-C297S cells were 1.3 ± 0.3 × 10^5 sites/cell and 5.7 ± 0.6 pmol · μl^{-1} · s^{-1}, respectively, giving a translocation rate of 68 ± 7 molecules · ENT1^{-1} · s^{-1}. When treated with MMTS, hENT1-C297S showed a 52 ± 4% enhancement in the B_{max} of [^{3}H]NBMPR binding with no significant change in K_d, as seen for the hENT1-WT (Fig. 2C; Table 2). However, MMTS did not affect [^{3}H]2-chloroadenosine uptake by hENT1-C297S (Fig. 2D; Table 2). Likewise, treatment with MTSET had no effect on [^{3}H]NBMPR binding to hENT1-C297S but still led to an increase in the V_{max} of [^{3}H]2-chloroadenosine uptake by these cells. MTSES did not affect [^{3}H]NBMPR binding or [^{3}H]2-chloroadenosine uptake by cells transfected with hENT1-C297S (Table 2).

Mutation of Cys333. hENT1-C333S-transfected cells bound [^{3}H]NBMPR with a K_d of 0.37 ± 0.06 nM (Table 1) to a maximum of 4.3 ± 0.4 × 10^5 sites/cell. Transport of [^{3}H]2-chloroadenosine by hENT1-C333S had a K_m and V_{max} of 39 ± 5 μM (Table 1) and 6.7 ± 0.7 pmol · μl^{-1} · s^{-1}, respectively, indicating that hENT1-C333S had a significantly higher affinity for [^{3}H]2-chloroadenosine than hENT1-WT. The substrate translocation rate was calculated as 24 ± 3 molecules · ENT1^{-1} · s^{-1}, which is similar to that determined for hENT1-WT (Park et al., 2011). MMTS treatment led to a 77 ± 13% enhancement of the B_{max} of [^{3}H]NBMPR binding to hENT1-C333S (Fig. 2E), similar to that seen for hENT1-WT (Table 2). In contrast, MMTS did not affect [^{3}H]2-chloroadenosine uptake by cells transfected with the hENT1-C333S mutant (Fig. 2F; Table 2) nor did MTSES or MTSET have any effect on [^{3}H]2-chloroadenosine uptake or [^{3}H]NBMPR binding by hENT1-C333S (Table 2).

Mutation of Cys378. hENT1-C378S-transfected cells bound [^{3}H]NBMPR with a K_d of 0.39 ± 0.03 nM (Table 1) to a B_{max} of 4.7 ± 0.4 × 10^5 sites/cell. [^{3}H]2-Chloroadenosine transport by hENT1-C378S had a V_{max} of 11 ± 1 pmol · μl^{-1} · s^{-1} and K_m of 57 ± 6 μM, providing a translocation rate of 35 ± 3 molecules · ENT1^{-1} · s^{-1}, similar to that of hENT1-WT. After treatment with MMTS, the B_{max} of [^{3}H]NBMPR binding to cells transfected with hENT1-C378S increased by 49 ± 14% and the V_{max} of [^{3}H]2-chloroadenosine transport decreased by 40 ± 12% (Table 2), again similar to that described for the hENT1-WT. However, unlike for hENT1-WT, MTSET and MTSES treatment of this mutant had no effect
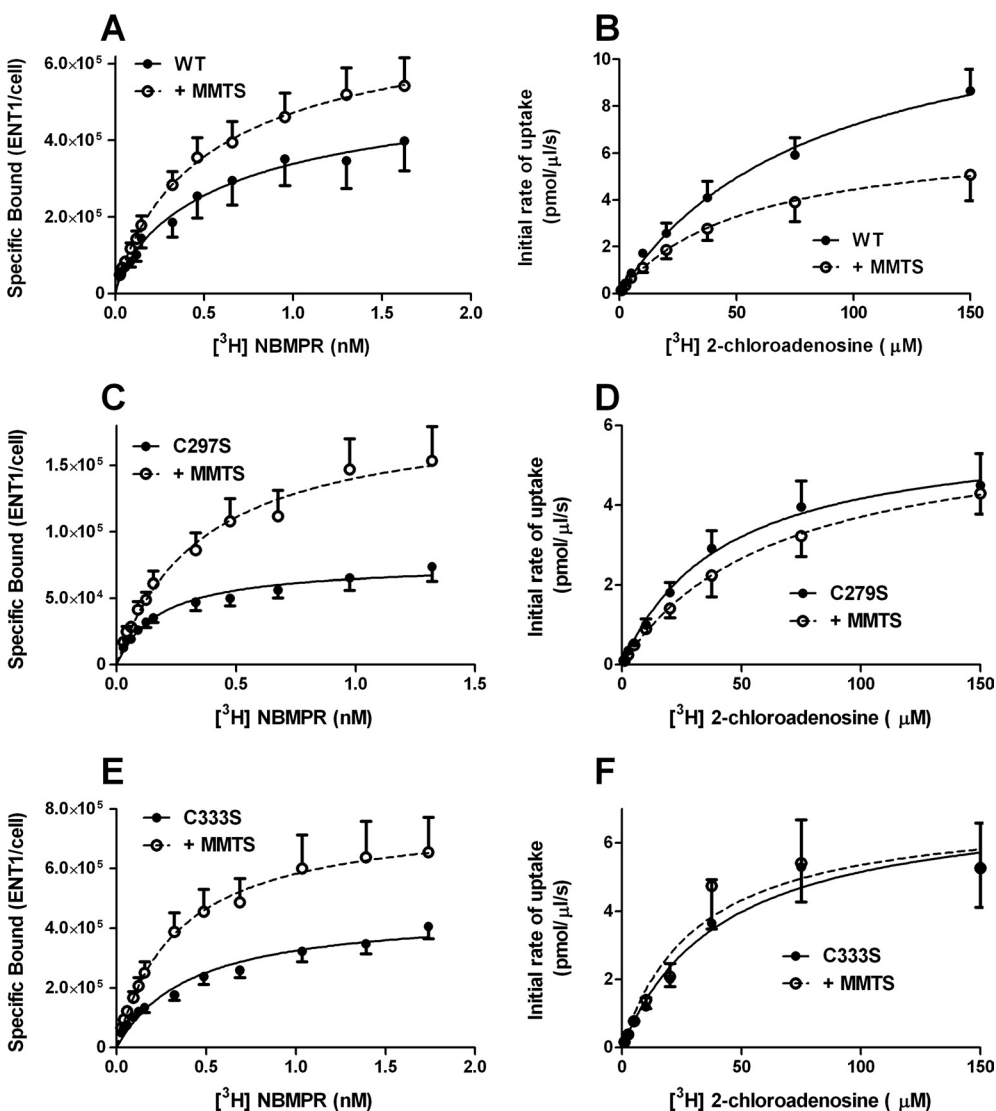
Fig. 2. Effects of MMTS on [³H]NBMPR binding and [³H]2-chloroadenosine uptake by hENT1-WT, hENT1-C297S, and hENT1-C333S expressed in PK15-NTD cells. Cells were incubated with either 1 mM MMTS or 0.1% DMSO (control) for 10 min and then washed three times before use in the following assays. A, C, and E, cells were incubated with a range of concentrations of [³H]NBMPR (abscissa) in the presence and absence of 10 μM NBTGR to define total and nonspecific binding. Specific binding (ordinate) was calculated as the difference between the total and nonspecific binding components. Each point represents the mean ± S.E.M. from at least five experiments done in duplicate. B, D, and F, cells were incubated with a range of concentrations of [³H]2-chloroadenosine (abscissa) for 5 s in the presence (nonmediated) or absence (total uptake) of 5 μM dipyridamole-NBTGR. Transporter-mediated uptake (ordinate) was calculated as the difference between the total and nonmediated uptake components. Each point represents the mean ± S.E.M. of the cellular accumulation of [³H]2-chloroadenosine from at least four independent experiments conducted in duplicate. The hENT1-WT cells were assessed in parallel with the hENT1-C297S transfected cells.

