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Cys^{294} is essential for the function of the human sodium-dependent multivitamin transporter☆

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ABSTRACT

The sodium-dependent multivitamin transporter (SMVT) plays an important role in biotin uptake in the intestine and other cell types. While significant knowledge has been gained with regard to regulation and cell biology of the SMVT system, there is little known about its structure-function relationships. Here we examined the role of each of the ten conserved (among species) cysteine residues in the function of the human SMVT (hSMVT) using site-directed mutagenesis. Our results showed a significant impairment in biotin uptake only in cells transfected with hSMVT mutated at Cys^{294}, but not at the other conserved cysteine residues; the impairment in biotin uptake caused by mutating Cys^{294} was not related to the polar status of substituting amino acid. The inhibition in hSMVT function upon mutating Cys^{294} was mediated via a significant reduction in the V_{max}, but not the apparent K_m, of the biotin uptake process, suggesting a decrease in the number (and/or activity) of hSMVT but not affinity. Biotinylation assay confirmed this suggestion by showing a marked reduction in the level of expression of the mutated protein at the cell membrane, without affecting total cellular level of induced hSMVT. These results show an important role for Cys^{294} in the function and cell biology of hSMVT.

1. Introduction

Biotin, a member of the B-family of water-soluble vitamins, functions as a coenzyme in critical metabolic reactions that include fatty acid biosynthesis and gluconeogenesis [1]. Deficiency of biotin in humans leads to serious clinical abnormalities that include neurological and dermatological disorders as well as growth retardation [2].

Mammals cannot synthesize biotin and obtain the vitamin from exogenous sources via intestinal absorption. Two sources of biotin are available to humans one being dietary and the other is bacterial, i.e., biotin that is supplied by the normal micro-flora of the large intestine [3–7]. Absorption of both of these sources of biotin in the small and large intestine, respectively, occurs via a carrier-mediated process that involves SMVT (the name was given to this system because it also transports the vitamin pantothenic acid and the metabolically important substrate, lipoate [8]. The gene that encodes the human SMVT (hSMVT), i.e., *SLC5A6*, contains 17 exons. Topologically, the hSMVT protein is predicted to have 12 trans-membrane domains (TMD) with both the N- and C-termini that are oriented toward the cell interior [9,10]. Studies from our laboratory utilizing gene silencing approach (gene-specific Si-RNA) have shown that hSMVT is the major biotin transporter in intestine [11]. We have also observed that the hSMVT protein is exclusively expressed at the apical membrane domain of the polarized enterocytes [12]. Other studies have shown that the activity of hSMVT is regulated by intracellular and extracellular factors via transcriptional and/or post-transcriptional mechanism [13–17].

Information concerning the structure-function relationships of the SMVT system is currently limited. We have previously shown an important role for His^{115} and His^{254} residues [18]. In another recent study from our laboratory we found hSMVT to be glycosylated at position Asn^{138} and Asn^{489}, and that mutating these residues affects functionality of hSMVT [13]. Furthermore, a role for PMA in modulating the regulatory effect of protein kinase C on hSMVT function was also studied [13].

Using a chemical approach, our previous studies have suggested involvement of cysteine residues in the function of SMVT [19,20]. Incubation of rabbit intestinal brush border membrane vesicles (BBMV) [19] and human liver HepG2 cells [20] with sulfhydryl group (−SH) modifying reagents showed significant inhibition in biotin uptake. The mammalian SMVT (i.e., that of human, bovine, rabbit, rat, and mouse) has ten conserved cysteine residues; which of these residues are the targets of inhibition by sulfhydryl group modifying agents is not clear. In this study we used site directed

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mutagenesis to identify the likely Cys targets. We tested the hypothesis by mutating conserved Cys residues and our results showed that mutation of Cys^{294}, but not the other conserved cysteine residues, leads to a significant inhibition in biotin uptake. This inhibition was mediated via a significant decrease in the V_{max} of the biotin uptake process with no change in the apparent K_m, and was the result of impairment in expression of the carrier protein at the cell surface. We also extended this study by mutating nonconserved cysteine residues oriented to the cell interior as potential disulphide bridge forming partner with Cys^{294}. Result showed that mutation at Cys^{628} to alanine or polar serine causes significant inhibition in biotin uptake suggesting potential disulfide bridge formation with Cys^{294}.

2. Materials and methods

2.1. Material

Radio labeled [^{3}H]-biotin (Specific activity > 30 Ci/mmol, radiochemical purity > 98%) was purchased from American Radiolabeled Chemicals (ARC, St. Louis, MO). All chemicals used are either of analytical or molecular biology grades and purchased from commercial sources.

