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Characterization of a Bioengineered Chimeric Na^+-Nucleoside Transporter

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ABSTRACT

Na^+-dependent nucleoside transporters mediate the intracellular uptake of purine and pyrimidine nucleosides. The N1, N2, and N3 Na^+-nucleoside transporters differ in substrate selectivity. N1 is purine-selective, N2 is pyrimidine-selective, and N3 is broadly selective. Recently, we created a chimeric transporter, T8, from the cloned rat N1 and N2 transporters. Whereas most chimeric proteins exhibit the characteristics of one of the two parent proteins, limited studies suggested that T8 possesses either a combined substrate selectivity of N1 and N2 or the selectivity of N3. The purpose of this study was to determine the substrate profile, transport mechanisms, and Na^+-coupling stoichiometry of T8 and to compare these measurements with those of wild-type N1, N2, and N3. In *Xenopus laevis* oocytes expressing T8, Na^+-dependent uptake of ^3H-labeled purine (adenosine, inosine, and guanosine) and pyrimidine nucleosides (uridine, thymidine, and cytidine) was significantly enhanced (3.5–18.6-fold), which suggests that T8 accepts both purine and pyrimidine nucleosides as permeants. T8-mediated uptake of [^3H]thymidine was competitively inhibited by inosine, and T8-mediated uptake of [^3H]inosine was competitively inhibited by thymidine, which suggests that purine and pyrimidine nucleosides share a common binding site. Base-modified ribo- and 2′-deoxyribonucleosides were potent inhibitors of T8. In contrast, 2′,3′-dideoxyinosine, 2′,3′-dideoxycytidine, and 3′-azidothymidine, which are known inhibitors of N1 or N2, did not inhibit T8-mediated uptake. These data suggest that the substrate profile of T8 is not a combination of those of N1 and N2; rather, it is similar to that of N3. However, the Na^+/nucleoside stoichiometric ratio of T8 was determined to be 1, consistent with both N1 and N2 but different from N3.

In mammalian cells, transmembrane flux of nucleosides is mediated by both equilibrative and Na^+-dependent nucleoside transporters. These processes are essential for nucleotide synthesis by salvage pathways and are the route of cellular uptake of many therapeutic nucleosides used in the treatment of cancer, viral infections, and cardiac arrhythmias (Cass, 1995; Griffith and Jarvis, 1996; Wang et al., 1997a).

Na^+-dependent nucleoside transporters mediate the active transport of nucleosides into cells by coupling the transmembrane flux of substrates to the physiological Na^+ gradient across the plasma membrane. These transporters exhibit distinct transport selectivity for purine and pyrimidine nucleosides and have been classified into several subtypes based on their substrate selectivity. The N1 system is purine-selective, the N2 system is pyrimidine-selective, and the N3 system is broadly selective, transporting both purine and pyrimidine nucleosides. Uridine, a pyrimidine nucleoside, and adenosine, a purine nucleoside, are ubiquitously transported by all Na^+-dependent nucleoside transport systems. A Na^+/nucleoside coupling ratio of 1:1 has been reported for N1 and N2 transporters, which indicates that the inward transport of each nucleoside molecule is driven by the interaction of one sodium ion (Cass, 1995; Griffith and Jarvis, 1996; Yao et al., 1996b). In contrast, a stoichiometry of 2:1 was observed for the N3 system, which indicates that two sodium ions are required for the translocation of one nucleoside molecule (Wu et al., 1992).

The N1 and N2 subtype Na^+-nucleoside transporters have now been cloned from [rat concentrative nucleoside transporter 1 (rCNT1) and sodium-dependent purine nucleoside transporter (SPNT)] and human [human CNT1 (hCNT1) and hSPNT1] (Huang et al., 1994; Che et al., 1995; Ritzel et al., 1997; Wang et al., 1997b). Although the cloned N1 and N2 transporters have distinct substrate selectivities for purine and pyrimidine nucleosides, they share a high sequence homology (60–70%) and a similar predicted membrane topology (14 putative transmembrane domains). They belong to a *CNT* gene family that also includes the NupC proton-nucleoside

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ABBREVIATIONS: TMD, transmembrane domain; 2CdA, 2-chloro-2′-deoxyadenosine; AZT, 3′-azidothymidine; ddC, 2′, 3′-dideoxycytidine; ddI, 2′, 3′-dideoxyinosine; rCNT1, rat concentrative nucleoside transporter 1; SPNT, sodium-dependent purine nucleoside transporter; hCNT1, human CNT1.

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symporter of *Escherichia coli* (Craig et al., 1994; Huang et al., 1994; Che et al., 1995). The broadly selective transporter N3 was characterized in rabbit choroid plexus, rabbit ileum, and rat jejunum (Wu et al., 1992, 1994; Huang et al., 1993; Redlak et al., 1996), and was also found in cultured human promyelocytic leukemia and colorectal carcinoma cells (Belt et al., 1993). However, the molecular identity of this transporter is currently unknown.