TABLE 1
Affinity of [³H]NBMPR and [³H]2-chloroadenosine for binding and uptake, respectively, by hENT1 cysteine mutants expressed in PK15-NTD cells

Data shown are the means ± S.E.M. from at least three independent experiments conducted as described in the legends to Figs. 2 to 6.

Cell Line	[³H]NBMPR Binding, K_d	[³H]2-Chloroadenosine Uptake, K_m
	nM	*μM*
hENT1-WT[a]	0.38 ± 0.02	71 ± 8
C297S	0.30 ± 0.04	62 ± 13
C333S	0.37 ± 0.06	40 ± 5.4*
C378S	0.39 ± 0.03	58 ± 6.2
C414S	0.45 ± 0.05	35 ± 5.0*
C378S/C414S	0.22 ± 0.03*	87 ± 18
C416A	0.14 ± 0.02*	N.D.
C439A	0.47 ± 0.10	165 ± 31*

N.D., not detected.
* Significant difference compared with hENT1-WT (Student's *t* test for paired samples, $P < 0.05$).
[a] Data from Park et al. (2011).

on either [³H]NBMPR binding (Fig. 3A) or [³H]2-chloroadenosine transport (Table 2).

Mutation of Cys414. hENT1-C414S-transfected cells bound [³H]NBMPR with a K_d of 0.45 ± 0.05 nM (Table 1) to a maximum of 2.1 ± 0.2 × 10⁵ sites/cell. [³H]2-Chloroadenos-

ine transport by these cells had a V_{max} and K_m of 6.3 ± 0.4 pmol · μl⁻¹ · s⁻¹ and 35 ± 5 μM (Table 1), respectively, providing a translocation rate of 45 ± 3 molecules · ENT1⁻¹ · s⁻¹. Treatment with MMTS increased [³H]NBMPR binding B_{max} by 98 ± 12%, which was significantly higher than this effect of MMTS on hENT1-WT or any of the other mutants tested (Table 2). MMTS treatment of hENT1-C414S cells also decreased the V_{max} of [³H]2-chloroadenosine transport by 30 ± 13%, which is similar to that seen for hENT1-WT (Table 2). MTSES did not affect [³H]NBMPR binding or [³H]2-chloroadenosine transport by the hENT1-C414S-transfected cells (Table 2). MTSET, on the other hand, had a significantly greater inhibitory effect on the binding of [³H]NBMPR to cells transfected with hENT1-C414S (50 ± 9% decrease in B_{max}) relative to hENT1-WT (Fig. 3B; Table 2).

Simultaneous Mutation of Cys378 and Cys414. Because mutation of the intracellular Cys414 led to an enhancement of the effect of membrane-impermeable MTSET on the binding of [³H]NBMPR and mutation of the predicted extracellular Cys378 eliminated the MTSET sensitivity of hENT1-WT (Fig. 3A; Table 2), we proposed that the accessibility of Cys378 to MTSET was being influenced by the Cys414 mutation via a conformation change in TM10. To test

TABLE 2
Effects of MTS reagents on [^{3}H]NBMPR binding and [^{3}H]2-chloroadenosine uptake by cells transfected with hENT1 cysteine mutants

Data shown are the means ± S.E.M. from at least three independent experiments conducted as described in Figs. 2 to 5.