2.2. Cell culture

The human retinal pigmented epithelial cells (ARPE19) were used for uptake studies. We used these cells because they are easy to transfect and they show robust efficiency in expressing transfected hSMVT [9,18]. The cells were obtained from ATCC (Manassas, VA) and were maintained in DMEM medium (Sigma, St. Louise, MO) supplemented with 10% fetal bovine serum and penicillin (100 units/ml) and streptomycin (100 μg/ml) as described by us previously [18].

2.3. Generation of hSMVT mutants and transient transfection

Quick Change II kit (Stratagen; La Jolla, CA) was used to perform site-directed mutagenesis in hSMVT, cloned into pcDNA 3.1 (−) [18] and GFP-N3 [12] vectors, respectively following manufacturer's protocol. Briefly, the corresponding template was PCR-amplified using specific mutant primers (Table 1). Following DpnI digestion, constructs were subsequently transformed into competent XL-1 blue cells. Correct clone carrying the corresponding mutation was identified by sequencing of the isolated plasmid (Laragen, Los Angeles, CA). Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as described before [18].

2.4. Real-time PCR analysis

At 48 h post-transfection total RNA was isolated using TRizol reagent (Invitrogen) and treated with DNAse I (Invitrogen). cDNA was prepared by using iScript cDNA synthesis kit (Bio-Rad) and the expression level was monitored in real-time PCR (Bio-Rad) using specific primers (Table 1). The expression level was normalized by using β-actin as an internal control and quantified by $2^{-\Delta\Delta CT}$ method [21].

2.5. Uptake studies

Forty eight hours after transfection, biotin uptake was measured in Krebs–Ringer (KR) buffer (in mM; 133 NaCl, 4.93 KCl, 1.23 $MgSO_4$, 0.85 $CaCl_2$, 5 glucose, 5 glutamine, 10 HEPES, 10 MES, pH 7.4) as described previously [18]. Endogenous biotin uptake was measured in control cell simultaneously transfected with pcDNA 3.1 (−) vector. Protein concentrations of the total cell lysate were measured by Dc protein assay kit (Bio-Rad) following the manufacturer's protocol, and uptake was expressed by fmol/mg protein/unit time.

Table 1
Combination of primers used for PCR.

Forward (F) and Reverse (R) Primers (5′-3′)		
hSMVT-RT	F	TGTCTACCTTCTCCATCATGGA;
	R	TAGAGCCCAATGGCAAGAGA
β-actin	F	AAATGGGTTCTAGACCGCGGAGA;
	R	CATGCTCGATGCGGTACTTCA
C68A	F	GACCGCAAAATGGGC**GCC**CTTCCGGTGGCACTG;
	R	CAGTGCCACCGGAAG**GGC**GCCCATTTTGCGGTC
C104A	F	TATTGGTTCCTGGGC**GCC**TGCTACTTTCTGGGG;
	R	CCCCAGAAAGTAGCA**GGC**GCCCAGGAACCAATA
C144A	F	AAAACTGTGGGAGTG**GCT**GGAACTGTGACCTTC;
	R	GAAGGTCACAGTTCC**AGC**CACTCGCACAGTTTT
C187A	F	GCCCTGGGCATTGTC**GCT**ACCGTCTATACAGCT;
	R	AGCTGTATAGACGGT**AGC**GACAATGCCCAGGGC
C294A	F	GCTGCTGTGCTCTCC**GCC**TATGCAGTGTTCCCC;
	R	GGGGAACACTGCATA**GGC**GGAGAGCACAGCAGC
C309A	F	TCCCTCTGCGTGGGC**GCC**CTCATTGGCCTGGTC;
	R	GACCAGGCCAATGAG**GGC**GCCCACGCAGAGGGA
C358A	F	GGGCTCTTCATTGCC**GCC**CTCTTCAGCGGCTCT;
	R	AGAGCCGCTGAAGAG**GGC**GGCAATGAAGAGCCC
C410A	F	GGCTATGGGCTGCTT**GCT**CTAGGAATGGCCTAT;
	R	ATAGGCCATTCCTAG**AGC**AAGCAGCCCATAGCC
C443A	F	CTGCTGGGACTCTTC**GCC**CTTGGAATGTTCTTT;
	R	AAAGAACATTCCAAG**GGC**GAAGAGTCCCAGCAG
C450A	F	GGAATGTTCTTTCCA**GCT**GCTAACCCTCCTGGT;
	R	ACCAGGAGGGTTAGC**AGC**TGGAAAGAACATTCC
C294S	F	GCTGCTGTGCTCTCC**TCT**TATGCAGTGTTCCCC;
	R	GGGGAACACTGCATA**AGA**GGAGAGCACAGCAGC
C294M	F	GCTGCTGTGCTCTCC**ATG**TATGCAGTGTTCCCC;
	R	GGGGAACACTGCATA**CAT**GGAGAGCACAGCAGC
C628A	F	GGGAGCAGCTCCACC**GCC**ATCCTCCAGGAGACC;
	R	GGTCTCCTGGAGGAT**GGC**GGTGGAGCTGCTCCC
C628S	F	GGGAGCAGCTCCACC**TCC**ATCCTCCAGGAGACC;
	R	GGTCTCCTGGAGGAT**GGA**GGTGGAGCTGCTCCC
C577A	F	CTCCTTCCGTTGTCC**GCT**CAGAAGCGGCTCCAC;
	R	GTGGAGCCGCTTCTG**AGC**GGACAACGGAAGGAG
C583A	F	CAGAAGCGGCTCCAC**GCC**AGGAGCTACGGCCAG;
	R	CTGGCCGTAGCTCCT**GGC**GTGGAGCCGCTTCTG

