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Original Article

Residue cysteine 232 is important for substrate transport of neutral amino acid transporter, SNAT4

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Abstract: SNAT4 is a system A type amino acid transporter that primarily expresses in liver and mediates the transport of L-alanine. To determine the critical amino acid residue(s) involved in substrate transport function of SNAT4, we used hydrosulfate cross-linking MTS reagents – MMTS and MTSEA. These two reagents caused inhibition of L-alanine transport by wild-type SNAT4. There are 5 cysteine residues in SNAT4 and among them; residues Cys-232 and Cys-345 are located in the transmembrane domains. Mutation of Cys-232, but not Cys-345, inhibited transport function of SNAT4 and also rendered SNAT4 less sensitive to the cross-linking by MMTS and MTSEA. The results suggested that TMD located Cys-232 is an aqueous accessible residue, likely to be located close to the core of substrate binding site. Mutation of Cys-232 to serine similarly attenuated the transport of L-alanine substrate. Biotinylation analysis showed that C232A mutant of SNAT4 was equally capable as wild-type SNAT4 of expressing on the cell surface. Moreover, single site mutant, C232A was also found to be more resistant to MTS inhibition than double mutant C18A,C345A, further confirming the aqueous accessibility of Cys-232 residue. We also showed that mutation of Cys-232 to alanine reduced the maximal velocity (Vmax), but had minimal effect on binding affinity (Km). Together, these data suggest that residue Cys-232 at 4th transmembrane domain of SNAT4 has a major influence on substrate transport capacity, but not on substrate binding affinity.

Keywords: Neutral amino acid transporter, SNAT4, Cys-232, substrate transport

Introduction

Amino acid transporters mediate the uptake and transport of amino acids present in the living organisms. They also regulate various cellular processes including uptake of nutrients, protein synthesis, neurotransmitter cycling, etc [1]. These transporters fall into different categories of gene families which consist of several members based on various properties, e.g. substrate specificity, tissue specificity, etc [2]. Sodium-coupled neutral amino acid transporters (SNAT) or the solute carrier 38 (SLC38) transporters are categorized in the amino acid/auxin permease (AAAP) gene family of anion-polyamine-organocation (APC) superfamily [3, 4]. These low affinity transporters mainly regulate transport of small neutral amino acids in a sodium- and pH-dependent manner [5]. SNAT group of transporters is further grouped into two subfamilies – systems A and N. System A subfamily coupled to sodium gradient mainly transports amino acids with aliphatic side chains and consists of SNAT1 (SLC38A1), SNAT2 (SLC38A2) and SNAT4 (SLC38A4). On the other hand, system N transporters which are coupled to both sodium and proton gradient transport amino acids with nitrogen in their side chain [5], for example SNAT3 (SLC38A3) and SNAT5 (SLC38A5). The sixth transporter, SNAT6 is not categorized into any sub-family as the function of the transporter is not yet known [5].

SNAT4 or SLC38A4 is a 547 amino acids member of the system A subfamily which mainly expresses in liver, muscle and placenta [6-9]. A previous study showed that SNAT4 is regulated by insulin mediated PI3-kinase signaling pathway and is likely to play a major role in liver function [7]. However, there is scarcity of information with regard to the structure and function of this transporter and SNAT family of transporters in general. SNAT4 prefers transport of L-alanine followed by L-histidine and L-glutamine

[6], but have also been reported to transport cationic amino acids independent of sodium gradient [10]. In comparison to SNAT1 and SNAT2, SNAT4 has low substrate affinity [10]. It shares 48% sequence homology with SNAT1 and 57% with SNAT2 [5]. Recent study by Shi et al [11], showed that SNAT4 consists of ten transmembrane segments with both N and C-termini facing the extracellular side. Cys-249 and Cys-321 are located in the same extracellular loop domain, likely forming disulfide bridge. However, the three-dimensional structure and the substrate binding sites of SNAT family of transporters are yet unknown.

The transmembrane domains (TMD) in the transporters have been shown to play an important role in substrate transport as they not only assist in substrate translocation but also harbor substrate binding sites [12]. In this study, we found that mutation of residue cysteine 232 to alanine leads to substantial decrease in substrate transport. This residue was not only found to be present in TMD4 but also found to be highly conserved in the SNAT transporter family. Interestingly, the cross-linking study with MTS reagents showed that this residue regulates transport capacity, but appears not to be directly involved in substrate binding.