Cell Line	MTS Reagent	[^{3}H]NBMPR Binding, B_{max}		[^{3}H]2-Chloroadenosine Uptake, V_{max}	
		Control	Treated	Control	Treated
		sites/cell $\times 10^5$		*pmol · μl^{-1} · s^{-1}*	
hENT1-WT[a]	MMTS	3.9 ± 0.4	5.6 ± 0.4*	10.8 ± 1.1	6.4 ± 1.0*
	MTSET	3.6 ± 0.4	3.2 ± 0.3*	9.1 ± 1.6	11.3 ± 1.7*
	MTSES	4.1 ± 0.5	4.0 ± 0.7	8.1 ± 1.2	7.6 ± 1.1
C297S	MMTS	1.2 ± 0.2	1.9 ± 0.3*	6.8 ± 0.8	6.6 ± 0.03
	MTSET	1.7 ± 0.4	1.3 ± 0.2	5.1 ± 1.6	8.1 ± 1.8*
	MTSES	1.0 ± 0.2	0.9 ± 0.1	6.5 ± 1.7	6.2 ± 0.2
C333S	MMTS	4.3 ± 0.5	7.7 ± 1.5*	7.8 ± 1.6	9.8 ± 2.4
	MTSET	3.5 ± 0.4	3.0 ± 0.8	5.8 ± 1.2	5.7 ± 0.8
	MTSES	5.0 ± 1.1	4.6 ± 1.4	6.2 ± 0.2	6.8 ± 0.9
C378S	MMTS	5.1 ± 0.7	7.6 ± 1.2*	17 ± 2.9	9.8 ± 1.3*
	MTSET	4.5 ± 0.5	4.3 ± 0.8	12 ± 1.5	12 ± 1.2
	MTSES	4.5 ± 0.6	4.2 ± 0.7	3.7 ± 0.6	3.2 ± 1.2
C414S	MMTS	1.5 ± 0.3	3.1 ± 0.4*	6.7 ± 0.9	4.6 ± 0.7*
	MTSET	2.0 ± 0.3	1.1 ± 0.2*	6.6 ± 0.7	5.8 ± 0.5
	MTSES	2.4 ± 0.4	2.4 ± 0.4	5.1 ± 0.2	5.7 ± 0.6
C378S/C414S	MMTS	6.1 ± 0.3	10 ± 0.8*	25 ± 4.2	7.8 ± 1.3*
	MTSET	3.2 ± 0.3	3.0 ± 0.3	24 ± 10	29 ± 18
	MTSES	5.6 ± 2.0	5.8 ± 2.0	15 ± 3.6	13 ± 2.5

* Significant difference from control (Student's *t* test for paired samples, $P < 0.05$).
[a] Data from Park et al. (2011).

this hypothesis, Cys378 and Cys414 were both mutated to serine and then were tested for MTS reagent sensitivity. Cells transfected with hENT1-C378S/C414S bound [^{3}H]NBMPR with a K_d of 0.22 ± 0.03 nM to a B_{max} of 4.6 ± 0.6 × 10^{-5} sites/cell (Table 1). hENT1-C378S/C414S was able to transport [^{3}H]2-chloroadenosine with a V_{max} and K_m of 21 ± 5 pmol · μl^{-1} · s^{-1} and 87 ± 18 μM (Table 1), respectively, providing a translocation rate for the double mutant of 69 ± 16 molecules · ENT1^{-1} · s^{-1}. After treatment with MMTS, the B_{max} of [^{3}H]NBMPR binding to the hENT1-C378S/C414S-transfected cells increased by 65 ± 15% and [^{3}H]2-chloroadenosine transport V_{max} showed a large decrease of 69 ± 8% (Table 2), similar to that for hENT1-WT. However, treatment of this mutant with MTSET had no effect on either [^{3}H]NBMPR binding or [^{3}H]2-chloroadenosine uptake (Fig. 3C; Table 2), supporting the hypothesis that Cys378 is the target for MTSET effects on hENT1 and that mutation of Cys414 was influencing the accessibility of Cys378 to MTSET.

Mutation of Cys416 and Cys439. Mutation of Cys416 or Cys439 to either serine or alanine led to no [^{3}H]2-chloroadenosine transport and no [^{3}H]NBMPR binding in stably transfected cells, nor could hENT1 protein be detected by immunoblotting. mRNA for the mutant hENT1 was clearly identified by PCR in these cells (data not shown), so the defect appeared to lie in the production and/or stability of the protein. We speculated initially that the cytoplasmically located Cys416 could be palmitoylated, and thus its removal interfered with proper processing to the plasma membrane. However, treatment of hENT1-WT cells with 100 μM 2-Br, a known blocker of palmitoylation, for 24 or 48 h in serum-free medium had no effect on [^{3}H]NBMPR binding or on [^{3}H]2-chloroadenosine transport (Fig. 4).

