Functional Identification of SLC5A8, a Tumor Suppressor Down-regulated in Colon Cancer, as a Na\(^+\)-coupled Transporter for Short-chain Fatty Acids*

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SLC5A8, a tumor suppressor gene down-regulated in human colon cancer, codes for a transporter in the Na\(^+\)/glucose cotransporter gene family, but the definitive functional identity of the transporter protein is not known. Since this gene is expressed abundantly in the colon where short-chain fatty acids are generated by bacterial fermentation, we tested the hypothesis that it codes for a Na\(^+\)-coupled transporter for these fatty acids. The coding region of SLC5A8 mRNA was amplified from human intestine and expressed heterologously in Xenopus laevis oocytes. Transport function was monitored by uptake of radiolabeled substrates and by substrate-induced currents under voltage-clamp conditions. Uptake of short-chain fatty acids (lactate, pyruvate, acetate, propionate, and butyrate) in oocytes was severalfold higher than in un.injected oocytes. Exposure of SLC5A8-expressing oocytes to these fatty acids induced inward currents under voltage-clamp conditions in a Na\(^+\)-dependent manner. These currents were saturable and the substrate concentrations needed for half-maximal induction of the current were in the range of 0.08–2.5 mM. The substrate-induced currents decreased as the carbon chain length of the substrates increased. The Na\(^+\)-activation kinetics indicated involvement of more than one Na\(^+\) ion in the activation process. Direct measurements of substrate (propionate) and charge transfer showed that three positive charges are transferred into oocytes per substrate molecule. These studies establish the functional identity of SLC5A8 as a Na\(^+\)-coupled transporter for short-chain fatty acids.

SLC5A8 was recently identified as a candidate tumor suppressor gene in humans that is silenced by methylation in colon cancer (1). The protein encoded by SLC5A8 is a putative transporter belonging to the Na\(^+\)/glucose cotransporter gene family

(2). SLC5A8 has been shown to transport Na\(^+\) when expressed in Xenopus oocytes (1), but the cotransported organic/inorganic substrate has not been identified. Interestingly, the cloning of an identical cDNA has been reported independently by Rodriguez et al. (3) who claimed that the cDNA codes for an uncoupled passive transporter for iodide. This reported functional feature of SLC5A8 as a passive iodide transporter has apparently led to the labeling of this transporter as SLC5A11 in a recent review by Wright and Turk (2). The findings by Li et al. (1) that SLC5A8 is a Na\(^+\)- transporter are in contradiction with those by Rodriguez et al. (3) that the same protein functions as an uncoupled (i.e. no Na\(^+\) involvement in the transport process) iodide transporter.

Since all members of the SLC5 gene family, with the exception of SLC5A13 which is a glucose-sensitive Na\(^+\) channel and SLC5A9 whose transport function has not yet been established (2), are Na\(^+\)-coupled cotransporters for organic or inorganic solutes, we hypothesized that SLC5A8 is most likely a Na\(^+\)- coupled transporter for a hitherto unidentified organic or inorganic solute. The abundant expression of SLC5A8 in the colon and its suggested role as a tumor suppressor (1) led us to question its purported role as a passive iodide transporter.

Based on the tissue expression pattern, we hypothesized that SLC5A8 is likely to be a Na\(^+\)-coupled transporter as suggested by Li et al. (1) and that short-chain fatty acids may be the cotransported substrates. Short-chain fatty acids such as acetate, propionate, and butyrate are generated at high levels in the colon by bacterial fermentation of dietary fiber and unab sorbed carbohydrates (4–6) and these fatty acids are the preferred metabolic fuel in colonic epithelial cells (4–6). Furthermore, it has been shown that short-chain fatty acids prevent colonic cell proliferation and reduce the incidence of colon cancer (7–9). It is conceivable that methylation-dependent silencing of the gene expression in the colon will decrease the availability of short-chain fatty acids to colonic epithelial cells and thus influence the metabolic profile and proliferation of these cells. These findings provided the rationale for our hypothesis that SLC5A8 may be a Na\(^+\)-coupled transporter for short-chain fatty acids. Here we provide evidence that human SLC5A8 does indeed function as a Na\(^+\)-coupled transporter for short-chain fatty acids.

