Serine 318 Is Essential for the Pyrimidine Selectivity of the N2 Na⁺-Nucleoside Transporter*

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Molecular cloning has isolated two subtypes of Na⁺-nucleoside transporters; one is pyrimidine-selective (N2), and the other is purine-selective (N1). Using chimeric rat N2/N1 transporters, we previously demonstrated that transmembrane domains (TM) 8 and 9 are the major sites for substrate binding and discrimination. Interestingly, when TM8 of N2 was replaced by that of N1, the resulting chimera, T8, lost the pyrimidine selectivity of N2 and accepted both purine and pyrimidine nucleosides. Five residues differ between rat N2 and N1 in TM8. To identify the critical residues responsible for transport selectivity, the five residues in N2 were systematically changed to their equivalents in N1. Replacing the serine residue at position 318 to its equivalent N1 residue, glycine, caused N2 to lose its selectivity for pyrimidine nucleosides and accept purine nucleosides as substrates. In contrast, replacing the other four residues did not change the pyrimidine selectivity of N2. Furthermore, when glycine 318 in chimeric T8 was changed back to serine, the chimeric transporter regained pyrimidine selectivity. These observations suggest that serine 318 is located in the nucleoside permeation pathway and is responsible for the substrate selectivity of N2. An adjacent residue, glutamine 319, was found to be important in modulating the apparent affinity for nucleosides.

In mammalian cells, transmembrane flux of purine and pyrimidine nucleosides is mediated by both facilitated and Na⁺-dependent nucleoside transporters (1–3). These transporters also play important roles in the cellular uptake of many therapeutic nucleoside analogs such as 2’-chlorodeoxyadenosine, azidothymidine, and 2’,3’-dideoxycytidine used in the treatment of cancer and viral infections (4–6). Na⁺-nucleoside transporters exhibit distinct transport selectivity for purine and pyrimidine nucleosides and have been classified into several subtypes based on their substrate selectivity (1–3). The N1 (or cif) system is selective for purine nucleosides, the N2 (or cit) system is selective for pyrimidine nucleosides, and the N3 (or cib) system is broadly selective (or nonselective), transporting both purine and pyrimidine nucleosides. Uridine, a pyrimidine nucleoside, and adenosine, a purine nucleoside, are ubiquitously transported by all three systems. Recently the N1 and N2 transporters were cloned from rat and human (5, 7–9). Although the cloned N1 and N2 transporters have distinct substrate selectivity for pyrimidine and purine nucleosides, they share a high sequence homology (60–70%) and a similar predicted membrane topology (14 putative transmembrane domains). The broadly selective transporter, N3, was characterized in a number of tissues and cells (10–12), but the molecular identity of this transporter is currently unknown.

To determine the structural basis for substrate recognition and discrimination in the Na⁺-nucleoside transporters, we previously took advantage of the high sequence similarity and yet distinct substrate selectivity of the cloned N1 and N2 transporters. By constructing and analyzing a series of chimeric rat N1/N2 transporters, we demonstrated that TMs 8 and 9 are the major sites for substrate binding and discrimination (13). Interestingly, when TM8 of N2 was replaced by that of N1, the resulting transporter, chimera T8, lost pyrimidine selectivity and became a broadly selective (or nonselective) transporter that accepts both purine and pyrimidine nucleosides as substrates (13). Sequence alignment revealed that 5 amino acid residues differ between rat N2 and N1 in the TM8 region (Fig. 1), suggesting that simultaneous replacement of the five divergent residues in TM8 of N2 with the corresponding residues in N1 would cause N2 to lose its selectivity for pyrimidine nucleosides. It is possible that individual residues in TM8 of N2 may gate the substrate permeation pathway, which would only allow pyrimidine nucleosides and the common substrate, adenosine, to pass through. Substitution of these residues removes the gating validity, resulting in nonselective transporters (e.g. chimera T8), which also allow the passage of purine nucleosides.