Experimental procedures

Materials

Quick Change Site Directed Mutagenesis Kit™ was purchased from Stratagene (La Jolla, CA). Leibovitz (L-15) medium, hydrogen peroxide, penicillin G sodium salt, streptomycin sulfate salt and gentamicin sulfate salt were obtained from Sigma (St. Louis, MO). Restriction enzymes and peptide N-glycosidase (PNGaseF) were from New England Biolabs (NEB) (Revere, MA). Peroxidase and FITC conjugated anti-rabbit antibody were obtained from GE Healthcare Amersham (Piscataway, NJ) and Invitrogen Corporation (Carlsbad, CA), respectively. SDS-polyacrylamide gel electrophoresis standards were purchased from Bio-Rad and the nitrocellulose membrane were from Schleicher and Schuell (Keene,NH). Enhanced Chemiluminescence (ECL) kit was obtained from Amersham Biosciences (Piscataway, NJ). NHS-SS-Biotin and Neutravidin beads were purchased from Pierce, now Thermo Fisher Scientific (Waltham, MA). mMESSAGE mMACHINE for *in vitro* transcription was obtained from Ambion (Austin, TX). [^{3}H]-L-alanine was purchased from American Radiolabeled Chemicals (St. Louis, MO). Protease inhibitors were obtained from Roche Molecular Biochemicals (Mannheim, Germany). MTS reagents were purchased from Anatrace (Santa Clara, CA) and Toronto Research Chemicals (Toronto, Canada). All other chemicals were either from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Construction of mutants

Subcloned mouse wild-type SNAT4 in *Xenopus* oocyte expression vector, pGEMHE [13]. Mutant C232A, C232S C345A and C18A, C345A were constructed using wild-type SNAT4 as template by using Quick Change Site Directed Mutagenesis Kit™ as per manufacturer's instructions and the identity of the mutants was confirmed by sequencing. The primers for PCR were designed to convert cysteine residue to serine or alanine. All the plasmids were linearized using *AflIII* enzyme and *in vitro* transcribed by T7 RNA polymerase using mMessage mMachine kit (Ambion, Austin, TX). cRNA was extracted and purified by lithium chloride and ethanol precipitation method according to manufacturer's instruction. RNA was resuspended in diethyl pyrocarbonated-treated water at a concentration of 1 μg/μl and stored at -80°C prior to use.

Transport assays

Xenopus laevis oocytes were injected with 40 nl of cRNA of wild-type or mutant SNAT4. Water injected oocytes were used as control. After incubating at 17°C for 72 hours, the uptake assays were performed. Oocytes were rinsed three times with the uptake buffer and then incubated in the same buffer for 30 minutes at room temperature. Amino acid uptake was measured by incubating the oocytes with 500 μl of 50 μM [^{3}H]-L-alanine for 30 minutes at room temperature. To study the effect of MTS reagents, the cRNA injected oocytes were incubated with appropriate amount of MTS reagents in both non-radioactive and radioactive uptake buffer. In some experiments, oocytes were also incubated with 0.02% of hydrogen peroxide (H_2O_2) along with MTS reagents to provide oxidative conditions [14]. To determine the substrate binding, the uptake of L-alanine was

measured at various concentrations, 0.2, 0.5, 1, 3, 6 and 8 mM. After incubation with radioactive substrate, cells were washed three times with the same cold uptake buffer to terminate the uptake and were lysed with 2% SDS. The lysate was used for measurement of radioactivity with a scintillation counter in 5 ml scintillation solution. The kinetic parameters (Km and Vmax) were analyzed using GraphPad prism. The results are presented as either percentage L-alanine uptake after normalization with the protein expression data or in pmol/oocyte/min (in kinetic studies) and are expressed as the means ± SEM. (n = 3).

Membrane protein preparation

Crude membrane extracts were prepared from *Xenopus* oocytes. Oocytes were homogenized in lysis buffer (5 mM Tris, 5 mM EDTA and 5 mM EGTA at pH 8.0) containing proteinase inhibitors (NEM, PMSF, leupeptin and sodium vanadate). The homogenate was centrifuged at 6.6g for 10 minutes at 4°C twice to remove the yolk and the supernatant was collected. The supernatant was then centrifuged at 100,000g for 30 minutes at 4°C. The membrane pellet was resuspended in lysis buffer containing 1% SDS and 5X sample buffer was added. The sample was then loaded on 10% SDS/PAGE for western blotting analysis.

Cell surface biotinylation

Biotinylation of cells was performed based on the modification of previously published procedures [15]. Seventy-two hours after cRNA injection, 20 oocytes were labeled twice with 1 mg/ml Sulfo-NHS-LC-biotin at 4°C for 30 min each. The oocytes were then washed three times with PBS plus 100 mM glycine to stop the biotinylation reaction. Later, the oocytes were lysed and crude membrane protein extract was prepared as described above. The pellet was dissolved in RIPA and TRIS buffer, and incubated with streptavidin beads for overnight at 4°C. The beads were washed three times with phosphate buffered saline, and the biotinylated proteins were eluted by boiling for 5 min in a SDS-containing sample loading buffer. The total lysate and biotinylated samples (eluted from the streptavidin beads) were separated on SDS/PAGE and then immunoblotted with affinity-purified anti-SNAT4 antibody. The band intensities for the biotinylated and total protein were quantified using Scion Image software (Scion Inc.). Percentage of biotinylated (representing the surface pool) *versus* total (preloaded) SNAT4 was calculated. Data was analyzed by ANOVA followed by the Student-Newman-Keuls test to compare wild type and mutant SNAT4 biotinylated fractions. Data is presented as mean ± SEM.