EXPERIMENTAL PROCEDURES

Materials—The following radiolabeled substrates were obtained from commercial sources: \([^{14}C]\text{-lactate}, [^{14}C]\text{-pyruvate, } [^{14}C]\text{-acetate, and [}^{3}H]\text{propionate from Moravek Biochemicals (Brea, CA).}

Amplification of the Coding Region of Human SLC5A8—To amplify the coding region of human SLC5A8 mRNA by RT\(^{-1}\)-PCR, we designed primers based on the published sequence (1) in GenBank\(^{TM}\) data base (accession number AF526216). The sense primer, containing the initiation codon (shown in bold), was 5’-GGATATAGCCATGGAACACGC- CACGGGCGAT-3’, and the antisense primer, located downstream of the stop codon, was 5’-CCCAAGCTTCAACAAAGGTTCCATTTGCT-3’. The underlined sequence in the antisense primer is an HindIII site, added to the 5’-end of the primer for cloning purpose. RT-PCR with these primers and human intestinal mRNA as the template yielded a \(1.9-kb\) product as expected from the positions of the primers in the published sequence (sense, 361–383; antisense, 2176–2204). This prod-
uct was subcloned into pGEM-T Easy vector. The insert was then released from the plasmid by digestion with EcoRI and HindIII and subcloned into the vector pGHL19 at the EcoRI/HindIII site. The pGHL19 vector (kindly provided by Dr. Peter S. Aronson, Yale University School of Medicine) contains the 3′-untranslated region of the Xenopus β-globin gene downstream of the cloning site. The cDNA were sequenced by the Tago DyeDeoxy terminator cycle method using an automated PerkinElmer Applied Biosystems 377 Prism DNA sequencer.

Functional Analysis of SLC5A8 in Xenopus Oocytes—The amplified human SLC5A8 cDNA was expressed heterologously in Xenopus oocytes by cRNA injection. Capped cRNA from SLC5A8 cDNA was synthesized using the mMESSAGE mMACHINE kit (Ambion Inc., Austin, TX). Mature oocytes (stage IV or V) from Xenopus laevis were injected with 25 ng cRNA. Uninjected oocytes served as controls. The oocytes were used for uptake and electrophysiological studies 3–6 days after cRNA injection. Uptake of radiolabeled substrates in uninjected and cRNA-injected oocytes was determined as described previously (10). Eight oocytes were used for each uptake measurement. Electrophysiological studies were performed by the two-microelectrode voltage-clamp method (11, 12). Oocytes were perfused with a NaCl-containing buffer (100 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 3 mM Hepes, 3 mM Mes, and 3 mM Tris, pH 7.5), followed by the same buffer containing different fatty acids. The membrane potential was clamped at −50 mV. The differences between the steady-state currents measured in the presence and absence of substrates were considered as the substrate-induced currents. To investigate the current-saturating potential (V–I) relationship, step changes in membrane potential were applied, each for a duration of 100 ms in 20-mV increments. The kinetic parameter K0.5 (i.e. the substrate concentration necessary for the induction of half-maximal current) was calculated by fitting the values of the substrate-induced currents to Michaelis-Menten equation. The Na+-activation kinetics was analyzed by measuring the substrate-specific currents in the presence of increasing concentrations of Na+. The data were analyzed by the Hill equation to determine the Hill coefficient (h; the number of Na+ ions involved in the activation process). The kinetic parameters were determined using the computer program Sigma Plot, version 6.0 (SPSS, Inc., Chicago, IL). Electrophysiological measurements of substrate-induced currents were repeated at least four times with separate oocytes. The data are presented as mean ± S.E. of these replicates.

Determination of Charge/Substrate Transfer Ratio—The charge-to-substrate transfer ratio was determined for propionate in four different oocytes as described previously (11, 12). The oocytes were perfused with 25 µM propionate (unlabeled plus radiolabeled propionate), and inward currents were monitored over a period of 8–10 min. At the end of the experiment, the amount of propionate transported into the oocytes was calculated by measuring the radioactivity associated with the oocytes. The area within the curve describing the relationship between the time and inward current was integrated to calculate the charge transferred into the oocyte during incubation with propionate. The values for substrate-transfer and charge transfer were used to determine the charge-to-substrate transfer ratio.

RESULTS

Amplification of the Coding Region of Human SLC5A8 mRNA for Functional Expression in Xenopus Oocytes—We amplified the coding region of SLC5A8 mRNA by RT-PCR using human intestinal mRNA as the template. The amino acid sequence of the amplified product was identical to the published sequences (1, 3) except for the amino acids at positions 193 and 251. Our amplified cDNA contains Ile at position 193 and Val at position 251. The corresponding amino acids are Ile and Phe in the clone reported by Li et al. (1) and Val and Phe in the clone reported by Rodriguez et al. (3).