In this study, we focused on determining the specific residues responsible for maintaining the substrate selectivity of N2. By site-directed mutagenesis, the five residues in N2 were systematically mutated to the corresponding residues in N1. The substrate selectivity of each mutant was subsequently evaluated in the Xenopus laevis oocyte expression system. The data suggest that a single residue, serine 318, is responsible for maintaining the pyrimidine selectivity of N2. An adjacent residue, glutamate 319, was found to be important in influencing the apparent affinity for nucleosides.

**EXPERIMENTAL PROCEDURES**

Site-directed Mutagenesis and Sequence Analysis—The cDNAs of wild-type rat N1 (SPNT) and N2 (CNT1) transporters were obtained by reverse transcriptase polymerase chain reaction (13). The mutagenic oligonucleotides were synthesized in the Biochemical Resource Center at the University of California, San Francisco. The Stratagene Chameleon™ and QuickChange™ site-directed mutagenesis kits (Stratagene) were used to construct mutant cDNAs following the manufacturer’s protocols. Mutants with single amino acid substitutions (S304T, T306A, S310A, S318G, and Q319M) were prepared using the cDNA of wild-type rat N2 as template. The double mutant S318G/Q319M was constructed by introducing a second mutation (Q to M) at position 319 of mutant S318G. The mutant T8.G318S was constructed by changing glyclne 318 to glycine 318.

*This work is supported by National Institutes of Health Grant GM42230. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The five residues that differ between N2 and N1 in TM8 are shown in wild-type N1 and N2 transporters (Fig. 1, A). The panel A, primary sequences of N1 and N2 transporters in the TM8 region (B), and amino acid substitutions in mutants and in chimera T8 (C). Panel A, wild-type N1 (659 amino acids), wild-type N2 (648 amino acids). Chimera T8 contains amino acid residues 1–300 of N2, 297–330 of N1, and 335–648 of N2. Panel B, the amino acid sequences of N2 and N1 in TM8 region. The five residues that differ between N2 and N1 in TM8 are shown in bold. Panel C, systematic substitutions of the divergent residues in mutants. The numbers refer to the sequence of rat N2. Chimera T8 has all five divergent residues in the TM8 of N2 replaced by the corresponding residues of N1.

Serine in a previously described chimeric transporter T8 (12). The sequence of each mutant was confirmed by direct DNA sequencing using an automated DNA sequencer (Applied Biosystems, Model 373A). Genetics Computer Group software package (Wisconsin Package, Version 9) was used for sequence alignment and helical wheel analysis.

Transport Assays in X. laevis Oocytes—cRNA of each mutant was synthesized and injected into defolliculated oocytes as described previously (5). Uptake was measured on groups of 10 oocytes 48–56 h post-injection at 25 °C in 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, pH 7.4) containing either 100 mM NaCl or 100 mM choline chloride and the respective 3H-labeled nucleoside (Moravek Biochemicals). The kinetic parameters (apparent Km and Vmax values) were determined by nonlinear least squares fits of substrate/velocity profiles to the Michaelis-Menten equation using Kaleidograp (Version 3.0, Synergy Software). Because of the intrinsic variability in the expression level of the transporters between batches of oocytes, the data are generally expressed as the mean ±S.E. from experiments performed in the same batch of oocytes. However, experiments were repeated at least twice in separate batches of oocytes.

RESULTS

Studies on chimeric transporters showed that replacing TM8 of N2 with that of N1 generated a chimera T8, which lost the pyrimidine selectivity of N2 (Fig. 1, A–B). To identify the specific residues responsible for maintaining the substrate selectivity of N2, we individually changed the five divergent residues (Fig. 1) in TM8 of N2 to the corresponding residues in N1. A double mutant (S310G/Q319M) and a reverse mutant (T8.G318S) were also constructed. Mutants were first screened with the common substrate, [3H]uridine, for activity. Significant Na+-dependent uptake of uridine (10–70-fold increase) was observed for all mutants (data not shown), suggesting that all mutants were expressed and functional.