To circumvent the probability that the inability to stably express these mutants was due to a cellular unfolded-protein response mechanism, we adopted a transient transfection approach. Transient transfection with hENT1-C416A led to the appearance of high-affinity [^{3}H]NBMPR binding with a K_d of 0.14 ± 0.02 and B_{max} of 4.6 ± 2.3 × 10^5 sites/cell,

similar to that seen for hENT1-WT (Fig. 5A; Table 1). However, these cells did not transport [^{3}H]2-chloroadenosine (Fig. 5B). Cell surface biotinylation analysis confirmed that hENT1-C416A was expressed at the plasma membrane (Fig. 6A). Furthermore, 79 ± 7% of the specific binding of [^{3}H]NBMPR by cells expressing hENT1-C416A could be inhibited by the membrane-impermeable FTH-SAENTA, again indicating that the majority of the hENT1-C416A protein was exposed on the plasma membrane(Fig. 6B). Furthermore, 2-chloroadenosine was able to inhibit the binding of [^{3}H]NBMPR to C416A membranes with a K_i of 37 ± 8 μM, which is similar to that seen for its inhibition of [^{3}H]NBMPR to membranes prepared from cells transfected with hENT1-WT (70 ± 19 μM). Cells transiently transfected with hENT1-C439A bound [^{3}H]NBMPR with an affinity (K_d) of 0.47 ± 0.10 and a B_{max} of 8.6 ± 1.4 × 10^5 sites/cell and, in contrast with C416A, transported [^{3}H]2-chloroadenosine with a V_{max} of 8.4 ± 0.8 pmol · μl^{-1} · s^{-1} and K_m of 165 ± 32 μM (Fig. 5; Table 1). The apparent translocation rate of hENT1-C439A was thus 15 ± 2 molecules · ENT1^{-1} · s^{-1}, which is significantly less than that determined for hENT1-WT (38 ± 3 molecules · ENT1^{-1} · s^{-1}) (Park et al., 2011) and the other mutants tested.

The effects of the Cys416 and Cys439 mutations on the sensitivity of hENT1 to MTSET and MTSES were then assessed in isolated membranes prepared from cells transiently transfected with hENT1-WT, C416A, or C439A. As shown previously (Park et al., 2011), MTSET and MTSES treatment of membranes prepared from hENT1-transfected cells led to a large decrease in the binding of [^{3}H]NBMPR (Fig. 7A; Table 3); similar decreases in binding were observed in MTS reagent-treated membranes prepared from C297S, C333S, C378S, and C414S stably transfected cells (data not shown). Likewise, MTSES and MTSET inhibited [^{3}H]NBMPR binding to the C439A mutant to a degree similar to that seen for hENT1-WT (Table 3). However, MTSES and MMTS treatment did not decrease [^{3}H]NBMPR binding in the C416A mutant (Fig. 7C; Table 3), and the effect of MTSET was attenuated significantly (Fig. 7B) relative to that for hENT1-WT.
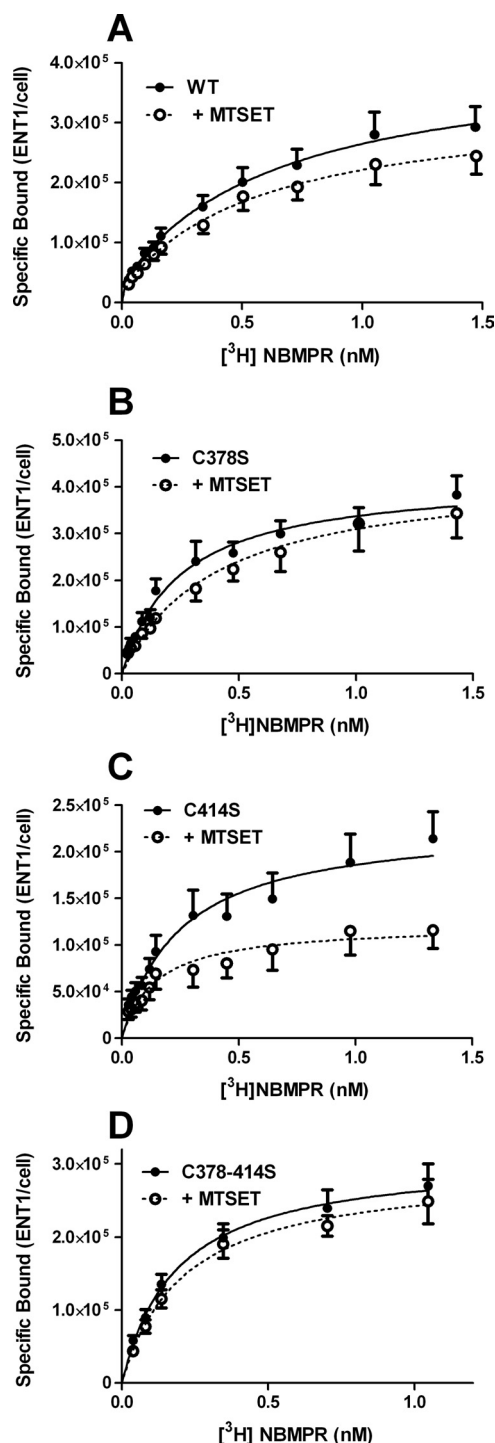
Fig. 3. Effects of MTSET on [³H]NBMPR binding by hENT1-WT (A), hENT1-C378S (B), hENT1-C414S (C), and C378–414S-hENT1 (D) expressed in PK15-NTD cells. Cells were incubated with either 5 mM MTSET or 0.5% DMSO (control) for 10 min, washed extensively, and then incubated with a range of concentrations of [³H]NBMPR in the presence and absence of 10 μM NBTGR to define total and nonspecific binding, respectively. Specific binding was calculated as the difference between the total and nonspecific binding components. Each point represents the mean ± S.E.M. from at least five experiments done in duplicate.

The inclusion of NBMPR (10 nM) or the hENT1 substrate adenosine (1 mM) in the MTSES treatment protocol did not affect the ability of MTSES to decrease [³H]NBMPR binding (Fig. 8).