Western blot and biotinylation analysis

Anti-SNAT4 IgG antibody was produced as described previously [16] and the purified protein was used to raise polyclonal antisera in rabbit (Pocono Rabbit Farm and Laboratory Inc., Canadensis, PA). The antisera generated were affinity-purified by passage through two Sepharose CL-4B columns, GST-conjugated and GST-SNAT4 fusion protein-conjugated, respectively. Membrane protein and biotinylation samples were loaded on 10% SDS/PAGE, transferred to the nitrocellulose membrane by semi-dry transfer apparatus (Bio-Rad) and the membrane was blocked with 10% non-fat dry milk for overnight. The membrane was probed with 1:1000 dilutions of affinity-purified anti-SNAT4 and 1:5000 dilutions of peroxidase conjugated anti-rabbit secondary antibody. The antibody was detected using chemiluminescence reagent (ECL kit) according to manufacturer's protocol. The membrane was then exposed to Phenix F-BX810 Blue X-Ray film and detected by autoradiography.

Results

MMTS and MTSEA inhibit L-alanine uptake of SNAT4

To determine if cysteine residues are directly involved in transport function of SNAT4, we used sulfhydryl crosslinking reagents to covalently react with cysteine residues and to study their effects on transport function [17-19]. Four methanethiosulfonate (MTS) reagents were used, MTSET, MTSES, MTSEA and MMTS. The reagents were selected on the basis of difference in charge, size and permeability so as to test accessibility of cysteine residues of SNAT4 and their ability to inhibit L-alanine uptake. Oocytes were injected with wild-type SNAT4 cRNAs and the transporter activity was determined in presence of MTS reagents. The result showed that L-alanine uptake was greatly inhibited by MMTS and MTSEA up to 90%, but not by

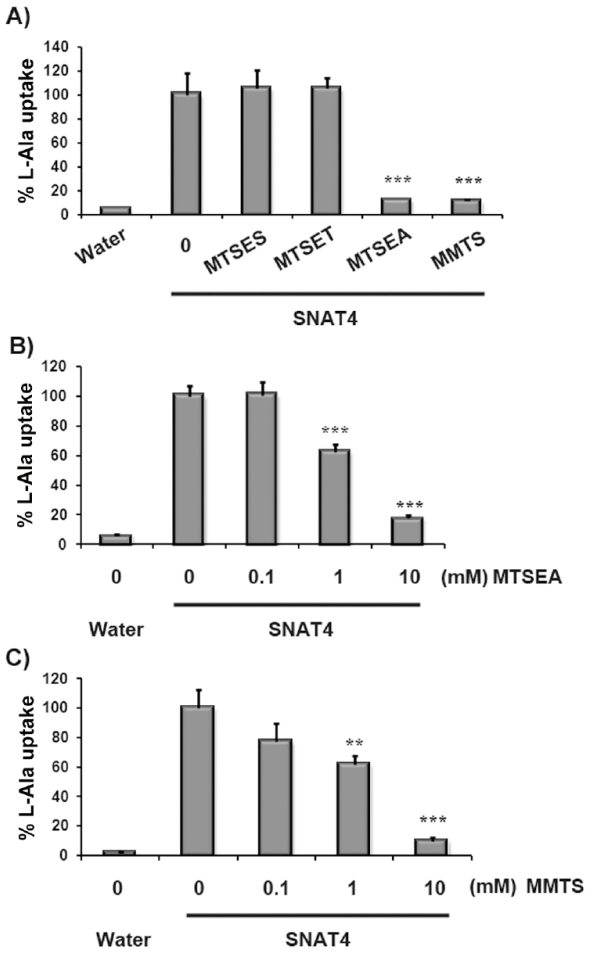

Figure 1. *Dose-dependent inhibition of SNAT4 transport activity by MTS reagents - MTSEA and MMTS*. (A) *Xenopus* oocytes microinjected with cRNA of wild-type SNAT4 were preincubated with MTS reagents, 10 mM MTSET, MTSES, MTSEA or MMTS for 30 minutes. [^{3}H] L-alanine uptake assay was performed in presence of MTS reagents. Water-injected oocytes were used as negative control. L-alanine uptake was significantly decreased by neutral reagents, MMTS and MTSEA, but not by charged reagents, MTSET and MTSES. *Xenopus* oocytes injected with wild-type SNAT4 cRNA were incubated with MTSEA (0-10 mM) (B) and MMTS (0-10 mM) (C), and the [^{3}H] L-alanine uptake assay was performed. L-alanine transport was significantly decreased in a dose-dependent manner in presence of MTS reagents compared to untreated control. Data is presented as mean ± SEM, n = 3. MTS at 0.1, 1 and 10mM *versus* untreated control of SNAT4, ***, $P < 0.001$.

MTSET and MTSES (**Figure 1A**). Former two reagents are neutral and small in size as compared to the latter two which are charged and bigger in size. Based on above data, MTSEA and MMTS were selected as the sulfhydryl reagents to determine the roles of cysteine residues of SNAT4. MMTS is reported to be membrane impermeable [20]. Dose response curve was obtained at 0.1, 1 and 10 mM concentrations of MTSEA (**Figure 1B**) and MMTS (**Figure 1C**). The L-alanine uptake was significantly decreased in a dose-dependent manner. This suggests that cysteine residues of SNAT4 are accessible to the neutral reagents.