Substrate Selectivity of Mutants—The substrate selectivity of each mutant was examined in uptake experiments using [3H]i-}

nosine as the model purine nucleoside and [3H]thyidine as the model pyrimidine nucleoside. The uptake of inosine (10 μM) and thymidine (10 μM) by N2, chimera T8, mutants S304T, T306A, S310A, S318G, Q319M, and S318G/Q319M is shown in Fig. 2. Compared with water-injected oocytes, significant Na+-dependent thymidine uptake (13–133-fold increase) was observed in oocytes expressing mutants S304T, T306A, S310A, and Q319M (Fig. 2, C–E and 2G). In contrast, there was no significant inosine uptake by these mutants (Fig. 2, C–E and 2G), suggesting that mutants S304T, T306A, S310A, and Q319M maintained the pyrimidine selectivity of N2. Therefore, single substitutions of these residues in N2 with the corresponding residues in N1 did not affect the substrate selectivity of N2.

In contrast, in addition to thymidine, the mutant S318G also transports inosine (Fig. 2F). In oocytes expressing mutant S318G, there was a 41-fold increase in Na+-dependent inosine uptake (3.27 ± 0.58 pmol/oocyte/30 min for S318G cRNA-injected oocytes versus 0.08 ± 0.01 pmol/oocyte/30 min for water-injected oocytes). These data suggest that changing serine 318 in N2 to its equivalent residue in N1 (glycine) causes N2 to accept inosine as a substrate. However, compared with the transport rate of (10 μM) thymidine (14.90 ± 2.17 pmol/oocyte/30 min), S318G transports inosine (10 μM) at a rate 4.6-fold lower (Fig. 2F). The data shown in Fig. 2F were from one representative experiment in which the same batch of oocytes was used. The experiment was performed several times. Although the rate of nucleoside uptake varied among experiments (ranging from 0.71 to 3.3 for inosine and from 4.30 to 15.10 for thymidine, pmol/oocyte/30 min) because of the intrinsic variability in the expression level between batches of oocytes, significant Na+-dependent inosine uptake (16- to 41-fold over water-injected oocytes) was observed in S318G cRNA-injected oocytes in all experiments. Within a single experiment, the thymidine uptake was consistently 4–6-fold higher than the inosine uptake. These data suggest that although mutant S318G accepts purine nucleosides as substrates, it still kinetically favors the transport of pyrimidine nucleosides at the tested concentration (10 μM).

Mutant Q319M maintained the substrate selectivity of wild-type N2; however it transports thymidine with a significantly decreased rate (Fig. 2A and 2G). Because glutamine 319 is also adjacent to serine 318, we suspect that this residue may play a role in substrate binding. Therefore we introduced a second Q to M mutation at position 319 of mutant S318G. This double mutant, S318G/Q319M, transports 10 μM inosine and thymidine at a comparable but slow rate (1.07 ± 0.20 pmol/oocyte/30 min for inosine and 1.25 ± 0.20 pmol/oocyte/30 min for thymidine), generating an uptake pattern similar to that of chimera T8 (Fig. 2, B and H). These data indicate that changing glutamine 319 to methionine in mutant S318G caused S318G to transport purine and pyrimidine nucleosides without much kinetic bias.

The data presented in Fig. 2 suggested that serine 318 is important for maintaining the pyrimidine selectivity of N2. Changing this residue in N2 to glycine causes N2 to lose its substrate selectivity. To investigate whether a reverse mutation can re-establish the pyrimidine selectivity in chimera T8, we changed the glycine 318 in chimera T8 back to the serine residue in N2. Interestingly, this mutant (termed T8.G318S) restored the pyrimidine selectivity of N2, transporting thymidine but not inosine (Fig. 3). These data strongly suggest that serine 318 is essential for maintaining the pyrimidine selectivity of the N2 transporter.

