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Residue 33 of Human Equilibrative Nucleoside Transporter 2 Is a Functionally Important Component of Both the Dipyridamole and Nucleoside Binding Sites

Frank Visser, Jing Zhang, R. Taylor Raborn, Stephen A. Baldwin, James D. Young, and Carol E. Cass

Canadian Institutes of Health Research Membrane Protein Research Group (F.V., J.Z., R.T.R., J.D.Y, C.E.C.), Departments of Oncology (F.V., J.Z., R.T.R., C.E.C.) and Physiology (J.D.Y.), University of Alberta and the Cross Cancer Institute (F.V., J.Z., R.T.R., C.E.C.), Edmonton, Alberta, Canada; and the School of Biochemistry and Microbiology, University of Leeds, Leeds, United Kingdom (S.A.B.)

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ABSTRACT

Human equilibrative nucleoside transporters 1 and 2 (hENT1 and hENT2) differ functionally in that hENT2 generally displays lower affinity for its nucleoside permeants and is less sensitive to inhibition by the coronary vasodilators dilazep and dipyridamole. In previous work, we demonstrated that mutation of residues 33 (Met versus Ile) of hENT1 and hENT2 altered sensitivity to dilazep and dipyridamole and that the hENT2 mutant (I33M) displayed a K_m value for uridine that was lower than that of hENT2 and similar to that of hENT1 (*J Biol Chem* **277:**395–401, 2002). In this study, we report results of an in-depth investigation of the role of residue 33 in hENT2. We found that hENT2-I33M displayed decreased K_m values for both pyrimidine and purine nucleosides and increased V_{max} values for purine nucleosides. Cys or Ser at position 33 had similar effects on the kinetic parameters of hENT2 as Met, indicating that hydrophobic (Met and Cys) or hydrogen-bonding energy (Ser) contributed to permeant binding by these residues. hENT2-I33M and I33C displayed increased sensitivities to dipyridamole compared with wild-type hENT2, hENT2-I33A, and hENT2-I33S, suggesting interaction of the sulfur atom of Met and Cys with aromatic moieties on dipyridamole. hENT2-I33C was inhibited by the membrane-impermeant sulfhydryl reactive reagent *p*-chloromercuribenzyl sulfonate, and uridine, adenosine, and dipyridamole protected against inhibition. Our results indicated that residue 33 resides in an extracellular domain as predicted by the current hENT2 topology model and suggested that it is a functionally important component of both the permeant and dipyridamole binding sites.

Integral membrane proteins mediate the transport of hydrophilic nucleosides and anticancer or antiviral nucleoside analogs across biological membranes (Cass et al., 1999). Equilibrative nucleoside transporters (ENTs) in mammalian cells mediate facilitated diffusion of nucleosides down their concentration gradients. Four ENT family members have been identified by molecular cloning: hENT1, hENT2, hENT3, and hENT4. hENT1 and hENT2 display equilibrative-sensitive (es) and equilibrative-insensitive (ei) transport activities, respectively, based on their differential sensitivities to the inhibitor nitrobenzylmercaptopurine ribonucleoside (NBMPR) (Griffiths et al., 1997a,b; Crawford et al., 1998). Neither hENT3 nor hENT4 has been functionally characterized, but hENT3 is believed to be a transporter of intracellular membranes (Hyde et al., 2001; Acimovic and Coe, 2002). Although hENT1 and hENT2 mediate the transport of a broad variety of nucleoside permeants, kinetic analyses have revealed that hENT2 generally displays lower affinities (higher K_m values) for its permeants, and, unlike hENT1, can also transport nucleobases (Ward et al., 2000; Yao et al., 2002). The amino acid residues responsible for these functional differences have not been identified.

ENT proteins control extracellular concentrations of aden-

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ABBREVIATIONS: ENT, equilibrative nucleoside transporter; ei, equilibrative insensitive; es, equilibrative sensitive; NBMPR, nitrobenzylmercaptopurine ribonucleoside; 6-[(4-nitrobenzyl)thiol]-9-β-D-ribofuranosyl purine; TM, transmembrane domain; hENT, human equilibrative nucleoside transporter; pCMBS, *p*-chloromercuribenzyl sulfonate; CMM, complete minimal medium.

osine, a ligand for cell surface adenosine receptors that facilitates a variety of physiological responses, such as coronary vasodilation, renal vasoconstriction, platelet aggregation, and neuromodulation (Van Belle, 1993). hENT1 and hENT2 are the pharmacological targets of the coronary vasodilators dilazep and dipyridamole and differ in their sensitivities to these inhibitors by 2 to 3 orders of magnitude, with hENT1 being more sensitive (Visser et al., 2002).

Despite limited sequence identities, all members of the ENT family share a common putative topology model consisting of 11 transmembrane domains (TMs), a large extracellular loop between TMs 1 and 2, and a large cytoplasmic loop between TMs 6 and 7 (Sundaram et al., 2001a). The current level of knowledge of the structure and function of these transporter proteins is limited. A number of studies on chimeric constructs involving domain swaps between different members of the ENT family have implicated TMs 3 to 6 as a region involved in permeant and inhibitor binding (Sundaram et al., 1998, 2001b; Yao et al., 2001b, 2002). In addition, Cys 140 in TM 4 of rat ENT2 has been demonstrated by sulfhydryl modification experiments to form part of the permeant translocation pore, and the corresponding residue of hENT1, Gly 154, is critical for NBMPR sensitivity (Yao et al., 2001a; SenGupta and Unadkat, 2004). Other mutagenesis studies have identified Gly 179 in TM 4 and Leu 92 in TM 2 of hENT1 as residues that, when mutated, impair inhibitor binding and transporter function (SenGupta et al., 2002; Endres et al., 2004). Single residues in TMs 5, 7, and 8 of the LdNT transporters, which are ENT family members from the parasitic protozoan *Leishmania donovani*, were demonstrated to play important roles in transporter function (Vasudevan et al., 2001; Arastu-Kapur et al., 2003). Furthermore, by use of the substituted cysteine accessibility method, TM 5 of LdNT1.1 was shown to line the permeant translocation pathway (Valdes et al., 2004).