Mutation of Cys-232 to alanine and serine leads to loss of transporter activity

There are five cysteine residues located in SNAT4 protein (**Figure 2**). To determine the role of cysteine residues located in the transmembrane domains, we generated single site mutants, C232A and C345A by replacing cysteine with alanine. L-alanine uptake assay was performed to determine the transport activity of these single site mutants. The transporter activity obtained was normalized with the level of SNAT4 protein expressed. The replacement of Cys-232, but not Cys-345, with alanine led to the loss in transporter activity of SNAT4 (**Figure 3A**). Cys-232 is located on TMD4 and it is possible that this site is present in the solvent accessible region of SNAT4 transporter. In order to confirm that the decrease in uptake by C232A is not due to cysteine to alanine mutation, we replaced the cysteine with serine residue. The result showed that the reduction in transporter activity of C232S mutant was even higher as compared to C232A (**Figure 3B**), although the structure of serine is more similar to cysteine than alanine. The data suggest that cysteine at position 232 is critical for transport function of SNAT4.

The difference between wild-type and C232A mutant of SNAT4 in L-alanine uptake could be due to the level of proteins expressed on the surface of oocytes. We conducted cell surface biotinylation assay to determine the level of wild-type and C232A mutant on the surface. The data showed that wild-type (WT) and C232A mutant was expressed on the cell surface at the similar level (**Figure 4**, quantitative data shown on the right panel).

Mutant C232A is more resistant to the inhibition of L-alanine uptake by MTS reagents

Previous studies have shown that transporters belonging to evolutionarily distinct protein families share the common features, such as their substrate binding sites reside on the transmembrane domains [12]. Furthermore, these

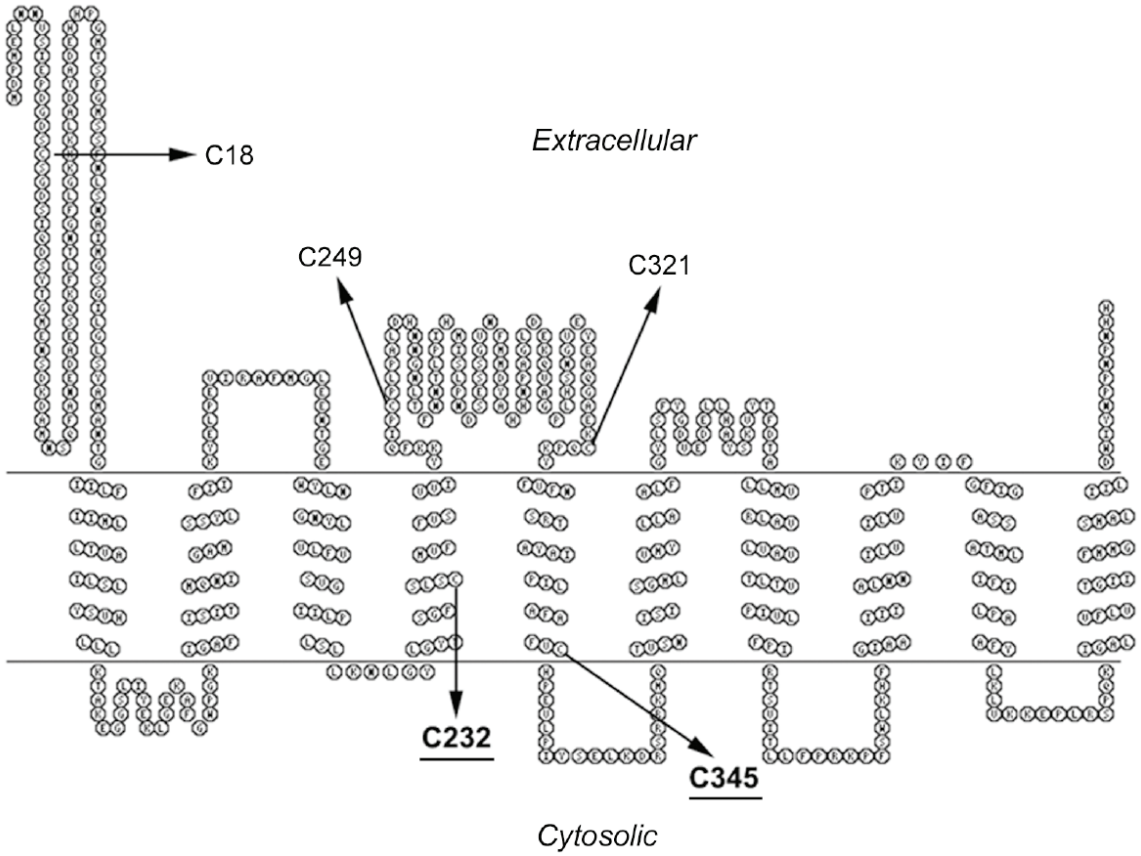

Figure 2. The locations of cysteine residues are indicated in the topological structure of SNAT4.