Identification of SLC5A8 as a Na+-coupled Transporter for Short-chain Fatty Acids—We tested our hypothesis that SLC5A8 is a Na+-coupled transporter for short-chain fatty acids by measuring the uptake of several fatty acids in uninjected oocytes and in oocytes injected with SLC5A8 cRNA (Table I). The uptake of all fatty acids tested (l-lactate, d-lactate, pyruvate, acetate, propionate, and butyrate) was higher in cRNA-injected oocytes than in uninjected oocytes. The cRNA-induced increase in uptake varied in the range of 2–45-fold depending on the fatty acid. This increase was not observed when the uptake was measured in the absence of Na+ (data not shown).

Electrophysiological Studies of SLC5A8—We then examined the transport function of SLC5A8 by electrophysiological methods. Exposure of cRNA-injected oocytes to various short-chain fatty acids induced marked inward currents under voltage-clamp conditions and the magnitude of these currents increased as the testing membrane potential was changed from positive values to negative values (Fig. 1A). Exposure of uninjected oocytes to these fatty acids did not induce detectable currents (data not shown). We then compared the substrate-induced currents in cRNA-injected oocytes among several fatty acids (5 mM). The currents, measured at −50 mV, were maximal for l-lactate, pyruvate, and propionate, intermediate for d-lactate, acetate, butyrate, and pentanoate and lowest for hexanoate, heptanoate, and octanoate (Fig. 1B). With propionate as the substrate, we analyzed the saturation kinetics and Na+-activation kinetics. Results from a representative oocyte are given in Fig. 2 and similar results were obtained in three different additional oocytes. Propionate-induced currents were saturable with a K0.5 value of 137 ± 14 µM (Fig. 2A). The relationship between propionate-induced currents and Na+ concentration was sigmoidal and the value for Hill coefficient (h) was 2.0 ± 0.1 (Fig. 2B). The K0.5 value was not influenced significantly by the testing membrane potential between −30 mV and −150 mV (Fig. 3A). But, the value increased dramatically when the testing membrane potential became more positive. The Imax value (i.e. the current induced by the substrate at maximal concentrations) was however influenced by the testing membrane potential profoundly, the values being higher at hyperpolarized membrane potentials than at depolarized membrane potentials (Fig. 3B).

Comparative Affinities of Short-chain Fatty Acids for SLC5A8—We determined the K0.5 values for l-lactate, d-lactate, pyruvate, acetate, propionate, and butyrate to compare the affinities of these fatty acids for interaction with SLC5A8. The affinities were found to be in the following order: butyrate (81 ± 17 µM) > propionate (127 ± 14 µM) > l-lactate (235 ± 24 µM) > d-lactate (742 ± 330 µM) > acetate (2.46 ± 0.89 mM). These data were from four different oocytes.

Charge-to-Substrate Transfer Ratio—The Na+-activation kinetics with propionate suggested that at least two Na+ are involved in the activation process. Propionate exists as a monovalent anion at pH 7.5 and cotransport of two Na+ with one propionate molecule would predict the transport process to be electrogenic and explain the observed propionate-induced inward currents under voltage-clamp conditions. However, the Hill coefficient is only an estimate and does not always predict the exact number of coupled ions involved in the transport.

| Substrate | Uninjected oocytes | cRNA-injected oocytes | Fold increase |
|-----------|--------------------|-----------------------|--------------|
| L-Lactate (50 µM) | 0.009 ± 0.001 | 0.402 ± 0.026 | 44.7 |
| l-Lactate (150 µM) | 0.028 ± 0.002 | 1.090 ± 0.041 | 38.9 |
| Pyruvate (500 µM) | 0.151 ± 0.017 | 2.622 ± 0.075 | 17.4 |
| Acetate (150 µM) | 0.032 ± 0.001 | 0.059 ± 0.003 | 1.8 |
| Propionate (50 µM) | 0.016 ± 0.001 | 0.255 ± 0.014 | 15.9 |
| Butyrate (150 µM) | 0.029 ± 0.001 | 0.116 ± 0.007 | 4.0 |

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process. Therefore, we directly measured the transfer of propionate and charge simultaneously in the same oocytes to determine the exact value for the charge-to-substrate ratio (Fig. 4A). The value for this ratio determined with four different oocytes for propionate was 3.1 ± 0.1. To determine whether any other anions such as Cl⁻ or OH⁻ are involved in the transport process, we examined the ionic dependence of propionate-induced currents (Fig. 4B). The substrate-induced currents were obligatorily dependent on the presence of Na⁺, but there appeared to be no involvement of Cl⁻ as there was no significant change in the magnitude of the currents when Cl⁻ was replaced by gluconate in the perfusion medium. We also investigated whether the currents were influenced by external pH. The propionate-induced currents remained the same over the pH range 6–8, suggesting that anions such as OH⁻ are not likely to be involved in the transport process (data not shown).