Recently, using a chimeric transporter approach, we demonstrated that transmembrane domains (TMDs) 8 and 9 of the cloned rat N1 and N2 transporters are the major sites for substrate binding and discrimination (Wang and Giacomini, 1997). As we constructed and analyzed a series of N1/N2 chimeric transporters, we noticed that one chimera exhibited an unusual substrate selectivity. This chimeric transporter, termed T8, is structurally identical with N2 except that the eighth TMD is replaced by that of N1 (Fig. 1). Surprisingly, unlike the wild-type N1 or N2, which are selective for either purine or pyrimidine nucleosides, T8 transports both inosine (a purine nucleoside) and thymidine (a pyrimidine nucleoside) (Wang and Giacomini, 1997). The T8-mediated uptake of uridine, a common substrate of both N1 and N2, was inhibited by naturally occurring purine and pyrimidine nucleosides (Wang and Giacomini, 1997). These data suggest that, unlike most chimeric proteins, which exhibit the characteristics of one of the two parent proteins, T8 may be a broadly selective transporter that accepts both purine and pyrimidine nucleosides. However, because only one purine and one model pyrimidine nucleoside were examined, and an inhibitor of a transporter may not be a substrate, it is not known whether T8 also transports other purine and pyrimidine nucleosides, such as guanosine and cytidine. Furthermore, if T8 is truly broadly selective, does the enlarged substrate profile result from the combination of a distinct purine-selective site derived from N1 and a distinct pyrimidine-selective site derived from N2 (i.e., the presence of two mutually exclusive recognition sites in the chimeric transporter)? Alternatively, is the broad substrate selectivity of chimera T8 attributable to a single engineered binding site that recognizes both purine and pyrimidine nucleosides? In the present study, we address these questions by determining the substrate profile, transport mechanism, and Na^+-coupling stoichiometry of T8. Information from this study may help us to gain further understanding of functional properties of Na^+-nucleoside transporters and may also pave the way for the bioengineering of nucleoside transporters for therapeutic purposes.

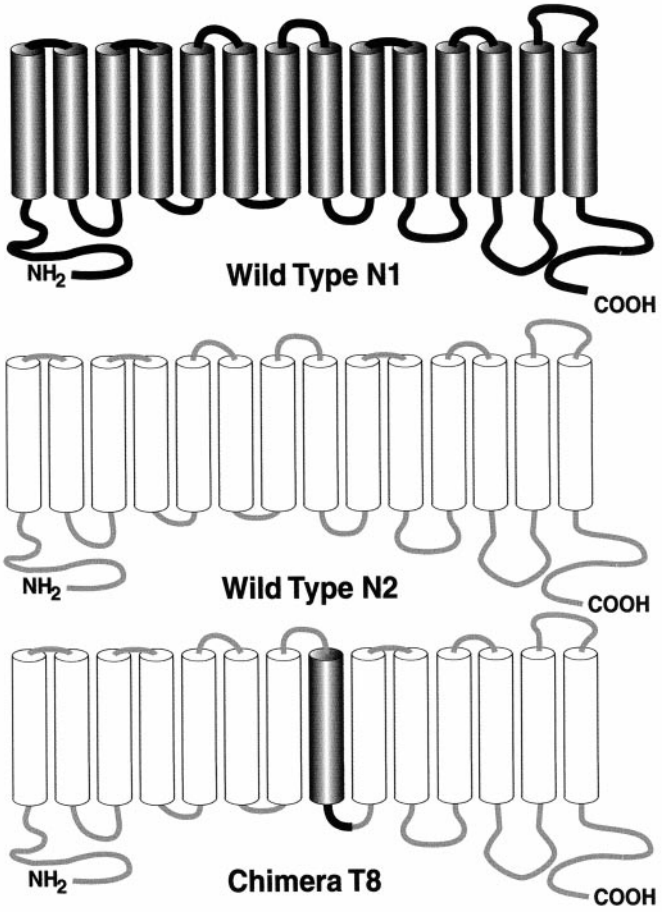

Fig. 1. Secondary structures of chimera T8 and wild-type N1 and N2 transporters. Wild-type N1 represents the rat N1 clone SPNT (659 amino acids). Wild-type N2 represents the rat N2 clone rCNT1 (648 amino acids). Chimera T8 contains amino acid residues 1 to 300 of N2, 297 to 330 of N1, and 335 to 648 of N2.

Materials and Methods

Chimera T8 cDNA. The methods used in the cDNA construction of chimeric transporters were described previously (Wang and Giacomini, 1997). In brief, the cDNAs of wild-type rat N1(SPNT) and N2 (rCNT1) were isolated by reverse transcription-polymerase chain reaction. The Genetics Computer Group software (Wisconsin Package, v. 8; Madison, WI) was used to align the nucleotides and the deduced amino acid sequences of N1 and N2. To construct chimera T8, a chimera, T8-14, consisting of TMD1 to TMD7 of N2 and TMD8 to TMD14 of N1 was first obtained by equivalent exchange at the internal *Nco*I sites. An equivalent *Afl*II site was then introduced into the N2 cDNA and chimera T8-14 cDNA at position 1158 by site-directed mutagenesis. Introducing the *Afl*II site in both clones did not change the encoded amino acids at these sites. T8 cDNA was then obtained from N2 and chimera T8-14 by equivalent exchange at the *Afl*II site. The sequence of T8 was confirmed by automated DNA sequencing in the Biochemical Resource Center at the University of California, San Francisco.