In previous work, we found that mutation of Met 33 of hENT1 to Ile, the corresponding residue in hENT2, resulted in ~10-fold reduced affinities for dilazep and dipyridamole, whereas the reciprocal mutation of Ile 33 of hENT2 to Met resulted in ~10-fold increased sensitivities to these inhibitors (Visser et al., 2002). hENT1-M33I displayed similar kinetic parameters for uridine transport to those of wild-type hENT1, whereas hENT2-I33M displayed kinetic parameters that were more similar to those of hENT1 than hENT2. In this work, we used an improved method for the functional characterization of recombinant hENT1 and hENT2 in yeast (Visser et al., 2002; Zhang et al., 2003; Vickers et al., 2004) to determine the kinetic properties of hENT1-M33I, hENT2-I33M, and a series of hENT2 mutants at residue 33 for a variety of different nucleoside permeants. These experiments revealed that hENT2-I33M had higher transport activities than wild-type hENT2 for all the permeants tested, whereas hENT1-M33I was functionally similar to wild-type hENT1. The residue 33 hENT2 mutants were also tested for their sensitivities to dipyridamole and the membrane-impermeant sulfhydryl-reactive reagent *p*-chloromercuribenzyl sulfonate (pCMBS). The results of these studies indicated that residue 33 was accessible from the extracellular side of the membrane and suggested that it is a common functional determinant of the nucleoside and dipyridamole binding sites.

Materials and Methods

Strains and Media. KY114 (*MATα*, *gal*, *ura3-52*, *trp1*, *lys2*, *ade2*, *hisd2000*) was the parental yeast strain used to generate fui1::TRP1, which contains a disruption in the gene encoding the endogenous uridine permease (FUI1) (Vickers et al., 2000). Other strains were generated by transformation of the *Saccharomyces cerevisiae/Escherichia coli* shuttle vector pYPGE15 (Brunelli and Pall, 1993) using a standard lithium acetate method (Gietz et al., 1992). cDNA inserts were under the transcriptional control of the constitutive phosphoglycerate kinase gene promoter. Yeast strains were maintained in complete minimal medium (CMM) containing 0.67% yeast nitrogen base (Difco, Detroit, MI), amino acids (as required to maintain auxotrophic selection), and 2% glucose. Plasmids were propagated in the *E. coli* strain TOP10F′ (Invitrogen, Carlsbad, CA) and maintained in Luria broth with 0.1 mg/ml ampicillin.

Plasmid Construction and Site-Directed Mutagenesis. The cDNAs encoding hENT1, hENT1-M33I, hENT2, and hENT2-I33M were subcloned into pYPGE15 to generate pYPhENT1, pYPhENT1-M33I, pYPhENT2, and pYPhENT2-I33M as described previously (Visser et al., 2002). pYPhENT2-I33C, pYPhENT2-I33A, and pYPhENT2-I33S were generated using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). All constructs were verified by DNA sequencing using an ABI PRISM 310 sequence detection system (Applied Biosystems, Foster City, CA).

Nucleoside Transport in *S. cerevisiae*. Yeast cells containing pYPhENT1, pYPhENT2, or plasmid with one of the constructs encoding the various mutant transporters were grown in CMM to A_{600} = 0.5 to 1.0, washed twice in fresh medium, and resuspended to A_{600} = 4.0. All transport assays were performed at room temperature and pH 7.4. All unlabeled nucleosides and nucleoside analogs, dilazep, dipyridamole, and NBMPR were obtained from Sigma-Aldrich (St. Louis, MO). The radiolabeled compounds [5,6-^{3}H]uridine, [5-^{3}H(N)]-cytidine, [methyl-^{3}H]thymidine, [2,8-^{3}H]adenosine, [2,8-^{3}H]inosine, [8-^{3}H]guanosine, [6-^{3}H]5-fluorouridine, [5-^{3}H]2′,2′-difluoro-2′-deoxycytidine (gemcitabine), [5-^{3}H]cytosine-β-D-arabinofuranoside (cytarabine), [8-^{3}H]2-chloro-2′-deoxyadenosine (cladribine), and [8-^{3}H]2-fluoroarabinofuranosyl adenine (fludarabine) were purchased from Moravek Biochemicals (Brea, CA). A final specific activity of 0.5 μCi/μl was used in all transport reactions. Fifty-microliter portions of yeast culture were added to 50-μl portions of 2× concentrated ^{3}H-nucleoside in 96-well microtiter plates. At a given time, the yeast cells were collected on a Filtermat using a Micro96 cell harvester (Skatron Instruments, Lier, Norway) and rapidly washed with deionized water. The individual filter circles corresponding to individual wells of microtiter plates were removed from the Filtermats using forceps and transferred to vials for liquid scintillation counting. Trace uridine transport activity in fui1::TRP1 yeast caused by the presence of the endogenous uracil/uridine permease FUR4 was subtracted by determining background uptake in the presence of 10 mM thymidine, which does not interact with any endogenous transport systems.