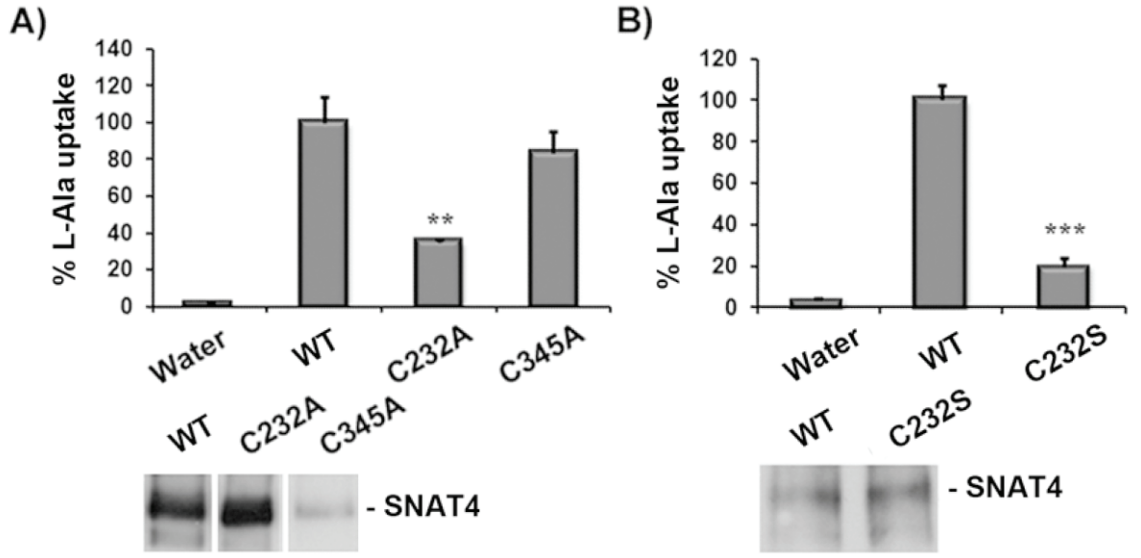

Figure 3. *Mutation of Cys-232 to alanine or serine decreases transport function of SNAT4* – The cRNAs of wild-type (WT), C345A (A) or C232S (B) SNAT4 mutants were injected into *Xenopus* oocytes and lysates were immunoblotted with anti-SNAT4 antibody (right panels). Water injected oocytes were used as control. The transport activity was normalized with the protein expression data. Both C232A and C232S showed about 60% and 80% decrease in uptake of L-alanine, respectively. Data is presented as mean ± SEM, n = 3. Mutant vs WT, ***, $P < 0.001$ and **, $P < 0.01$.

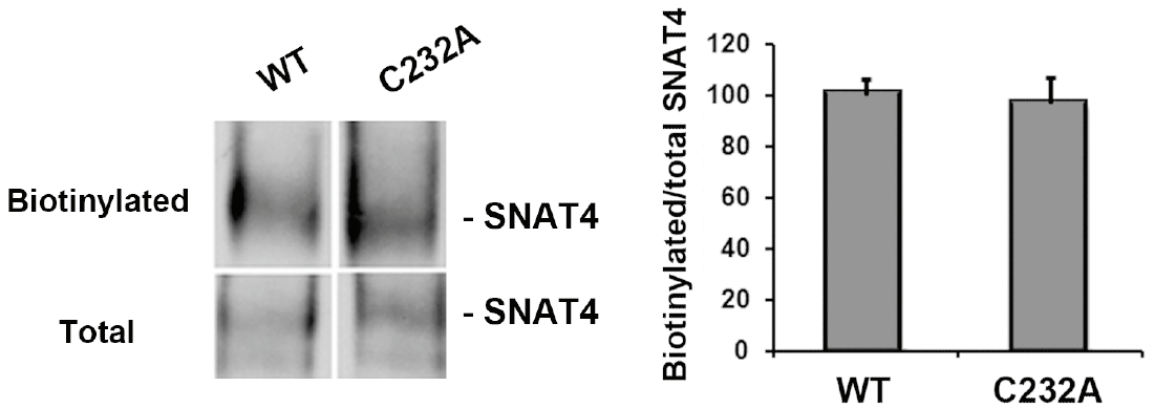

Figure 4. *Mutation of Cys-232 to alanine does not affect cell surface expression of SNAT4 – Xenopus* oocytes injected with cRNAs of wild-type (WT) and C232A mutant were biotinylated, and biotinylated samples and preloaded controls (Total) were immunoblotted with anti-SNAT4 antibody (left panel). The intensity of the bands was quantified (right panel). Data is presented as mean ± SEM, n = 3.