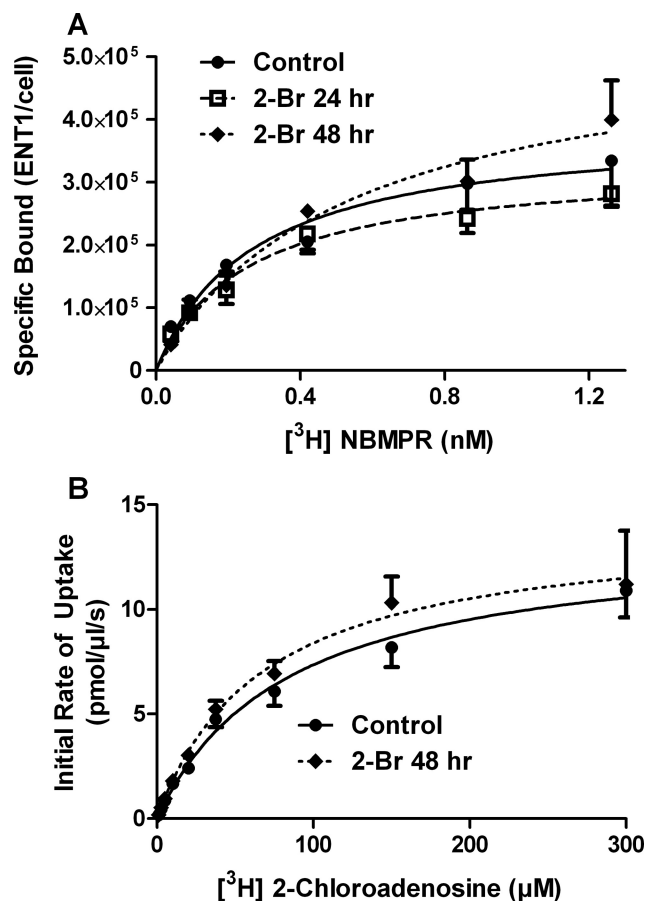**Fig. 4.** Effect of palmitoylation inhibition on [³H]NBMPR binding and [³H]2-chloroadenosine uptake by hENT1 expressed in PK15-NTD cells. Cells transfected with hENT1-WT were treated with the palmitoylation inhibitor 2-Br (100 μM) or DMSO (control) for 24 or 48 h in serum-free medium. Cells were harvested, washed, and subjected to either [³H]NBMPR binding (A) or [³H]2-chloroadenosine uptake (B) assays. Each point represents the mean ± S.E.M. from at least four independent experiments conducted in duplicate.

Discussion

This study, in conjunction with a previously published study by our laboratory (Park et al., 2011), has identified the cysteine residues responsible for the effects of sulfhydryl reagents on NBMPR binding and hENT1 transporter function. Treatment of intact cells with the positively charged MTSET reagent caused an inhibition in NBMPR binding to hENT1-WT, and mutation of Cys378 to serine abolished this effect. Furthermore, Cys416 appears to be the residue primarily responsible for the inhibitory effects of MTSET and MTSES on [³H]NBMPR binding when these MTS reagents are allowed access to the cytoplasmic side of the protein. The localization of Cys378 at the extracellular interface of TM9 and Cys416 in fifth intracellular loop (IL5), along with the interaction between Cys414 and Cys378, indicates that TM10 and its extracellular and cytoplasmic extensions play an important role in defining the NBMPR binding conformation. We established previously the importance of cysteines located in TM5 and TM6 of hENT1, in which Cys222 in TM6 was identified as the residue involved in the enhancement of NBMPR binding by the neutral thiol modifier MMTS, and Cys193 in TM5 was suggested to play a role in the effects of MMTS inhibition of 2-chloroadenosine uptake (Park et al.,
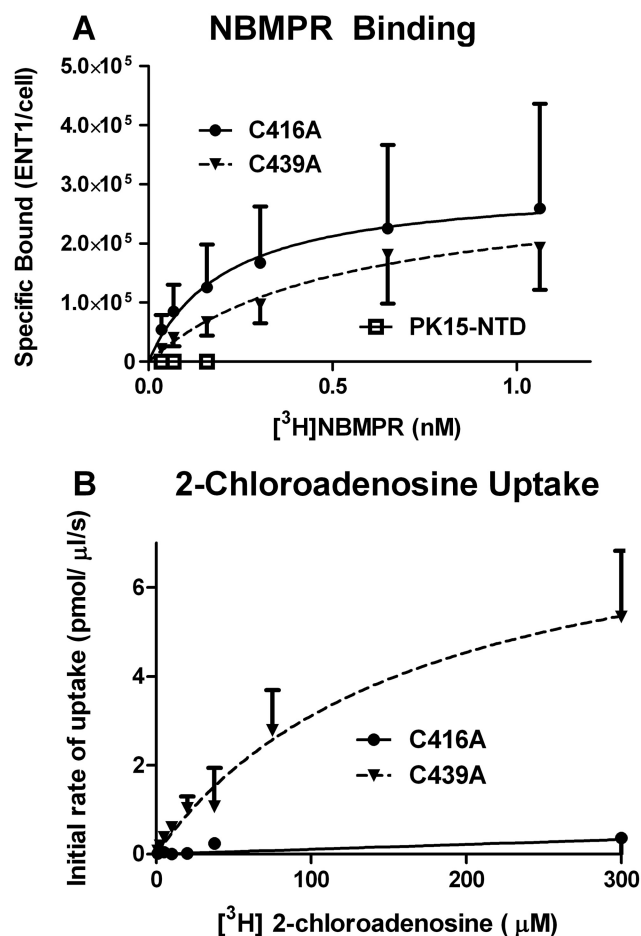
Fig. 5. [³H]NBMPR binding and [³H]2-chloroadenosine uptake by PK15-NTD cells transfected with hENT1-C416A or hENT1-C439A. A, cells were incubated with a range of concentrations of [³H]NBMPR in the absence (total binding) and presence (nonspecific binding) of 10 μM NBTGR. Specific binding was calculated as the difference between the total and nonspecific binding components. Each point represents the mean ± S.E.M. from at least five experiments done in duplicate. B, cells were incubated with a range of concentrations of [³H]2-chloroadenosine for 5 s in the presence (nonmediated) or absence (total uptake) of 5 μM dipyridamole-NBTGR. Transporter-mediated uptake was calculated as the difference between the total and nonmediated uptake components. Each point represents the mean ± S.E.M. of the cellular accumulation of [³H]2-chloroadenosine from at least four independent experiments conducted in duplicate.