Transport Kinetics of Mutants S318G and S318G/Q319M—To investigate whether mutant S318G transports thymi-
dine more favorably than inosine at 10 μM (Fig. 2F) and how substitution of Q319M in mutant S318G neutralized this imbalance (Fig. 2H), we examined the kinetics of thymidine and inosine uptake mediated by mutant S318G and mutant S318G/Q319M (Fig. 4). Uptake of both nucleosides via mutant S318G was saturable (Fig. 4A). The apparent $K_m$ of inosine was 273 ± 62 μM, whereas that of thymidine was 27.5 ± 4.3 μM. The $V_{max}$ of inosine was 28.8 ± 2.5 pmol/oocyte/30 min, whereas that of thymidine was 24.6 ± 0.9 pmol/oocyte/30 min (Fig. 4A). These data suggest that mutant S318G has a much lower (<10-fold) apparent affinity for inosine than for thymidine, whereas the apparent maximal rate of transport, $V_{max}$, for inosine is close to that for thymidine. Therefore at low substrate concentrations (e.g. 10 μM), mutant S318G will favor the transport of thymidine over that of inosine. The uptake of inosine and thymidine mediated by mutant S318G/Q319M was also saturable (Fig. 4B). For this mutant, the $K_m$ of inosine was 54.8 ± 14.7 μM, and the $K_m$ of thymidine was 79.3 ± 11.9 μM. The $V_{max}$ of inosine was 10.8 ± 0.7 pmol/oocyte/30 min and that of thymidine was 12.3 ± 0.5 pmol/oocyte/30 min (Fig. 4B). These data suggest that mutant S318G/Q319M has similar apparent $K_m$ and $V_{max}$ values for inosine and thymidine, transporting these two compounds without much kinetic bias. Compared with the mutant S318G (Fig. 4A), the apparent affinity for inosine in the double mutant is greatly enhanced ($K_m = 54.8$ versus $K_m = 273$ μM,
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FIG. 4. Michaelis-Menten studies of thymidine and inosine uptake mediated by mutant S318G (A) and mutant S318G/Q319M (B). Oocytes were injected with 20 ng of cRNA of mutant S318G or S318G/Q319M. The initial velocities (30 min) of [3H]thymidine uptake (solid circles) or [3H]inosine uptake (squares) were determined in NaCl buffer containing the respective nucleoside at concentrations ranging from 1 to 1000 μM. Each point represents the mean ± S.E. (n = 8–10). Apparent $K_m$ and $V_{max}$ values were determined by fitting the data to the Michaelis-Menten equations using a nonlinear fitting routine of Kaleidagraph.

p < 0.01), whereas the apparent affinity for thymidine is significantly decreased ($K_m = 79.3$ versus $K_m = 24.6$ μM, p < 0.01).

Helical Wheel Analysis of TM8—To analyze the position of serine 318 and glutamine 319 on TM8, helical wheels at a fixed angle of 100° are drawn for TM8 of N2 (Fig. 5). The five substitutions in N1 are indicated by arrows. Distinct amphipathic patterns (one side of the helix being hydrophobic, and the other side hydrophilic) are observed, suggesting that one side of TM8 may face an aqueous pore (e.g. the substrate binding pore), whereas the other side may face the hydrophobic lipid. The residue serine 318 is located in the center of hydrophilic side, suggesting its side chain may directly interact with the substrates. The residue glutamine 319 is located near the boundary of the amphipathic interface. Its side chain may interact directly with the substrates or may contribute indirectly to the conformation of the substrate recognition sites.

DISCUSSION

Our previous studies showed that replacing TM8 of N2 with that of N1 caused N2 to lose its substrate selectivity (13). In this study, we focused on determining the specific residues responsible for maintaining the substrate selectivity of N2. By site-directed mutagenesis, the five divergent residues in N2 were systematically mutated to the corresponding residues in N1. Replacing serine 318 in N2 to its equivalent residue, glycine, resulted in a mutant (S318G), which lost the pyrimidine selectivity of N2 and began to accept purine nucleosides as substrates (Fig. 2F). In contrast, replacing the other four residues with their equivalents did not alter the selectivity of N2 (Fig. 2). Importantly, when the glycine residue in the broadly selective chimera T8 was changed back to serine, the resulting transporter (T8.G318S) regained pyrimidine selectivity (Fig. 3). These data strongly suggest that serine 318 is essential for conserving the pyrimidine selectivity of wild-type N2.