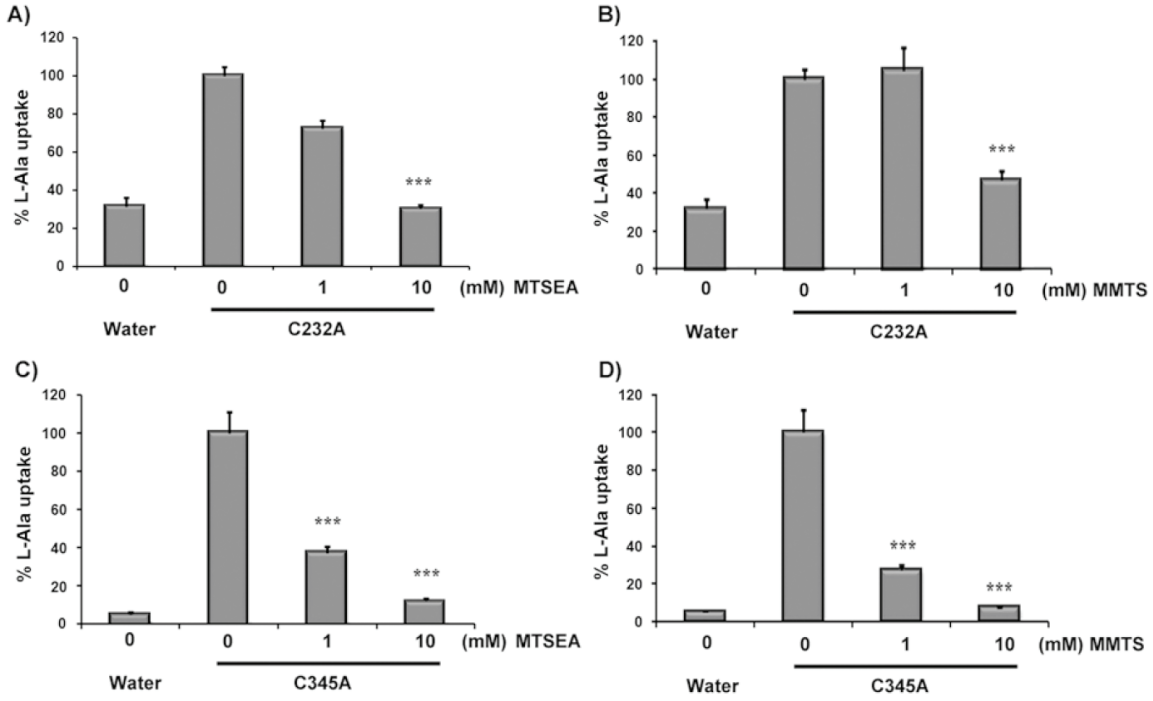

Figure 5. *C232A mutant of SNAT4 is more resistant to MTS reagents than C345A.* The cRNAs of mutants C232A or C345A were injected in *Xenopus* oocytes and the L-alanine transport activity was determined in presence of MMTS (1 mM) or MTSEA (1 mM). Contrary to mutant C345A, L-alanine uptake of mutant C232A (A and B) was not significantly inhibited by MTS reagents at 1 mM. Data is presented as mean ± SEM, n = 3. MTS at 1 and 10 mM *versus* untreated control of SNAT4, ***, $P < 0.001$.

binding sites are the most conserved residues in the transporters [12]. Hence, it is possible that highly conserved cysteine residue 232 of TMD4 is present around the substrate binding region of SNAT4 and thus is accessible to aqueous MTS reagents. To test this possibility, C232A mutant cRNA was injected in oocytes and the transporter activity was determined in presence of 1 and 10 mM MTSEA and MMTS. The result showed that in presence of 10 mM MTSEA or MMTS the L-alanine transport was completely abolished in both C232A (**Figure 5A** and **5B**) and C345A (**Figure 5C** and **5D**) mutants. Inhibition was not observed with 1