Expression in *Xenopus laevis* Oocytes. Plasmid that contained chimera T8 was linearized with *Xba*I. cRNA was synthesized with T7 polymerase in the presence of ^{m7}GpppG cap using the mCAP RNA Capping kit (Stratagene, La Jolla, CA). Oocytes were harvested from *X. laevis* (Xenopus, Ann Arbor, MI) and defolliculated as described previously (Giacomini et al., 1994; Zhang et al., 1997). Healthy stage V and VI oocytes were injected with 50 nl of T8 cRNA (0.4 ng/nl) or 50 nl of water using a semiautomatic injector (PL1-188; Nikon, Melville, NY). Injected oocytes were maintained for 2 to 3 days at 18°C in Barth's medium (88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.4 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 2.4 mM $NaHCO_3$, and 10 mM HEPES/Tris, pH 7.4) before the assay of transport activity. Uptake experiments were carried out 48 to 56 h after injection. To minimize variability, each experiment used oocytes from a single animal.

Transport Assays. Uptake of nucleosides by oocytes was traced with the respective ^{3}H-labeled nucleosides (Moravek Biochemicals, Brea, CA). Assays were performed at 25°C on groups of 10 oocytes in 150 μl of transport buffer containing 100 mM NaCl or 100 mM choline chloride and 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.4. At the end of the incubation, uptake was terminated by removing the incubation medium followed by six rapid washes in ice-cold choline chloride buffer. Individual oocytes were dissolved in 10% SDS; the radioactive content of each oocyte was assayed by liquid scintillation counting. For inhibition studies, non-

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radioactive compounds (Sigma Chemical, St. Louis, MO) were also included in the reaction mixture at concentrations indicated in the figure legends. Chimera T8-mediated thymidine and inosine uptake was linear up to 1 to 3 h (data not shown); therefore, initial rates of uptake in kinetic studies were measured using an incubation period of 30 min. For studies designed to determine the Na^+ stoichiometric coupling ratio, oocytes were preincubated in choline buffer at 25°C for 30 min and washed three times with choline buffer before uptake to remove extracellular Na^+. ^{3}H-labeled nucleoside (10 μM) uptake was then measured in transport buffer containing 0 to 100 mM NaCl, using choline chloride to maintain iso-osmolality.

Data Analysis. Uptake values are presented as mean ± S.E. for 8 to 10 individual oocytes. The kinetic parameters were determined by fitting velocity/substrate versus velocity to the equation obtained from the Eadie-Hofstee linear transformation of the Michaelis-Menten equation. In particular, the data were fit to the equation $V = V_{max} - K_m \cdot V/S$, where V is the initial rate of uptake, V_{max} is the maximal transport rate, K_m is the concentration of nucleoside when the initial rate is at half the maximum, and S is the nucleoside concentration in the reaction mixture. Apparent V_{max} and K_m values were obtained from the slopes ($-K_m$) and vertical intercepts (V_{max}) of the Eadie-Hofstee plots. To ascertain the stoichiometric coupling ratio between Na^+ and nucleoside, the data were fit to the following Hill equation: $V = V_{max} \cdot C_{Na+}{}^n/(K_d{}^n + C_{Na+}{}^n)$, where V is the initial rate of uptake, V_{max} is the maximal rate of nucleoside transport at saturating concentrations of Na^+, C_{Na+} is the concentration of Na^+, K_d is the concentration of Na^+ that is able to produce half the maximum rate of nucleoside transport, and n is the Hill coefficient. The fits were carried out using a nonlinear, least-squares, regression-fitting program (Kaleidagraph, v. 3.0; Abelbeck/Synergy Software, Reading, PA). Statistical analysis was carried out by comparing the tested compounds with the controls from the same experiments using an unpaired Student's t test. Results were considered statistically different with a probability of $p < .05$.

Results

Transport of Naturally Occurring Nucleosides. The first evidence of the broad substrate selectivity of T8 came from the observation that both inosine and thymidine are transported by this chimeric transporter (Wang and Giacomini, 1997). However, it is not known whether the transporter is truly "broadly selective" (i.e., whether other nucleosides are transported by T8). To investigate whether T8 is truly a broadly selective transporter that also accepts other purine and pyrimidine nucleosides as substrates, we examined the uptake, by oocytes injected with T8 cRNA, of ^{3}H-labeled naturally occurring purine (adenosine, inosine, and guanosine) and pyrimidine nucleosides (uridine, thymidine, and cytidine). Compared with water-injected oocytes, a Na^+-dependent increase (3.5–9.6-fold) in the uptake of ^{3}H-labeled adenosine, inosine, and guanosine was observed in T8 cRNA-injected oocytes (Fig. 2A). For ^{3}H-labeled pyrimidine nucleosides (uridine, thymidine, and cytidine), a 10.6- to 18.6-fold increase was observed (Fig. 2B). These data suggest that T8 is a Na^+-dependent, broadly selective nucleoside transporter that accepts both purine and pyrimidine nucleosides as substrates.