Interaction of Iodide with SLC5A8—Since studied by Rodriguez et al. (3) have shown that SLC5A8 functions as a passive transporter for iodide, we examined the potential interaction of this inorganic anion with SLC5A8 in cRNA-injected oocytes. The presence of 5 mM NaI did not induce any detectable current (inward or outward) in these oocytes (data not shown).

**DISCUSSION**

The present studies have established the functional identity of human SLC5A8 as a Na⁺-coupled transporter for short-chain fatty acids with a Na⁺:substrate stoichiometry of 4:1. This is evident from the uptake of radiolabeled substrates as well as from substrate-induced inward currents. The inward currents induced by short-chain fatty acids are obligatorily dependent on Na⁺. Neither Cl⁻ nor OH⁻ is involved in the transport process. The magnitude of the substrate-induced currents is maximal for fatty acids containing 2–5 carbon atoms and the currents decrease as the carbon chain length increases. Under the experimental conditions employed in our studies, we could not detect any interaction of iodide with SLC5A8. The reasons for the differences between our studies and those of Rodriguez et al. (3) are not known. Li et al. (1) have shown that SLC5A8-expressing oocytes had higher levels of Na⁺, leading to the conclusion that SLC5A8 is a Na⁺ transporter even though their studies did not establish the identity of the cotransported ion. Sodium pyruvate is normally added to the medium used in the maintenance of oocytes following cRNA injection, and incubation of SLC5A8-expressing oocytes in pyruvate-containing medium during the maintenance of the oocytes is expected to increase intracellular Na⁺ based on the functional identity of the transporter established in the present studies. However, it was not readily apparent whether or not the oocytes were maintained in the presence of pyruvate in the studies reported by Li et al. (1). Our studies show unequivocally that SLC5A8 is a Na⁺-coupled transporter for short-chain fatty acids.

The substrate specificity of SLC5A8 is similar to that of H⁺-coupled monocarboxylate transporters (MCTs) (13). Therefore, based on the functional identity of SLC5A8 as the Na⁺-coupled transporter for monocarboxylates, we refer to this transporter as SMCT (for sodium-coupled monocarboxylate transporter). However, there is no sequence similarity between SMCT and MCTs. The functional identity of SMCT as a Na⁺-coupled transporter for short-chain fatty acids is very important with regard to the abundant expression of the transporter...
short-chain fatty acids in the colon (14, 15), but surprisingly none of these studies has identified the presence of a Na⁺-coupled absorptive mechanism for these fatty acids. However, a Na⁺-coupled lactate transport system has been demonstrated in renal brush border membrane vesicles (16, 17), but the molecular identity of the transport system has not yet been established. Since SLC5A8 transcripts are expressed in kidney (1), we suggest that this transporter is likely to be responsible for Na⁺/lactate cotransport in kidney. The ability of SMCT to transport ß-lactate is of physiological relevance, because while mammalian metabolism generates exclusively ß-lactate, bacterial metabolism generates not only ß-lactate but also 3-lactate (18). Therefore, SMCT is most likely responsible for the absorption of ß-lactate from the intestinal tract. ß-Lactate is found in human blood and elevated levels of ß-lactate in blood (ß-lactic acidosis) result from bacterial overgrowth in the small intestine under various clinical and pathological conditions (19).

The functional identification of SMCT as a Na⁺-coupled transporter for short-chain fatty acids is also relevant to recent studies by Li et al. (1). Their studies suggested that this transporter may function as a tumor suppressor because its expression is silenced in colon cancer. This suggests that the absorption of short-chain fatty acids may be impaired in colonocytes at the site of cancer. Several studies have shown that short-chain fatty acids in the colon are effective in reducing the incidence of colon cancer (7–9). The potential mechanisms of tumor suppression by these fatty acids include inhibition of histone deacetylases (20), induction of apoptosis (21, 22), modulation of gene expression (23), and induction of cell cycle arrest (24). These fatty acids may also be involved in the prevention of colon cancer metastasis (25).

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