For determination of nucleoside concentration-effect relationships, unlabeled nucleosides and [^{3}H]adenosine were added simultaneously to yeast suspensions. For dipyridamole concentration-effect relationships, the yeast suspensions were first incubated for 15 to 30 min with dipyridamole to allow for equilibration of the inhibitor with its binding sites before the addition of radiolabeled permeant as described previously (Visser et al., 2002).

pCMBS Experiments. Yeast containing pYPhENT1, pYPhENT2 or one of the various mutant plasmids were grown in CMM to A_{600} of 0.5 to 1.0, washed twice in ice-cold fresh medium, pH 7.4, and resuspended to an A_{600} of 2.0. All reactions were performed on ice (Yao et al., 2001a). The yeast cells were distributed to microcentrifuge tubes to which pCMBS (Toronto Research Chemicals, Toronto, ON, Canada) was added alone or together with uridine, adenosine, dilazep, dipyridamole, or NBMPR. After a 30-min incubation period, the cells were centrifuged and washed 3× with ice-cold medium to remove unreacted pCMBS, nucleosides and inhibitors. The cells were

resuspended to an A_{600} of 4.0 and distributed to 96-well microtiter plates for nucleoside transport assays.

Results

Initial Rates of Nucleoside Transport by Recombinant hENT1 and hENT2 Produced in Yeast. Fui1::TRP1 yeast cells containing either pYPhENT1, pYPhENT2, or pYPGE15 (vector without insert) were incubated in the presence of 1 or 1000 μM ^{3}H-labeled uridine, cytidine, thymidine, adenosine, inosine, or guanosine for various intervals within 0 to 30 s and 0 to 60 min (data not shown). For yeast with recombinant hENT1, the rates of uptake of 1 or 1000 μM uridine, cytidine, adenosine, inosine, and guanosine from 0 to 30 s were linear and not significantly different from the rates observed from 0 to 10 min, and for uridine and adenosine from 0 to 60 min. For yeast with hENT2, rates of uptake of 1 or 1000 μM uridine, adenosine, and inosine were linear from 0 to 30 s and not significantly different from the rates observed at times up to 10 min. Uptake of [^{3}H]cytidine and [^{3}H]guanosine by yeast producing hENT2 was significant but with poor signal-to-noise ratios, and subsequent kinetic experiments did not yield reproducible data. Uptake of [^{3}H]thymidine by yeast producing either hENT1 or hENT2 was very poor, even though thymidine is a known permeant of both transporters (Griffiths et al., 1997a,b) and unlabeled thymidine was a potent inhibitor of nucleoside transport in yeast with either transporter (data not shown). The low uptake of thymidine was likely because fui1::TRP1 yeast do not possess thymidine kinase and thus cannot metabolically "trap" thymidine once inside the cell. However, metabolism did not seem to be rate-limiting for uptake of the other nucleoside permeants because functional differences were observed between hENT1, hENT2, and mutants thereof, indicating that the transported permeants were rapidly trapped and that the intracellular concentrations of free nucleosides were therefore negligible. For yeast containing pYPGE15, the rates of uptake for all ^{3}H-labeled nucleosides were low and similar to those of yeast producing recombinant hENT1 or hENT2 in the presence of 10 mM unlabeled thymidine or uridine. For all subsequent experiments, initial rates of nucleoside transport were estimated from values of total uptake at 10 min minus values observed in the presence of 10 mM unlabeled thymidine.

Nucleoside Transport by hENT1, hENT1-M33I, hENT2, and hENT2-I33M. Yeast cells containing either pYPhENT1, pYPhENT1-M33I, pYPhENT2, pYPhENT2-I33M, or pYPGE15 were incubated in the presence of 10 μM ^{3}H-labeled uridine, cytidine, adenosine, inosine, or guanosine for 10 min (Fig. 1A). hENT1 and hENT1-M33I displayed similar rates of uptake for all of the nucleosides, whereas hENT2-I33M displayed rates of uptake that were higher than those of wild-type hENT2 and similar to those of hENT1. This experiment demonstrated total uptake observed after 10 min and did not distinguish between mediated and nonmediated uptake.

Uptake of the ^{3}H-labeled nucleoside analog drugs (5-fluorouridine, gemcitabine, cytarabine, cladribine, and fludarabine) into yeast cells containing pYPhENT1, pYPhENT2, pYPhENT2-I33M, or pYPGE15 was also determined (Fig. 1B). Consistent with what was observed for the naturally occurring nucleosides, hENT2-I33M displayed rates of uptake that were higher than those of hENT2 and similar to those of hENT1, with the exception of the cytidine analogs gemcitabine and cytarabine of which hENT2-I33M-mediated uptake was higher than that of either wild-type protein.

Inhibition of Adenosine Transport Mediated by Recombinant hENT1 and hENT2 by Physiological Permeants. Concentration-effect relationships for inhibition of 1 μM [^{3}H]adenosine transport by recombinant hENT1 and hENT2 in yeast by graded concentrations of either uridine, cytidine, thymidine, adenosine, inosine, or guanosine were determined. The resulting IC_{50} values were used to calculate K_i values using the Cheng and Prusoff (1973) equation: $K_i = IC_{50}/(1 + [S]/K_m)$, where [S] is the permeant concentration. The results are given in Tables 1 (hENT1 series) and 2 (hENT2 series). The K_i values obtained for inhibition of adenosine transport were similar to the observed K_m values for transport of the same permeant, indicating that a common transporter (hENT1 or hENT2) was responsible for uptake of the permeants tested. Furthermore, the K_i values served as surrogate measures of the affinities of the transporter for its permeants, which enabled assessment of hENT2 interactions with cytidine and guanosine (Table 2).