Mechanism of Broad Selectivity. Because T8 is derived from wild-type N1 and N2 transporters, there are two potential mechanisms for its broad substrate selectivity. It is possible that T8 possesses two binding sites: one purine-binding site obtained from N1 and one pyrimidine-binding site obtained from N2, therefore exhibiting an apparent broad selectivity for both purine and pyrimidine nucleosides. Alternatively, it is also possible that introducing TMD8 of N1 into N2 altered the binding site of N2, expanding its transport capacity to purine nucleosides. To investigate the mechanism (two mutually exclusive binding sites or a single, engineered binding site) by which T8 transports purine and pyrimidine nucleosides, we studied the effect of purine nucleosides on T8-mediated pyrimidine nucleoside uptake and the effect of pyrimidine nucleosides on T8-mediated purine nucleoside uptake using inosine as a model purine nucleoside and thymidine as a model pyrimidine nucleoside.

If T8 interacts with purine and pyrimidine nucleosides through two mutually exclusive recognition sites, inosine should not inhibit the transport of thymidine and vice versa. However, if inosine and thymidine share a common binding site, inosine should be able to inhibit the transport of thymidine and vice versa. The data show that thymidine uptake was completely inhibited by (1 mM) inosine (Fig. 3A) and inosine uptake was completely inhibited by (1 mM) thymi-

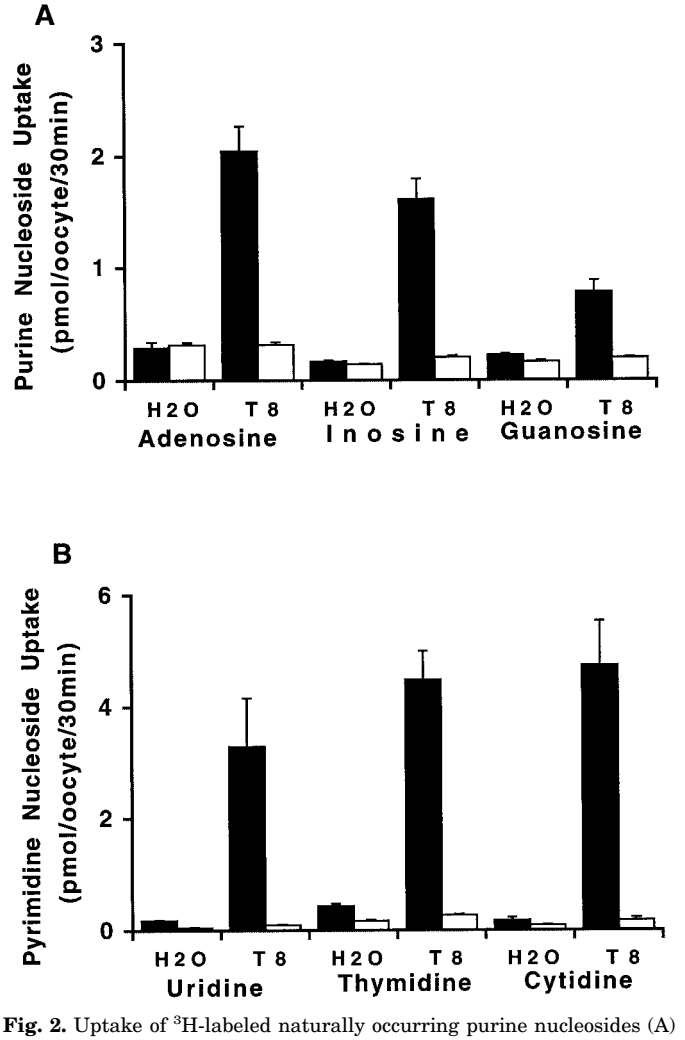

Fig. 2. Uptake of ^{3}H-labeled naturally occurring purine nucleosides (A) and pyrimidine nucleosides (B) by chimera T8. *X. laevis* oocytes were injected with water (as control) or 20 ng of cRNA of chimera T8. Two days after injection, uptake was measured on groups of 10 oocytes at 25°C in 150 μl of transport buffer (2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4) in the presence (100 mM NaCl, ■) or absence of Na^+ (100 mM choline chloride, □), and the respective ^{3}H-labeled nucleoside (10 μM). Each value represents the mean ± S.E. of data obtained from 8 to 10 individual oocytes from one representative experiment performed on oocytes obtained from a single animal.

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dine (Fig. 3B), which suggests that the two compounds share a single recognition site in T8.