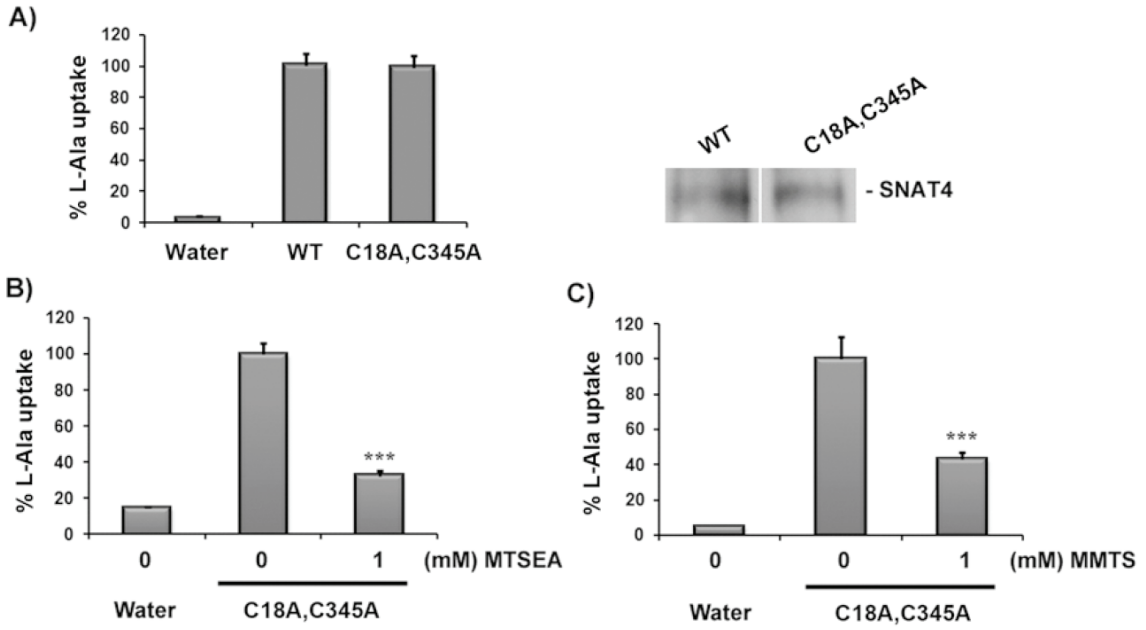

Figure 6. *Double mutant C18A,C345A has no effect on transport activity, but transport activity is reduced in presence of MTS reagents* – (A) cRNA of double mutant, C18A,C345A, was injected into the oocytes, the L-alanine transport activity was determined and the data was normalized with the protein expression. No significant difference in L-alanine transport was observed in comparison to wild-type SNAT4. (B and C) The cRNA of C18A,C345A mutant-injected oocytes were incubated with MTSEA (1 mM) or MMTS (1 mM), and L-alanine uptake assay was performed. The result showed that the transport in mutant significantly decreased in presence of MTS reagents. Data is presented as mean ± SEM, n = 3. MTS at 1 mM *versus* untreated control of SNAT4, ***, $P < 0.001$.

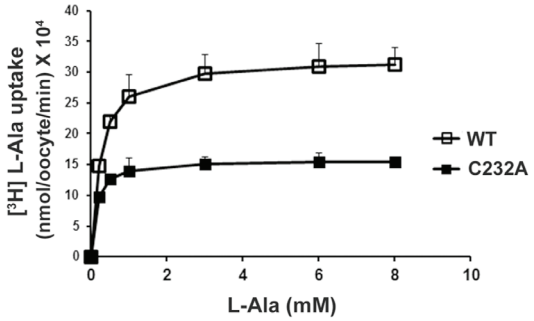

Figure 7. *Mutation on Cys-232 decreases transport velocity, but has no effect on substrate binding affinity*. cRNAs of wild-type (WT) or C232A mutant was injected in *Xenopus* oocytes. The transport activity was determined with various concentrations of L-alanine and the Km and Vmax values were calculated. The result showed that Cys-232 has impact on substrate transport velocity, but not affinity.

mM MMTS in C232A mutant (**Figure 5B**) whereas C345A activity was dramatically reduced (**Figure 5D**). Consistent with MMTS, the activity of C232A was also not blocked by 1 mM MTSEA, but a significant reduction was observed for C345A mutant. The MTSEA had lesser degree of inhibition as compared to MMTS in both C232A (**Figure 5A**) and C345A (**Figure 5C**) possibly due to structural constraints of MTSEA. This data suggests that TMD of Cys-232 is more accessible to the MTS reagent than Cys-345 and this residue may play a role in substrate binding or translocation.

Binding of MTS reagents to Cys-232 inhibits transport function

To further confirm that MTS reagents bind to Cys-232 to inhibit transport by SNAT4, besides relatively non-reactive disulfide bond, we only retained Cys-232 by mutating other two cysteine residues Cys-18 and Cys-345 to alanine. The cRNA of the double site mutant, C18A,C345A, was injected into the oocytes along with wild-type SNAT4 cRNA, and L-alanine uptake assays were performed. The result showed that the transporter activity of mutant C18A,C345A was completely preserved, similar to the wild-type SNAT4 (**Figure 6A**). The mutant was then tested for activity in presence of 1 mM MTSEA and MMTS. The result showed that there was a significant loss in transporter activity in presence of MTSEA (**Figure 6B**) or

MMTS (**Figure 6C**). This results suggests that the binding of MTS reagents to Cys-232 leads to inhibition of L-alanine uptake.

Residue Cys-232 regulates transport velocity, but not substrate affinity in SNAT4 transporter

Oocytes injected with wild-type or mutant C232A were incubated with various concentrations of L-alanine and the kinetic characteristics were analyzed (**Figure 7**). Compared to wild-type, C232A showed significant reduction in maximal velocity (Vmax). However, no significant change in the substrate affinity was observed in the mutant (Km ~ 0.17 ± 0.04 mM) compared to wild-type (Km ~ 0.3 ± 0.07 mM, $P = 0.2$). This result suggests that the residue cysteine 232 present at a solvent accessible position regulates the substrate translocation of SNAT4.

Discussion

In this study, we identified a critical amino acid residue involved in substrate transport of SNAT4, a member of SNAT family. Cys-232 is one of the four highly conserved cysteine residues across the SNAT family of transporters [11] and is present on the TMD4 of SNAT4. Our data suggests that Cys-232 plays a role in substrate translocation and is likely to be one of the critical residues in substrate permeation pathway of SNAT4 transporter.