Fig. 6. Analysis of the plasma membrane expression of hENT1 via biotinylation and FTH-SAENTA competition assays. A, untransfected PK15-NTD or cells transiently transfected with hENT1-WT or hENT1-C416A were cell surface-biotinylated and isolated via avidin-linked agarose columns as described under *Materials and Methods*. Recombinant hENT1-WT and hENT1-C416A were visualized with mouse anti-FLAG antibodies (1:2500), and blots were then stripped and probed with anti-Na⁺/K⁺-ATPase antibody (1:2500) to assess plasma membrane loading levels. B, cells transfected with hENT1-WT, C416A, or C439A were incubated with 5 nM [³H]NBMPR for 45 min in the absence or presence of either 100 nM FTH-SAENTA or 10 μM NBTGR. The total number of ENT1-associated NBMPR binding sites (specific binding) was calculated as the total binding of [³H]NBMPR (absence of inhibitors) minus nonspecific binding (in presence of the membrane-permeable 10 μM NBTGR). Cell surface binding was calculated as the difference in specific binding with and without coincubation with the membrane-impermeable inhibitor FTH-SAENTA. Data are shown as the ratio of cell surface binding to total ENT1-associated binding of [³H]NBMPR. Each bar represents the mean ± S.E.M. of three independent experiments.

2011). It is noteworthy that all of the mutants in that previous study (C87S, C193S, C213S, and C222S) retained high-affinity NBMPR binding. Likewise, all mutants in the present study (C297S, C333S, C378S, C414S, C416A, and C439A) bound NBMPR with high affinity (Table 1). These data suggest that none of the cysteines are involved directly in the binding of NBMPR but rather collectively play a role in maintaining the transporter in a conformation compatible with high-affinity NBMPR binding.

Mutation of Cys297, Cys333, and Cys378 resulted in the loss of MTSET-mediated inhibition of NBMPR binding, whereas mutation of the cytoplasmic Cys414 located in IL5 enhanced the ability of MTSET to inhibit the binding of NBMPR. MTSET, as a large membrane-impermeable charged reagent, would not gain access to the cytoplasmic Cys414 or probably to the transmembrane region-located Cys297 and Cys333. Thus, it was Cys378, located at the extracellular interface of TM9, that was probably being modified by MTSET. In support of this hypoth-
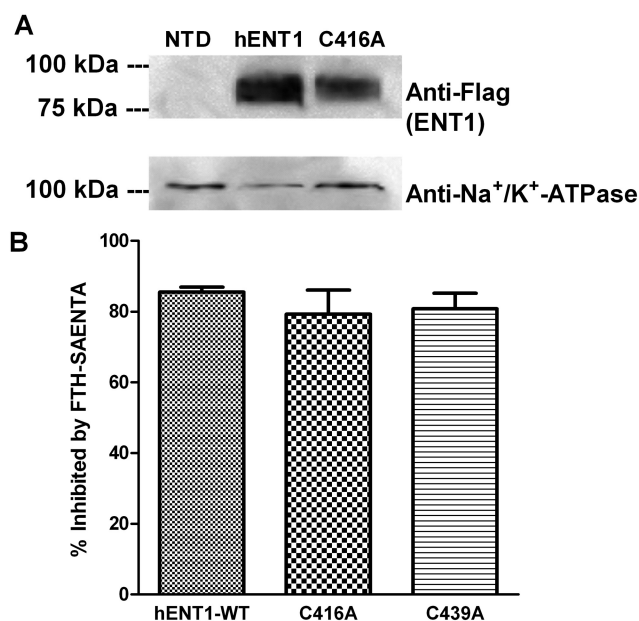
esis, mutation of Cys378 countered the effect of the Cys414 mutation on MTSET modification of NBMPR binding, with the double mutant being insensitive to MTSET. These results suggested that a mutation in the cytoplasmic portion of the transporter can change the conformation of TM10, leading to enhanced accessibility of the extracellular Cys378 to MTS reagents. A recent study by Yao et al. (2011) also highlighted a role for Cys414 in defining the nucleobase transport capability of hENT1 expressed in a *Xenopus laevis* oocyte model. It is possible that the conformation change induced in TM10 by the mutation of Cys414 affects the extracellular binding domain for ENT1 substrates and may involve Cys378. It must also be noted that we have not found any evidence for nucleobase (hypoxanthine) transport by recombinant hENT1 expressed in the PK15-NTD cells at concentrations of nucleobases less than 400 μM (J.R. Hammond and D.B.J. Bone, unpublished data). Thus, the nucleobase transport observed by hENT1 by Yao et al. may reflect the expression model used, or higher concentrations of nucleobases than we used are required to measure observable transporter-mediated uptake.