We further determined the mechanism of interaction between inosine and thymidine. The effect of inosine at different concentrations (0, 20, and 40 μM) on the initial rate of thymidine uptake (Fig. 4A) and the effect of thymidine at different concentrations (0, 20, and 40 μM) on the initial rate of inosine uptake (Fig. 4B) were determined in the presence of Na^+. The Na^+-driven transport of thymidine was saturable (K_m = 41 ± 5 μM; V_{max} = 7.0 ± 0.6 pmol/oocyte/30 min). In the presence of 20 and 40 μM inosine, the apparent K_m values of thymidine (79 ± 9 and 122 ± 33 μM, respectively) increased significantly ($p < .05$), whereas the apparent V_{max} values (8.6 ± 0.7 and 9.6 ± 2.0 pmol/oocyte/30 min, respectively) were not significantly different. The Na^+-driven transport of inosine was also saturable (K_m = 14.5 ± 2.1 μM; V_{max} = 3.6 ± 0.3 pmol/oocyte/30 min). In the presence of 20 and 40 μM thymidine, the apparent K_m values of inosine increased significantly (34 ± 3 and 36 ± 6 μM versus 14.5 ± 2.1 μM) ($p < .05$), whereas the apparent V_{max} values were not significantly different (3.7 ± 0.2 and 3.5 ± 0.4 pmol/oocyte/30 min versus 3.6 ± 0.3 pmol/oocyte/30 min). These data suggest that inosine competitively inhibits the transport of thymidine and that thymidine competitively inhibits the transport of inosine. An Eadie-Hofstee plot of these data generated inhibition patterns consistent with a competitive mechanism (Fig. 4, insets). These data further support the notion that inosine and thymidine compete for the same substrate binding site in T8.

Interaction with Nucleoside Analogs. Because our data suggested that the broad substrate selectivity of T8 may result from changes within the binding site of N2, we hypothesized that T8 may possess novel selectivity for synthetic nucleoside analogs, which include a wide array of therapeutic

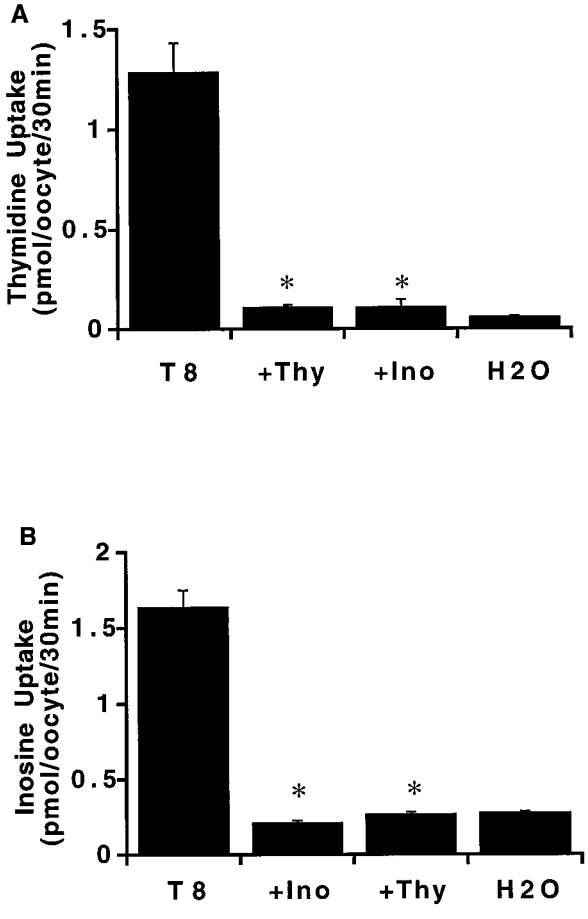

Fig. 3. A, effect of inosine on [^{3}H]thymidine uptake. B, effect of thymidine on [^{3}H]inosine uptake. Oocytes were injected with water or 20 ng of T8 cRNA. ^{3}H-nucleoside uptake was determined in Na^+-containing buffer in the absence or presence of 1 mM inosine (Ino) or thymidine (Thy). Uptake by water-injected oocytes in Na^+ buffer was used as the baseline reference. Each value represents the mean ± S.E. of data obtained from 8 to 10 oocytes from one representative experiment. Both inosine and thymidine significantly inhibited the uptake of each other (*$p < .05$).