According to previous studies, cysteine residues have been reported to play a critical role in expression and function of transporter. A previous study shows that a conserved cysteine residue at the N-terminus of human multidrug resistance-associated protein 1 (MRP1) plays a critical role in the MRP-1 mediated drug resistance and leukotriene C(4) transport activity [21]. Similar to our observation, mutation of this residue leads to the decrease of Vmax due to the conformational change of the N-terminus. Study of a GABA transporter, gabP in *E.Coli* identifies a 'signature cysteine' residue Cys-300, which has the ability to sustain wild-type transporter properties in a cysteine-less gabP mutant [22]. Another study also reports the significance of cysteine residues in organic anion transporter (OAT1) [23]. According to this study, mutation of the cysteines leads to decreased expression of OAT1 in HeLa cells. Furthermore, the involvement of cysteine residues in protein expression and function has also been reported for other transporters, such as serotonin transporter [24] and Na^+/dicarboxylate co-transporter [25]. However, the significance of cysteine residues in SNAT family of transporters has not yet been investigated.

In our initial study we treated wild-type SNAT4 with four MTS reagents differing in their size and charge, and analyzed the effect on transporter activity. No significant inhibition on SNAT4 transport activity was observed with charged reagents, MTSES and MTSET. On the other hand, the smaller size, neutral reagents MTSEA and MMTS significantly inhibited L-alanine transport by wild-type SNAT4 in a dose-dependent manner. This data suggests that the MTS accessible cysteine residue is likely to be present in the substrate translocation pore. It is possible that the covalent attachment of neutral MTS reagents with cysteine residue(s) present towards the water accessible region leads to block in L-alanine transport by SNAT4. The lack of reactivity with larger size thiol reagents could be due to smaller pore/cavity and/or inaccessible location of cysteine residues. Overall, this data provides a strong indication that cysteine residue(s) are likely to play an important role in substrate transport by SNAT4.

We showed that mutation of Cys-232 to alanine lead to substantial loss of transporter activity. A more significant decrease in alanine transport by mutant C232S compared to mutant C232A confirmed that this residue is indispensable for the transport function of SNAT4. Interestingly, structure-wise, cysteine is more analogous to serine than to alanine; however, mutation to serine leads to even more reduction in transport function. One possible interpretation is that addition of serine residue may form an undesirable hydrogen bond that somehow hinders substrate translocation. Moreover, we also showed that Cys-232 did not play a role in cell surface expression which strongly indicates that Cys-232 is likely to be a critical residue in SNAT4 transport activity.

Previous studies have shown that residues of amino acid transporters involved in substrate binding or translocation are highly conserved within the transporter family [12]. Furthermore, these residues have been found to be present on the TMD of the transporters [12]. In the

same context, Cys-232 is also highly conserved and present on the TMD which offers a strong clue suggesting involvement of Cys-232 in substrate translocation or binding. If Cys-232 is involved in substrate binding or translocation, it is possibly a water accessible residue capable of interaction with MTS reagents. A strong evidence for the above assumption was obtained by the decreased sensitivity of mutant C232A to the inhibitory effect of MMTS and MTSEA on L-alanine uptake. However, the transport activity of another cysteine mutant C345A was inhibited in presence of MTS reagents although the C345 residue is also present on a TMD. But in this case, the mutant also had C232 residue intact on the TMD which is the possible reason for inhibition of C345A mutant transport activity by MTS reagents. This confirmed that Cys-232 is not only accessible but also responsible for SNAT4 inhibition by MTS reagents. Furthermore, inhibition in transport activity of the double site mutant C18A,C345A under the same conditions provides further clue that Cys-232 is exclusively responsible for inhibition in L-alanine uptake observed in presence of MTS reagents. Hence, this clearly suggests the possibility of MTS reagents directly binding to Cys-232 to exert their effect on SNAT4.

Previous study on gab permease, a GABA transporter suggested presence of a signature cysteine residue in the consensus amphipathic region or a CAR domain of the transporter [22]. Interestingly, residue Cys-232 and its TMD4 share several structural features with the CAR domain. First, TMD4 is also amphipathic. Second, it consists of highly conserved cysteine residue. Third, the residue is capable of sustaining the wild-type properties although not to the extent of the signature cysteine of GABA transporter. Finally, the cysteine is a target of inhibitory sulfhydryl reagents that can modify the functional properties. According to the result, removal of cysteine 232 led to drastic reduction in rate of [^{3}H]-alanine transport but did not affect the substrate binding or Km. This suggests that Cys-232 may be present in substrate permeation pathway and is involved in regulating the rate limiting step (transition state) of substrate translocation. However, since no change in substrate affinity was observed compared to wild-type SNAT4, Cys-232 is unlikely a direct substrate binding site. Interestingly, similar results were also obtained on removal of signature cysteine in GABA transporter [22]. In the GABA transporter mutation of cysteine 300 to alanine leads to diminished rate of GABA transport but does not affect the substrate affinity. It is possible that, this feature is not only conserved within the SNAT family but also might be extended to other transporter families with no sequence identity. Further studies will be undertaken to identify functionally significant amino acid residues to delineate the substrate binding site(s) in SNAT family of transporters.

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