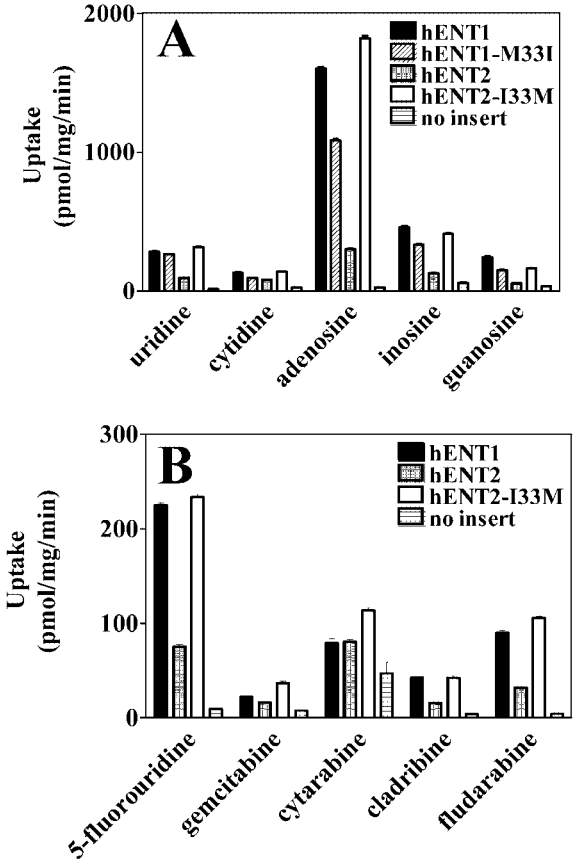

Fig. 1. Nucleoside and nucleoside analog uptake rates by hENT1, hENT1-M33I, hENT2, and hENT2-I33M. Yeast cells containing pYPhENT1, pYPhENT1-M33I, pYPhENT2, pYPhENT2-I33M, or pYPGE15 (no insert) were incubated for 10 min with the following ^{3}H-labeled nucleosides or nucleoside analogs: uridine, cytidine, adenosine, inosine, or guanosine (A) and 5-fluorouridine, gemcitabine, cytarabine, cladribine, or fludarabine (B) at a concentration of 10 μM. The representative uptake values are presented as the means ± S.E. of triplicate determinations. Three separate experiments gave similar results. For each single experiment, all five yeast strains were assayed simultaneously for all the permeants indicated so that direct uptake rate comparisons could be made.

Kinetic Parameters of hENT1 and hENT1-M33I. The concentration dependence of ^{3}H-labeled uridine, cytidine, adenosine, inosine, and guanosine transport was determined for yeast cells containing either pYPhENT1 or pYPhENT1-M33I (Table 1). Both wild-type and mutant hENT1 conformed to simple Michaelis-Menten kinetics for all nucleosides tested. Recombinant hENT1-M33I displayed apparent K_m values that were similar to those of hENT1, whereas the mutant consistently displayed lower V_{max} values than hENT1, which probably reflected a lower abundance of the mutant protein in the plasma membrane. The V_{max}/K_m ratios, which reflect transporter efficiencies for the various nucleoside permeants, for hENT1-M33I were similar to those for hENT1. These results suggested that there were no apparent functional differences between mutant and wild-type hENT1.

Kinetic Parameters of hENT2 and Various Residue 33 Mutants. The concentration dependence of ^{3}H-labeled uridine, cytidine, adenosine, inosine, and guanosine transport was determined for yeast cells containing pYPhENT2 or pYPhENT2-I33M (Table 2). As was observed for hENT1, both wild-type hENT2 and hENT2-I33M conformed to simple Michaelis-Menten kinetics for all the nucleoside permeants tested. Recombinant hENT2-I33M displayed K_m values for the pyrimidine nucleosides uridine and cytidine that were similar to those of hENT1 and ~25% of those of wild-type hENT2. Although the K_m values of hENT2-I33M for adenosine, inosine, and guanosine were lower than those of wild-type hENT2, they were higher than those of hENT1. The V_{max} values of hENT2-I33M for the purine nucleosides, particularly adenosine, were significantly higher than those of either hENT1 or hENT2. The V_{max}/K_m ratios of hENT2-I33M for all the nucleoside permeants tested were higher than those for hENT2 and similar to those for hENT1, providing an explanation for the differences in nucleoside uptake results observed in Fig. 1A.

To test the effects of substituting different amino acid side chains at residue 33 of hENT2, additional mutations were generated (hENT2-I33A, hENT2-I33C, and hENT2-I33S), and the kinetic parameters of uridine and adenosine transport were determined (Table 2). Representative rate versus concentration plots and Eadie-Hofstee plots for adenosine transport by hENT2 and all the residue 33 mutants are presented in Fig. 2. hENT2-I33A displayed similar K_m and V_{max} values to those of hENT2, whereas the values for hENT2-I33C and I33S were similar to those of hENT2-I33M in that they displayed K_m values for uridine that were 20 to 30% of wild-type and V_{max} values for adenosine that were 2- to 3-fold higher. hENT2-I33M and hENT2-I33C displayed modestly lower K_m values for adenosine compared with

TABLE 1
Kinetic properties of hENT1 and hENT1-M33I
The K_m, V_{max}, and K_i values shown are the means ± S.E. of three to five separate experiments.

Protein	Permeant	K_m	V_{max}	V_{max}/K_m	K_i
		μM	*pmol/mg/min*	*pmol/mg/min/μM*	*μM*
hENT1	Uridine	44.1 ± 2.6	1060 ± 20	24.0	51.9 ± 2.8
	Cytidine	234 ± 47	1280 ± 70	5.4	346 ± 49
	Thymidine	N.D.	N.D.	N.D.	81.6 ± 3.1
	Adenosine	17.8 ± 0.8	1330 ± 20	74.7	10.3 ± 0.4
	Inosine	28.5 ± 2.6	1300 ± 30	45.6	34.6 ± 1.9
	Guanosine	47.5 ± 4.8	1080 ± 30	22.7	48.6 ± 3.6
hENT1-M33I	Uridine	30.0 ± 1.4	707 ± 10	23.6	
	Cytidine	150 ± 38	814 ± 68	5.4	
	Adenosine	12.2 ± 0.5	1010 ± 10	82.8	
	Inosine	24.0 ± 4.0	804 ± 36	33.5	
	Guanosine	49.8 ± 4.7	784 ± 36	15.7	

N.D., not determined.