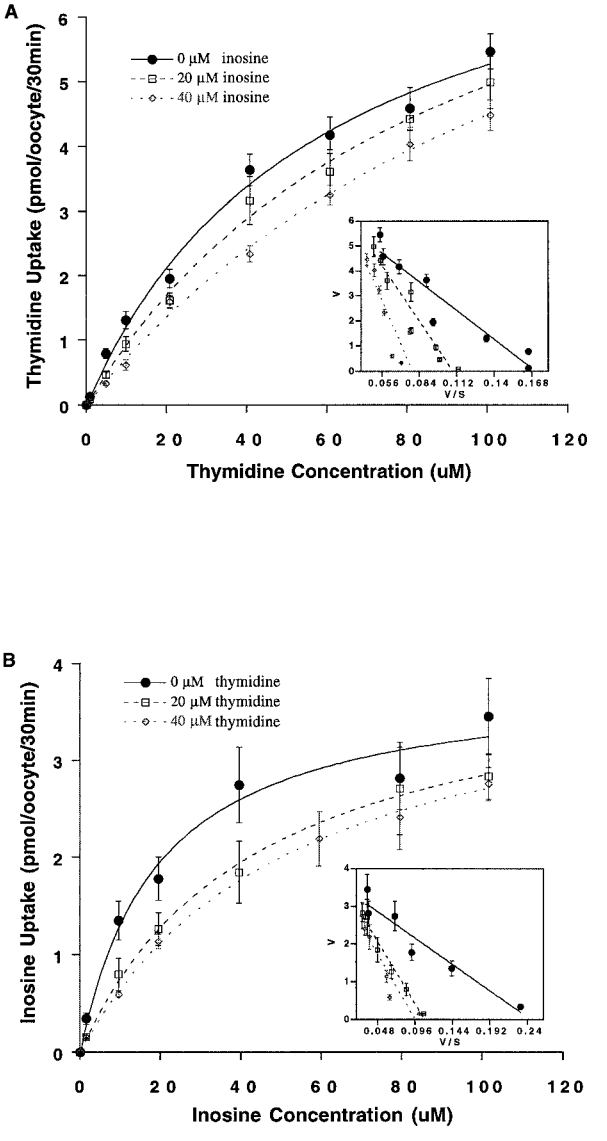

Fig. 4. A, the inhibition mechanism of thymidine uptake by inosine. B, The inhibition mechanism of inosine uptake by thymidine. Oocytes were injected with water or 20 ng of T8 cRNA and [^{3}H]-thymidine uptake was determined in Na^+-containing buffer in the absence (●) or presence of 20 μM (□) or 40 μM (◇) of inhibitors. Each value represents the mean ± S.E. of data obtained from 8 to 10 oocytes. *Inset*, Eadie-Hofstee plots. V, rate of uptake; V/S, rate of uptake/substrate concentration. Apparent K_m and V_{max} values were determined from the slopes and the vertical intercepts of the Eadie-Hofstee plots and are presented in the text.

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agents. The effect of various compounds on the Na^+-driven transport of thymidine was studied to further define the substrate profile of T8 (Fig. 5). At 1 mM, inosine, thymidine, formycin B, 2-chloro-adenosine and 5-fluoro-uridine, 2-chloro-2′-deoxyadenosine (2CdA), and 5-iodo-2′-deoxyuridine, significantly inhibited ($p < .05$) Na^+-driven thymidine uptake (Fig. 5). At the same concentration (1 mM), ribose, thymine, xanthine, L-thymidine, 5′-thymidine monophosphate, 3′-thymidine monophosphate, 2′, 3′-dideoxyinosine (ddI), 2′, 3′-dideoxycytidine (ddC), 3′-azidothymidine (AZT), cytosine arabinoside, and acycloguanosine were unable to significantly inhibit Na^+-driven thymidine uptake (Fig. 5). Two ^{3}H-labeled compounds, 2-CdA and L-thymidine, were further tested in uptake studies (Fig. 6). For ^{3}H-labeled 2CdA, significantly increased uptake was observed (Fig. 6) and, as expected, there was no significant ^{3}H-labeled L-thymidine uptake (Fig. 6). These data suggest that chimera T8 is a Na^+-dependent nucleoside transporter that selectively transports naturally occurring nucleosides or synthetic nucleosides that have been modified on the base and/or on the 2′-position of the ribose. Interestingly, the substrate profile of T8 is very similar to the well characterized, broadly selective transport system N3 (Wu et al., 1992, 1994). In contrast, ddI, ddC, and AZT, which contain modifications on the 3′-position of the ribose and are known inhibitors of N1 or N2, did not inhibit T8-mediated uptake, further suggesting that T8 does not exhibit the combined characteristics of N1 and N2.

Na^+ Stoichiometry. To determine the Na^+ stoichiometry of T8, the effect of Na^+ concentrations (ranging from 0 to 100 mM) on the initial uptake of thymidine and inosine (10 μM) was examined. The uptake of thymidine (Fig. 7A) and inosine (Fig. 7B) was sensitive to Na^+ concentration. The data were fit to a Hill equation as described in *Materials and Methods*. The predicted Na^+/nucleoside coupling stoichiometry of T8, determined from Hill coefficients, was not significantly different from 1 for both thymidine and inosine (Hill coefficients, 1.16 ± 0.34 and 1.05 ± 0.27, respectively). Previous studies have established a 1:1 coupling ratio for the wild-type N1 and N2 transporters (Cass, 1995; Griffith and Jarvis, 1996; Yao et al., 1996b). In contrast, the coupling ratio for the N3 system was determined to be 2:1 (Wu et al., 1992).