Kinetic studies revealed that mutant S318G has a much lower (−10-fold) apparent affinity for inosine than for thymidine (Fig. 4A), suggesting that although the ability of a mutant to accept purine nucleosides is determined by whether a serine or a glycine residue is present at position 318, other residues may contribute to the kinetic differences in the transport of purine and pyrimidine nucleosides by mutant S318G. Indeed, a second Q to M mutation at position 319 following the S318G substitution resulted in a mutant (S318G/Q319M), which has comparable apparent $K_m$ values for inosine and thymidine (Fig. 4B). These data suggest that although a single substitution of glutamine 319 with methionine would not affect the pyrimidine selectivity of N2 (Fig. 2G), changing it after the serine to glycine substitution at position 318 would greatly enhance the apparent affinity of mutant S318G toward purine nucleosides. In transport kinetic analysis, the apparent affinity (i.e. apparent $K_m$) reflects not only substrate affinity for the binding site but is also influenced by rate constants of substrate translocation and dissociation, which occur subsequent to recognition (14). Therefore, the observed changes in apparent affinity introduced by the Q to M mutation in the S318G mutant may reflect changes in any of these processes.

Studies of a number of membrane transporters suggest that the substrate permeation pathway in a transporter is a channel-like structure formed by several transmembrane helices (15–17). Charged and polar residues, often found on one side of these helices, play critical roles in interacting with the substrates (15, 18–20). Helical wheel analysis of TM8 revealed a distinct amphipathic pattern (Fig. 5), suggesting that one side of TM8 may face an aqueous channel for substrate permeation. Serine 318 is located in the center of the hydrophilic side (Fig. 5), suggesting that its side chain may protrude into the channel and act as a gating residue through specific interactions with the substrates. Substitution of a serine residue with a smaller residue, glycine, will result in a loss of a methylene and a hydroxyl group on the side chain. These changes may cause a gain in the size of the substrate permeation channel and a loss of some specific chemical interactions (e.g. a hydrogen bond), allowing the resulting transporter S318G to tolerate the bulkier purine nucleosides as substrates. On the helical wheel diagram, the residue glutamine 319 is located near the boundary of the amphipathic interface (Fig. 5). Its side chain may interact directly with the substrates or may affect the apparent affinity by indirect influence of the conformation of the substrate recognition site in the channel. Substitution of this residue with a methionine in mutant S318G may induce changes that make the transporter interact with purine nucleosides with an increased apparent affinity. However, it should be noted that the proposed topology of N2 consisting of 14 transmembrane domains with an internal C and N termini needs experimental validation. In addition, with the crystal structure of N2 unknown, the possibility that serine 318 may influence the selectivity of N2 through indirect interactions with other sites in the protein cannot be excluded.
In summary, we identified a single residue, serine 318, that is primarily responsible for determining the substrate selectivity of the N2 Na\(^{+}\)-nucleoside transporter. The data suggest that serine 318 may be located in the nucleoside permeation pathway and act as a gating residue that is important for the pyrimidine selectivity of N2. An adjacent residue, glutamine 319, was found to be important in influencing the apparent affinity of the transporter for purine nucleosides. These studies provide important information about the molecular mechanisms that govern the functional characteristics of Na\(^{+}\)-nucleoside transporters. Furthermore, the finding that a few residues along the solute permeation pathway are responsible for the substrate selectivity and affinity of N2 may reflect a common molecular mechanism for substrate discrimination in some membrane transporters.

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J. Biol. Chem. 1999, 274:2298-2302.
doi: 10.1074/jbc.274.4.2298

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