TABLE 2
Kinetic properties of hENT2, hENT2-I33M, hENT2-I33A, hENT2-I33C, and hENT2-I33S
The K_m, V_{max}, and K_i values shown are the means ± S.E. of three to five separate experiments. Representative curves for adenosine are presented in Fig. 2.

Protein	Permeant	K_m	V_{max}	V_{max}/K_m	K_i
		μM	*pmol/mg/min*	*pmol/mg/min/μM*	*μM*
hENT2	Uridine	195 ± 14	1940 ± 60	9.9	216 ± 17
	Cytidine	N.D.	N.D.	N.D.	1380 ± 170
	Thymidine	N.D.	N.D.	N.D.	129 ± 9
	Adenosine	106 ± 6	3420 ± 60	32.2	93.7 ± 8.1
	Inosine	180 ± 37	2020 ± 150	11.2	192 ± 29
	Guanosine	N.D.	N.D.	N.D.	394 ± 70
hENT2-I33M	Uridine	49.0 ± 2.3	1110 ± 20	22.6	
	Cytidine	393 ± 77	1700 ± 70	4.3	
	Adenosine	52.0 ± 2.0	10,000 ± 110	231	
	Inosine	95.6 ± 6.5	3420 ± 70	35.8	
	Guanosine	81.2 ± 15.6	2300 ± 110	28.3	
hENT2-I33A	Uridine	213 ± 28	1410 ± 70	6.6	
	Adenosine	104 ± 11	2010 ± 50	19.3	
hENT2-I33C	Uridine	39.1 ± 5.0	1640 ± 40	41.9	
	Adenosine	43.6 ± 2.2	6830 ± 80	157	
hENT2-I33S	Uridine	67.1 ± 5.7	2080 ± 40	31.0	
	Adenosine	98.5 ± 4.4	10,200 ± 130	104	

N.D., not determined.

hENT2, whereas hENT2-I33S only displayed an increase in its V_{max} value. These results suggested that Met, Cys, and Ser side chains at residue 33 of hENT2 were all capable of promoting similar interactions with uridine and adenosine.

Concentration-Effect Relationships for Dipyridamole Inhibition of hENT2 and Various Residue 33 Mutants. hENT2-I33M was previously shown to be more sensitive to dipyridamole than wild-type hENT2 (Visser et al., 2002). Yeast cells producing either hENT2 or one of the residue 33 mutants were incubated in the presence of 1 μM [^{3}H]uridine in the absence (control) or presence of graded concentrations of dipyridamole (Fig. 3). The resulting IC_{50} values were used to calculated K_i values using the equation of Cheng and Prusoff as was done in Tables 1 and 2 (Table 3). hENT2 and hENT2-I33A displayed similar K_i values, whereas hENT2-I33M and hENT2-I33C were 14- and 18-fold more sensitive, respectively, and hENT2-I33S was only one-fifth as sensitive to dipyridamole inhibition as wild type. The K_i values were used to calculate the Gibbs free energy for dipyridamole binding by hENT2 and the residue 33 mutants using the equation $\Delta G^o = -RT\ln(K_i)$, where R is the gas constant and T is the temperature (Table 3). In hENT2-I33M and hENT2-I33C, the Met and Cys side chains each contributed an additional 7 kJ/mol to dipyridamole binding.

Sulfhydryl Modification of hENT2 and Various Residue 33 Mutants. Yeast cells producing hENT2, hENT2-I33M, hENT2-I33C, hENT2-I33A, or hENT2-I33S were incubated with graded concentrations of pCMBS, a membrane-impermeant sulfhydryl-reactive reagent, followed by measurement of 1 μM [^{3}H]uridine uptake (Fig. 4). hENT2, hENT2-I33A, hENT2-I33S, and hENT2-I33M-mediated uridine uptake was insensitive to concentrations of pCMBS up to 3 mM, whereas hENT2-I33C-mediated uridine uptake was inhibited in a dose-dependent manner to a maximum inhibition of 64% and an average IC_{50} value of 8.8 $\pm$ 1.0 μM (n = 3). The average Hill slope of hENT2-I33C inhibition by pCMBS was 1.08 $\pm$ 0.14, suggesting that modification of a single Cys residue was responsible for the observed effect. The observation that hENT2-I33A and I33S were insensitive to pCMBS suggested that the substitution of an amino acid residue with a small side chain did not induce a conformational change leading to the exposure of an endogenous pCMBS-reactive Cys residue. Furthermore, the observation that wild-type hENT2 was insensitive to inhibition by pCMBS was consistent with previously published work (Yao et al., 2001a). The current putative topology model of hENT2 places residue 33 as the last position on the extracellular end of TM 1 (Griffiths et al., 1997b; Sundaram et al., 2001a,b; Yao, 2001), and the observation that residue 33 was accessible to pCMBS supported this model.

Permeant and Inhibitor Protection of hENT2-I33C from pCMBS Modification. Yeast cells producing hENT2-I33C were incubated in the presence or absence of 0.1 mM pCMBS either alone or together with 1) 1 mM adenosine or uridine, 2) 10 μM dilazep or dipyridamole, or 3) 1 μM NBMPR and then assayed for [^{3}H]uridine uptake (Fig. 5A). The presence of either adenosine, uridine, or dipyridamole protected hENT2-I33C from pCMBS inhibition, whereas dilazep and NBMPR did not protect against the pCMBS-dependent inhibition. Yeast cells producing hENT2-I33C that had been incubated with 0.1 mM pCMBS and subsequently incubated with 1 mM dithiothreitol exhibited full recovery of uridine uptake activity, demonstrating involvement of a sulfhydryl group in the pCMBS-dependent inhibition.