Discussion

In this study, we functionally characterized a bioengineered chimeric Na^+-nucleoside transporter, T8. The structure of T8 is identical with the pyrimidine-selective Na^+-nucleoside transporter N2, except that TMD8 was replaced with that of N1 (Fig. 1). Previously, using chimeric N1/N2 transporters, we demonstrated that TMD8 to TMD9 determined the substrate selectivity of N1 and N2 transporters and may constitute a major part of the substrate-binding site in these transporters (Wang and Giacomini, 1997). The integrity of TMD8 to 9 may be necessary for chimeric transporters to maintain the substrate selectivity of wild-type transporters, because chimeras with junction sites within TMD8 to TMD9 seemed to exhibit novel properties (Wang and Giacomini, 1997). In this study, we functionally characterized chimera T8 and specifically determined its substrate profile and transport mechanism. Data from this study suggest that chimera T8 is a broadly selective nucleoside transporter that transports both purine and pyrimidine nucleosides (Fig. 2). This broad substrate selectivity may be attributable to structural alterations within the binding pocket of N2 (i.e., the presence of a single engineered binding site that recognizes both purine and pyrimidine nucleosides). Alternatively, the purine-selective site of N1 may be located in TMD8 and the pyrimidine-selective site of N2 may be located in TMD9; the broad substrate selectivity of T8 is simply caused by the presence of these two mutually exclusive binding sites. To investigate whether T8 transports purine and pyrimidine nucleosides after interaction with one binding site or two independent binding sites, we studied the effect of inosine on T8-mediated thymidine uptake and the effect of thymidine on T8-mediated inosine uptake. The data showed that thymidine uptake was completely inhibited by inosine (Fig. 3A) and inosine uptake was completely inhib-

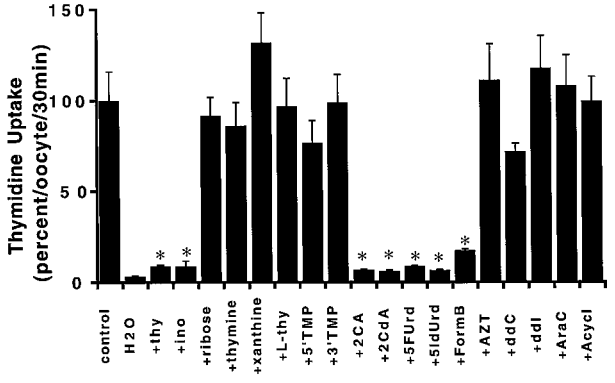

Fig. 5. Effects of nucleoside and nucleoside analogs on [^{3}H]thymidine uptake in oocytes injected with T8 cRNA. Uptake was determined in Na^+-containing buffer in the presence and absence (control) of 1 mM various compounds (thy, thymidine; ino, inosine; L-thy, L-thymidine; 5′TMP, 5′-thymidine monophosphate; 3′TMP, 3′-thymidine monophosphate; 2CA, 2-chloroadenosine; 5FUrd, 5-fluorouridine; 5IdUrd, 5-iodo-2′-deoxyuridine; FormB, formycin B; AraC, cytosine arabinoside; and Acycl, acycloguanosine). Compounds with an * significantly inhibited the T8-mediated uptake of thymidine ($p < .05$).

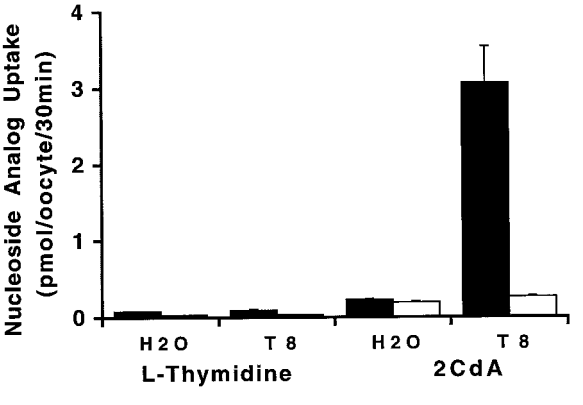

Fig. 6. Uptake of ^{3}H-labeled L-thymidine and 2CdA by chimera T8. *X. laevis* oocytes were injected with H_2O or 20 ng cRNA of chimera T8. Uptake was measured on groups of 10 oocytes in the presence of Na^+ (■) or in the absence of Na^+ (□). Each value represents the mean ± S.E. of data obtained from 8 to 10 individual oocytes obtained from a single animal.

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ited by thymidine (Fig. 3B). Furthermore, the inhibition mechanisms were found to be competitive (Fig. 4), which suggests that inosine and thymidine compete for the same substrate-binding site in T8. Collectively, these data suggest that transplanting TMD8 of N1 into N2 altered the structure of the substrate-binding pocket of N2 and subsequently expanded the transport capacity of N2 to purine nucleosides.

The potential of T8 to interact with synthetic nucleoside analogs was evaluated in inhibition studies. The data in Fig. 5 indicated that base-modified and 2′-ribose-modified nucleosides are potent inhibitors of T8. The uptake study with ^{3}H-labeled 2-CdA (Fig. 6) further demonstrated that this anticancer nucleoside is also a true permeant of T8. Previously, Yao et al. studied the interaction of rat N2 with the antiviral drug AZT and ddC in the *X. laevis* oocyte expression system (Yao et al., 1996a). They found both drugs were inhibitors as well as permeants of N2 (K_m = 550 and 503 μM, respectively). Recently, Schaner et al. demonstrated, in a HeLa cell expression system, that another commonly used antiviral agent, ddI, was a potent inhibitor of the wild-type rat N1 transporter (IC_{50} = 46 μM) (Schaner et al., 1997). In contrast, none of these compounds (AZT, ddC, and ddI) at 1 mM concentration was able to inhibit T8-mediated thymidine uptake (Fig. 5). These data also suggest that the substrate selectivity of T8 is not a simple combination of those of N2 and N1; rather, it is a novel property resulting from an engineered binding site.