The present study found that mutation of Cys416 or Cys439 was incompatible with the stable expression of a functional nucleoside transport protein in PK15-NTD cells. However, transient expression of these constructs did result in the expression of hENT1 protein as defined by immuno-
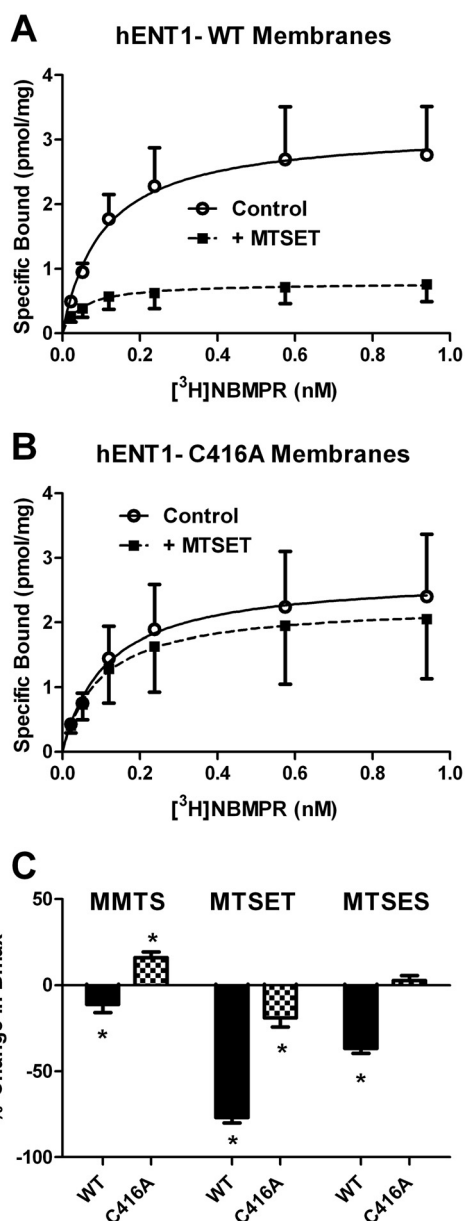
A   hENT1- WT Membranes

B   hENT1- C416A Membranes

C

Fig. 7. Effect of MTS reagents on [3H]NBMPR binding by PK15-NTD cells transiently transfected with hENT1-WT and hENT1-C416A. Membranes prepared from hENT1-WT or hENT1-C416A were incubated for 10 min in the absence or presence of 1 mM MMTS, 5 mM MTSET, or 5 mM MTSES for 10 min at room temperature, washed extensively, and then incubated with a range of concentrations of [3H]NBMPR in the presence and absence of 10 μM NBTGR to define total and nonspecific binding. A and B, binding profile ± MTSET in hENT1-WT and hENT1-C416A transfected cells, respectively. Each point represents the mean ± S.E.M. from at least five experiments done in duplicate. C, results obtained as described for A and B for each of the MTS reagents with data presented as the percentage change in specific binding ± S.E.M. upon treatment with each sulfhydryl modifier. Data for MTSES and MMTS are from three experiments conducted in duplicate. *, significant effect of the MTS reagent (Student's t test, $P < 0.05$).

blotting and NBMPR binding. This finding suggests that chronic expression of these mutant proteins was deleterious to cell function and were probably degraded via cellular unfolded-protein response mechanisms. Because cysteines can be palmitoylated and this contributes to protein trafficking and membrane tethering (Linder and Deschenes, 2007), we tested the hypothesis that Cys416 and Cys439 were targets

TABLE 3
Effects of MTS reagents on [3H]NBMPR binding to membranes prepared from cells transfected with hENT1-WT, C416A, or C439A
Membranes were prepared from cells 24 h after transfection with the indicated hENT1 construct. The isolated membranes were then assessed for their level of site-specific [3H]NBMPR binding as described for Fig. 7. Values shown are the means ± S.E.M. from at least three independent experiments conducted in duplicate.

Cell Line	MTS Reagent	[3H]NBMPR Binding, B_{max}	Treated
		pmol / mg	
hENT1-WT	MMTS	1.24 ± 0.34	1.03 ± 0.17
	MTSET	3.15 ± 0.94	0.78 ± 0.28*
	MTSES	3.19 ± 1.51	1.92 ± 0.85*
C416A	MMTS	1.23 ± 0.19	1.42 ± 0.29
	MTSET	2.73 ± 1.17	2.30 ± 1.14
	MTSES	2.33 ± 0.97	2.34 ± 0.97
C439A	MMTS	0.99 ± 0.23	0.78 ± 0.08
	MTSET	4.29 ± 1.78	1.13 ± 0.42*
	MTSES	1.57 ± 0.61	0.71 ± 0.25*

* Significant difference from control (Student's t test for paired samples, $P < 0.05$).

for this post-translational modification. Although Cys439 was predicted to be palmitoylated (CSS-Palm 3.0) (Ren et al., 2008), the palmitoylation inhibitor 2-Br had no effect on hENT1 function, suggesting that palmitoylation was not a factor. Additional motifs surrounding the areas of Cys416 and Cys439 include the GxxxG motif known for helix-helix interactions (Russ and Engelman, 2000; Polgar et al., 2004, 2010) and the endoplasmic reticulum retention signal KKVK (Cosson and Letourneur, 1994; Letourneur et al., 1994). A previous study on *Plasmodium falciparum* ENT1 indicated that the GxxxG motif in TM11 is important for folding and/or assembly (Riegelhaupt et al., 2010); we have also found that mutation of Gly445 (of the TM11 GxxxG motif of hENT1) led to a construct that did not result in any protein expression upon stable transfection in PK15-hENT1 cells (J. R. Hammond and F. K. Cunningham, unpublished data). It is noteworthy that mutation of Cys439 (one helix turn N-terminal of the GXXXG motif) to alanine led to a significantly lower K_d for NBMPR binding and K_m for 2-chloroadenosine uptake relative to those observed for hENT1-WT and the other cysteine mutants.