To determine the extent to which either adenosine, uridine, dipyridamole, or dilazep protected yeast cells producing hENT2-I33C from pCMBS inhibition, graded concentrations of compound were tested in the experiments of Fig. 5B. hENT2-I33C was protected from pCMBS inhibition in a dose-dependent manner by uridine (EC_{50} = 320 $\pm$ 50 μM, n = 3), adenosine (EC_{50} = 67.1 $\pm$ 8.9 μM, n = 3), and dipyridamole ($EC_{50} \geq 10$ μM, n = 3), whereas dilazep had no protective effects at concentrations up to 1 mM. The highest dipyridamole concentration used for protection from pCMBS modification was 10 μM because this was the solubility limit on ice, the temperature at which the reactions were performed. The data suggested that binding of adenosine, uridine, or dipyridamole prevented pCMBS from inhibiting hENT2-I33C and implied that residue 33 was involved in the binding of these compounds or at the least forms part of the immediate binding environment of these compounds.

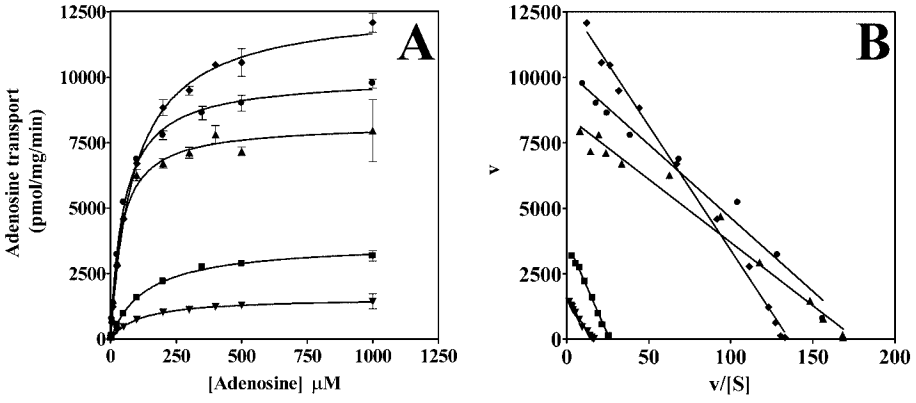

Fig. 2. Concentration dependence of adenosine transport by hENT2 and various residue 33 mutants. A, yeast cells containing pYPhENT2 (squares), pYPhENT2-I33M (circles), pYPhENT2-I33C (triangles), pYPhENT2-I33A (inverted triangles), or pYPhENT2-I33S (diamonds) were incubated for 10 min with increasing concentrations of [^{3}H]adenosine. The transport rates presented were derived from the difference between the uptake observed in the absence and presence of 10 mM unlabeled thymidine at each adenosine concentration. K_m and V_{max} values were obtained by nonlinear regression analysis using GraphPad Prism version 4.0 software, and the average values from three to five separate experiments are presented in Table 2. B, Eadie-Hofstee plot of the data presented in A. Each point is presented as the mean $\pm$ S.E. (n = 4–9), and where the size of the point is larger than the standard error, it is not shown.

Discussion

In this study, we have demonstrated that fui1::TRP1 yeast cells display little or no endogenous transport activity for uridine, cytidine, thymidine, adenosine, inosine, and guanosine and are therefore a powerful heterologous expression system for the comprehensive functional analysis of recombinant hENT1 and hENT2. The observed affinity parameters K_i or K_m for uridine were similar to those reported in other studies for recombinant hENT1 and hENT2 produced in yeast (Osato et al., 2003; Endres et al., 2004; Sen-Gupta and Unadkat, 2004; Vickers et al., 2004). However, these parameters differed from those obtained in other recombinant expression systems such as *Xenopus laevis* oocytes and transfected mammalian cells (Griffiths et al., 1997a,b; Yao et al., 1997; Ward et al., 2000). These discrepancies are probably the result of differences in post-translational processing of the transporter protein and in the lipid composition of plasma membranes. Nonetheless, the relative affinities of hENT1 and hENT2 for their permeants were consistent with those reported in transfected cells with the exception of inosine and thymidine for hENT2 (Ward et al., 2000). Recombinant hENT1 and hENT2 were reported previously to have similar apparent affinities for uridine (Griffiths et al., 1997a,b; Ward et al., 2000), although earlier studies of endogenous es and ei transport systems in cultured cells and rat erythrocytes had demonstrated lower affinities of the ei transporter (i.e., ENT2) for uridine (Jarvis and Young, 1986; Boleti et al., 1997).

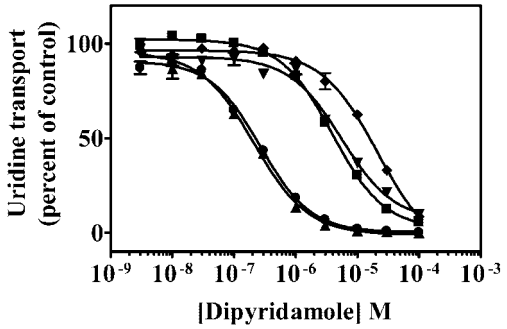

Fig. 3. Concentration dependence of dipyridamole inhibition of hENT2, hENT2-I33M, hENT2-I33C, hENT2-I33A, and hENT2-I33S. Yeast cells containing pYPhENT2 (squares), pYPhENT2-I33M (circles), pYPhENT2-I33C (triangles), pYPhENT2-I33A (inverted triangles), or pYPhENT2-I33S (diamonds) were incubated with 1 μM [^{3}H]uridine for 10 min in the absence (control) or presence of graded concentrations of dipyridamole. IC_{50} values were determined by nonlinear regression analysis using GraphPad Prism version 4.0 software, and the average values from three separate experiments are presented in Table 3. Each point is presented as the mean ± S.E. (n = 4), and where the size of the point is larger than the standard error, it is not shown.