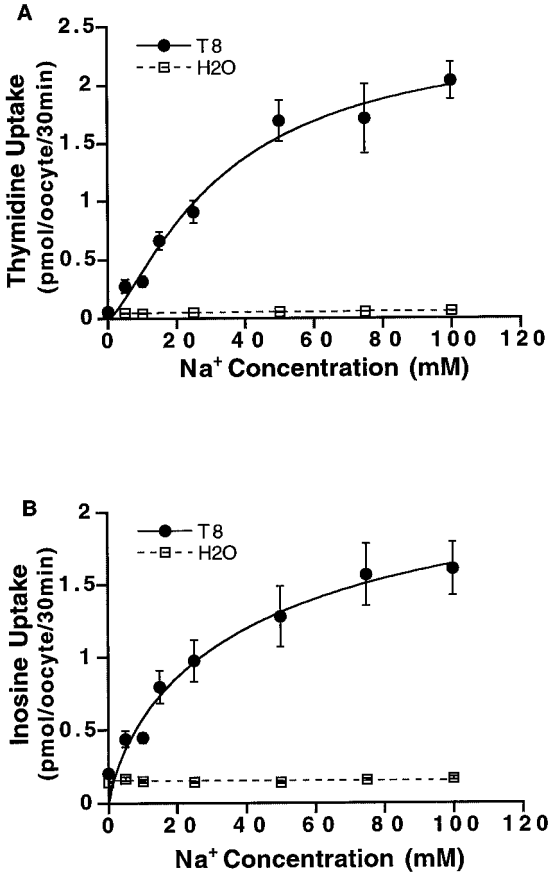

Fig. 7. Sodium dependence of T8-mediated uptake of (A) thymidine and (B) inosine. Uptake was measured in water-injected (□) or T8 cRNA-injected (●) oocytes in transport buffer containing 0 to 100 mM NaCl, using choline chloride to maintain iso-osmolality. Each value represents the mean ± S.E. of data obtained from 8 to 10 individual oocytes. Hill coefficients were determined by fitting the data to a Hill equation using nonlinear regression analysis (Kaleidagraph). In the fitting, values for inosine were corrected for endogenous inosine uptake (i.e., uptake by water-injected oocytes). A corresponding correction for thymidine was not performed because of the small (<1%) contribution of endogenous thymidine uptake observed in water-injected oocytes.

In nature, Na^+-dependent, broadly selective nucleoside transporters have been well documented in rabbit choroid plexus, rabbit ileum, and rat jejunum (Wu et al., 1992, 1994; Huang et al., 1993; Redlak et al., 1996). These transporters, classified as N3 subtypes, were also described in cultured human promyelocytic leukemia and colorectal carcinoma cells (Belt et al., 1993). As yet, however, no typical N3 transporter has been cloned, and the molecular identity of N3 is still unknown. Interestingly, the substrate profile of T8 is amazingly similar to that of N3. Both are broadly selective nucleoside transporters that accept ribo- and 2′-deoxyribopurine and pyrimidine nucleosides (Fig. 2) as substrates (Wu et al., 1992, 1994). Both interact with synthetic base-modified ribo- or 2′-deoxyribonucleosides (e.g., 2-chloroadenosine, 5-fluorouridine, and 5-iodo-2′-deoxyuridine) but not with ribose-modified nucleosides, such as AZT and cytosine arabinoside, or 2′,3′-dideoxynucleosides, such as ddC and ddI (Fig. 5) (Wu et al., 1992, 1994). It is possible that the sequence of the uncloned N3 transporter may be very similar to the cloned N2 transporter, because only a few amino acid substitutions during evolution in the TMD8 region may transform N2 into an N3 subtype transporter. Alternatively, the N2 and N1 transporters may evolve from a common N3-type ancestor and gain their substrate selectivity by a few amino acid substitutions in the TMD8 to 9 region. However, the Na^+-coupling ratio of T8 was determined to be 1 (Fig. 7), identical with the coupling ratios of N1 and N2 but different from the 2:1 ratio of N3 determined in choroid plexus. Therefore, the Na^+-coupling ratio of these transporters may not be necessarily linked to their substrate selectivity, or, in other words, the Na^+-binding site in these transporters may be a distinct domain, separated from but energetically coupled to the substrate-binding domain.

In this study, we present functional characteristics of a bioengineered chimeric Na^+-nucleoside transporter. Consistent with our previous finding that TMD8 toTMD9 are the major structural components of the substrate-binding site in Na^+-nucleoside transporters (Wang and Giacomini, 1997), the unique transport characteristics of chimera T8 reflected the intrinsic changes within this binding site. The finding that chimera T8 possesses novel substrate selectivity unique to a third subtype of nucleoside transporters suggests that novel transporters can be engineered from known transporters. A thorough understanding of the molecular mechanisms governing the functional properties of the Na^+-nucleoside transporters may help us to use these transporters or bioengineer new transporters for site-specific drug targeting and delivery.

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