A striking finding of this study is that mutation of Cys416 in IL5 to alanine resulted in the transient expression of an hENT1 protein that could bind [3H]NBMPR with high affinity but did not show any [3H]2-chloroadenosine transport activity. We considered the possibility that this dissociation between transport and binding may reflect a redistribution of ENT1 proteins to intracellular membranes where they can bind NBMPR (which is membrane-permeable) but not contribute to the cellular uptake of nucleosides. However, cell surface biotinylation and FTH-SAENTA competition assays determined that mutation of Cys416 did not change the relative distribution of hENT1 between the plasma membrane and intracellular compartments. Likewise, 2-chloroadenosine was able to inhibit the binding of [3H]NBMPR with similar affinity in both the hENT1-WT and hENT1-C416A transfectants. Given that the ability of a substrate to inhibit the binding of [3H]NBMPR generally reflects its affinity as an hENT1 substrate, this finding suggests that the loss of [3H]2-chloroadenosine transport by hENT1-C416A was not due to a decline in transporter substrate affinity. Therefore, the C416A mutant may be compromised in terms of its substrate
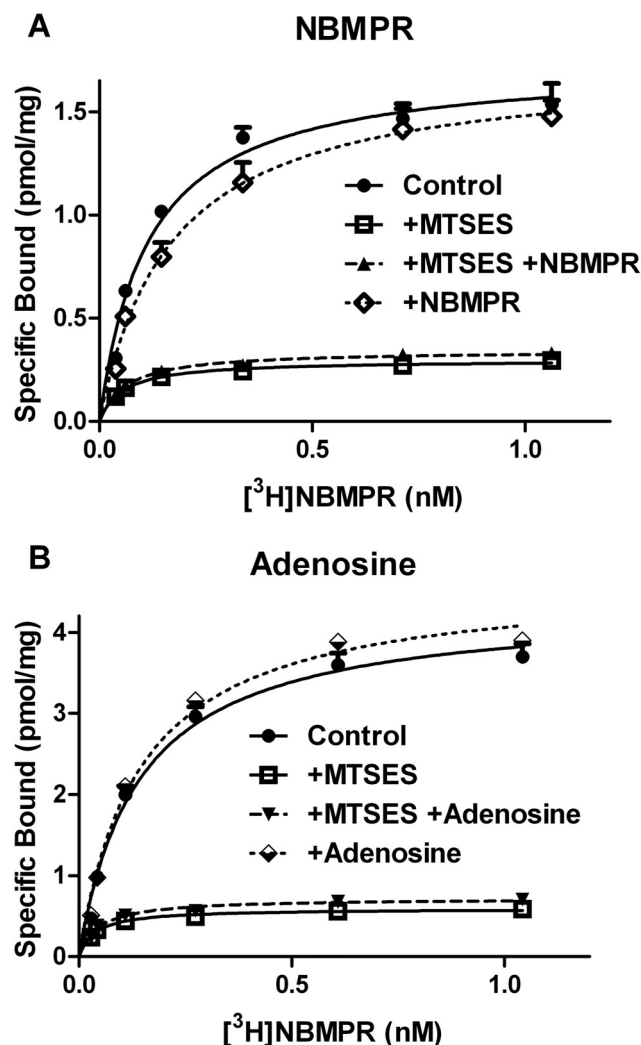
Fig. 8. NBMPR and adenosine are unable to protect against MTSES-induced inhibition of [3H]NBMPR binding in cell membranes. Membranes isolated from PK15-NTD cells transfected with hENT1-WT were incubated for 10 min at room temperature with 0.5% DMSO (control) or 5 mM MTSES in the absence and presence of 10 nM NBMPR (A) or 1 mM adenosine (B). After extensive washing to remove NBMPR/adenosine and unreacted MTSES, membranes were exposed to a range of concentrations of [3H]NBMPR in the presence and absence of 10 μM NBTGR to define the site-specific binding. Each point is the mean ± S.E.M. from at least four experiments conducted in duplicate.

translocation mechanism. An ab initio structural model of LdNT1.1, a *Leishmania* nucleoside transporter belonging to the ENT family (Valdés et al., 2009) predicts an inner bundle of TM helices (1, 2, 4, 5, 7, 8, 10, and 11) encompassing a hydrophilic cavity and the remaining TMs (3, 6, and 9) encircling the inner bundle. On the basis of that model, our results indicate that the cytoplasmic loop between TM10 and TM11 where Cys416 is located may be crucial to maintaining the integrity of the translocation pore. Although mutation of Cys416 to an alanine did not affect the binding of [3H]NBMPR, modification of this residue with bulky reagents such as MTSES did inhibit binding, giving further credence to the important role of this part of the protein in transporter function. Given that NBMPR is known to bind to the exofacial side of ENT1 and our finding that coincubation with the ENT1 substrate adenosine or the ENT1 inhibitor NBMPR did not prevent the effects of MTSES on [3H]NBMPR binding

to cell membranes, it is postulated that modification of Cys416 by the sulfhydryl reagents allosterically disrupts the conformation of the NBMPR binding pocket that is associated with the substrate translocation mechanism.

To conclude, our study has identified Cys378 as an extracellular-located residue modified by MTSET to inhibit the binding of NBMPR. The functional association of Cys378 and Cys414 indicates an allosteric interaction between TM9, TM10, and intracellular loop 5. Conjointly, this study has established Cys416 as the cytoplasmically located cysteine that is modified by charged sulfhydryl reagents to inhibit the binding of [3H]NBMPR. Cys416 may also have a central role in the substrate translocation mechanism of hENT1.

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