TABLE 3

Inhibition of hENT2, hENT2-I33M, and hENT2-I33C-mediated [^{3}H]uridine transport by dipyridamole

K_i values are the mean ± S.E. calculated using the equation of Cheng and Prusoff (1973) with the IC_{50} values obtained by nonlinear regression analysis of the curves presented in Fig. 5 using GraphPad Prism version 4.0 software.

Protein	K_i	Ratio[a]	ΔG°
	μM		kJ/mol
hENT2	3.77 ± 0.25	1.00	31
hENT2-I33M	0.263 ± 0.011	14.3	38
hENT2-I33A	4.95 ± 0.55	0.76	30
hENT2-I33C	0.206 ± 0.018	18.3	38
hENT2-I33S	18.6 ± 4.7	0.20	27

ΔG°, Gibb's free energy.
[a] Ratio = K_i hENT2/K_i mutant.

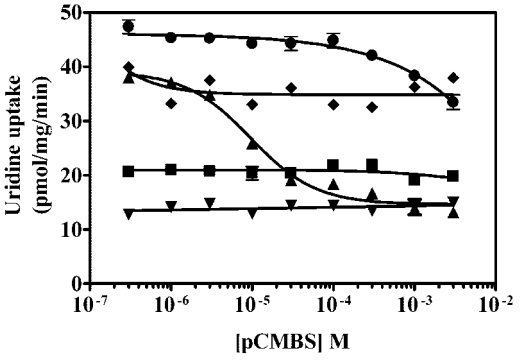

Fig. 4. Concentration dependence of pCMBS inhibition of hENT2 and various residue 33 mutants. Yeast cells containing pYPhENT2 (squares), pYPhENT2-I33M (circles), pYPhENT2-I33C (triangles), pYPhENT2-I33A (inverted triangles), or pYPhENT2-I33S (diamonds) were incubated in the absence or presence of graded concentrations of pCMBS followed by incubation with 1 μM [^{3}H]uridine for 10 min. Each point represents the mean ± S.E. (n = 4), and where the size of the point is larger than the standard error, it is not shown. IC_{50} values were determined by nonlinear regression using GraphPad Prism version 4.0 software, and average values are presented in the text. Three separate experiments gave similar results.

hENT2-I33M, hENT2-I33C, and hENT2-I33S all displayed increased affinities for uridine (Table 2), suggesting that residue 33 is an important functional determinant for the binding of uridine and other pyrimidine nucleosides. Furthermore, the observation that hENT2-I33M displayed increased apparent V_{max} values for all purine nucleoside permeants and that hENT2-I33M, hENT2-I33C, and hENT2-I33S displayed notably increased apparent V_{max} values for adenosine also suggested that residue 33 was an important functional determinant for the purine nucleoside transport activity of hENT2. That increased V_{max} values were observed with adenosine and not with uridine suggested that the observed effects were not caused by an increase in the plasma membrane abundance of the protein but rather to an increase in catalytic activity brought about by increases in the rate of conformational turnover and/or increases in protein flexibility. However, hENT2-I33S did not display a reduced K_m value for adenosine (Table 2), as was observed for hENT2-I33M and hENT2-I33C, suggesting that residue 33 contributed to permeant recognition and transport. The Met and Cys side chains contain highly polarizable sulfur atoms (Gellman, 1991) that probably interacted with hydrophobic moieties on the base portions of uridine and adenosine, whereas the Ser side chain evidently contributed hydrogen-bond interactions to uridine binding. Furthermore, Met was favored over Ile in this regard, probably because of its relatively high degree of conformational flexibility. The apparent ability of this residue to interact with different parts of the permeant molecule probably stems from the conformational flexibility in TM 1 brought about by the highly conserved glycine residues G22 and G24 (Hyde et al., 2001).

The observation that hENT1 and hENT1-M33I did not display functional differences suggested that residue 33 did not contribute to permeant interactions in hENT1 (Table 1). This was probably caused by minor structural differences between hENT1 and hENT2. In particular, the large extracellular loop that extends from TM 1 is considerably more hydrophobic in hENT1 than hENT2 and may affect the con-

formation and solvent accessibility of residue 33. Nonetheless, we have observed that mutations of W29 have specific effects on the permeant selectivity of hENT1 (our unpublished observations). Because TM 1 is probably α-helical, as would be predicted from the crystal structures of other major facilitator superfamily proteins such as lac permease (Abramson et al., 2003), W29 is predicted to be in proximity to residue 33, suggesting that this region of hENT1 is involved in permeant recognition and transport.

The substitution of Cys or Met at residue 33 increased sensitivity to dipyridamole by 18- and 14-fold, respectively, whereas the substitution of Ile (wild type), Ala, or Ser was less favorable for dipyridamole inhibition (Fig. 3; Table 3). That hENT2-I33S exhibited increased transport activity but decreased dipyridamole sensitivity was consistent with the conclusion that this residue could not engage in hydrogen bond interactions with the more hydrophobic dipyridamole molecule. Met or Cys at residue 33 probably participated in interactions with the pi electron cloud of the aromatic moieties on the dipyridamole structure, which was supported by the fact that the strength of the interaction (7 kJ/mol) is consistent with the strength of similar interactions observed in α-helices (3–8 kcal/mol) and is based on the high degree of polarizability of the sulfur atom (Viguera and Serrano, 1995; Pal and Chakrabarti, 2001).

hENT2-I33C was the only hENT2 mutant that displayed a dose-dependent sensitivity to the membrane-impermeant sulfhydryl reactive reagent pCMBS. These results independently confirmed the location of this residue on the putative topology model of hENT2, which places it as the last residue on the extracellular end of TM 1 (Sundaram et al., 2001). hENT2-I33C was protected from pCMBS modification by uridine, adenosine, and dipyridamole. These results suggested that the binding of permeants or dipyridamole prevented pCMBS from reacting either by directly blocking access to residue 33 or by altering accessibility to residue 33 by a long-range conformational effect on the tertiary structure of hENT2. Given the functional significance of residue 33, we postulate a direct interaction between residue 33 and either nucleoside permeants or dipyridamole. Previous studies had provided contradictory evidence for both competitive and allosteric binding of dipyridamole to hENT1 (Griffith and Jarvis, 1996). However, the current results, which suggested that dipyridamole and permeants bind to the outward-facing conformation of mammalian ENTs, support the conclusion that dipyridamole and nucleoside permeant bind to the same or an overlapping site (Paterson et al., 1980; Jarvis et al., 1983; Jarvis, 1986). That Met was favored at residue 33 for permeant and inhibitor binding is consistent with the ability of ENTs to bind a large variety of chemically unrelated permeants and inhibitors. Met residues of the signal-recognition particle 45 and calmodulin have been implicated as critical for recognition of a variety of unrelated protein targets (Gellman, 1991).

Although our previous study (Visser et al., 2002) had suggested an important role for residue 33 in dilazep binding, dilazep was unable to protect against pCMBS inhibition of hENT2-I33C, even though sensitivity to dilazep inhibition was retained. Dilazep and dipyridamole are believed to bind to the same or overlapping sites of hENT1 (Koren et al., 1983; Sundaram et al., 1998), which was supported by our previous study in which residue 33 mutations similarly affected the potency of these two inhibitors (Visser et al., 2002). The results of the current study suggest that dilazep may bind adjacent to residue 33 in a manner that does not occlude this residue.

In conclusion, our in-depth study of the impact of mutations of residue 33 in hENT2 in the yeast expression system has yielded novel information about the role of this residue in permeant and inhibitor interactions with hENT2 and hENT1. We confirmed our previous conclusion (Visser et al., 2002) that residue 33 is a determinant of the overall func-

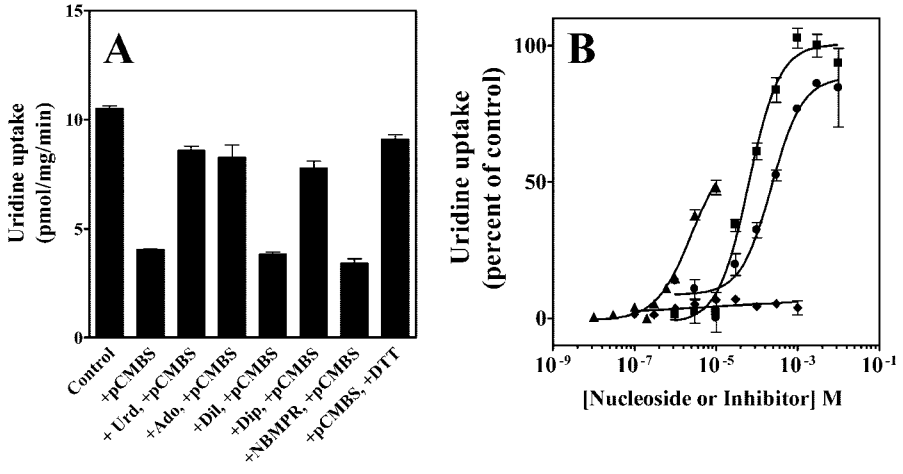

Fig. 5. Protection of hENT2-I33C from pCMBS modification by various permeants and inhibitors. A, yeast cells producing hENT2-I33C were incubated in the absence (control) or presence of 0.1 mM pCMBS with or without 1 mM uridine (Urd), 1 mM adenosine (Ado), 10 μM dilazep (Dil), 10 μM dipyridamole (Dip), or 1 μM NBMPR. Some of the yeast cells incubated with 0.1 mM pCMBS alone were subsequently incubated with 1 mM dithiothreitol. The yeast cells were then incubated for 10 min with 1 μM [^{3}H]uridine in the absence of the test protecting agents. Uridine uptake is presented as the mean ± S.E. (n = 4) and was analyzed using GraphPad Prism version 4.0 software. Three separate experiments gave similar results. B, yeast cells producing hENT2-I33C were incubated in the absence or presence of 0.1 mM pCMBS with or without graded concentrations of uridine (circles), adenosine (squares), dilazep (diamonds), or dipyridamole (triangles) followed by incubation for 10 min with 1 μM [^{3}H]uridine in the absence of the test protecting agents. Uridine transport rates in the absence of 0.1 mM pCMBS were set as 100% of control, whereas rates in the presence of 0.1 mM pCMBS were set as 0% of control. EC_{50} values were determined by nonlinear regression using GraphPad Prism version 4.0 software, and average values are presented in the text. Each point represents the mean ± S.E. (n = 4), and where the size of the point is larger than the standard error, it is not shown. Three separate experiments gave similar results.

tional differences between hENT1 and hENT2. We demonstrated that residue 33 is extracellular, thereby validating the predicted topology model for hENT2. Our results established residue 33 as a common exofacial determinant of the binding sites for nucleosides and inhibitors, providing molecular evidence that dipyridamole competes with nucleosides for binding to hENT2. Although these conclusions are consistent with the data presented in this study, a crystal structure of the transporter would be necessary to further address the role of residue 33 in permeant and inhibitor interactions with hENT2.

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Address correspondence to: Dr. Carol E. Cass, Department of Oncology, Cross Cancer Institute, 11560 University Ave., T6G 1Z2, Edmonton, AB T6G 1Z2, Canada. E-mail: carol.cass@cancerboard.ab.ca