"hSMVT-RT" and "β-actin" represents the primer sequences used for quantitative real-time PCR for hSMVT and β-actin respectively. The bold faced nucleotides represent the mutation in respective primers used for site-directed mutagenesis.

2.6. Cell surface biotinylation assay

Cell surface expression of hSMVT (tagged to GFP) was quantified using a cell surface biotinylation kit (Pierce Biotechnology, Rockford, IL) as described by the manufacturer. Briefly, 48 h following transfection cells were treated with sulfo-NHS-SS-biotin and the biotinylated proteins were isolated by incubating the lysate with streptavidine-agarose beads. Isolated protein was subjected to Western blot analysis and membrane expression of hSMVT (i.e., hSMVT-GFP) was quantified using specific antibody. Total cellular hSMVT-GFP protein was used for normalization as described previously [13].

2.7. Western blotting

Forty eight hours after transfection with wild-type or mutant hSMVT-GFP construct cells were lysed in Ripa buffer (Sigma) supplemented with protease inhibitors cocktail (Roche). Equal amount of protein was loaded in NuPAGE 4-12% Bis-Tris gradient minigels (Invitrogen). Following electrophoresis, the gel was electro blotted onto PVDF membrane (Bio-Rad) and blocked overnight with blocking buffer (LI-COR Biosciences, Lincoln, NE). The blot was simultaneously probed with anti-GFP monoclonal antibodies (Clontech, Mountain View, CA) (raised in mouse) and anti-β-actin antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) (raised in rabbit) for 90 min. The blot was washed and incubated simultaneously with anti-mouse IR dye, 800 and anti-rabbit IR, 680 dye (Li-Cor) in 1:20,000 dilutions for 1 h at room temperature. Followed by washing the fluorescent

intensity was quantified in Odyssey Infrared imaging system (LI-COR Biosciences) using odyssey application software (version 3.0).

2.8. Statistical analysis

Data shown are means ± SE of at least three independent determinations. Uptake was expressed as fmol per milligram protein per 10 min; statistical significance was determined by the Student's *t*-test. Diffusion component was determined from the slope of the uptake line between the point of origin and uptake at high biotin concentration (1 mM); carrier-mediated uptake was determined by subtracting diffusion component from total uptake. Kinetic parameters of the uptake process, i.e., apparent K_m and V_{max}, were determined by non-linear regression analysis using Graph Pad Prism software (Version 5.03) by fitting data into Michaelis–Menten equation.

3. Results

3.1. Effect of mutating conserved cysteine residues of hSMVT on uptake function

The hSMVT protein has ten cysteine residues that are conserved in other mammals (bovine, rabbit, mouse, and rat). These residues are located at positions 68, 104, 144, 186, 294, 309, 358, 410, 443 and 450 of the hSMVT polypeptide. According to topology prediction, Cys^{68} and Cys^{144} of the hSMVT polypeptide are extracellular, those at position 294 and 450 are cytoplasmic, and Cys^{104}, Cys^{186}, Cys^{309}, Cys^{358}, Cys^{410} and Cys^{443} are located in the hydrophobic TMDs of the hSMVT polypeptide (Fig. 1). We mutated each of the conserved cysteine residues of the SMVT polypeptide to alanine and examined the effect of the individual mutation on the function of hSMVT in biotin uptake in transiently expressing ARPE 19 cells. The results showed that mutating Cys^{294} to lead to a significant ($p<0.01$) inhibition in biotin uptake, while mutating the other conserved cysteine residues to have no effect on hSMVT transport function (Fig. 2A). To determine whether the dramatic loss of hSMVT function upon mutating Cys^{294} to the non- polar alanine is due to lose of the

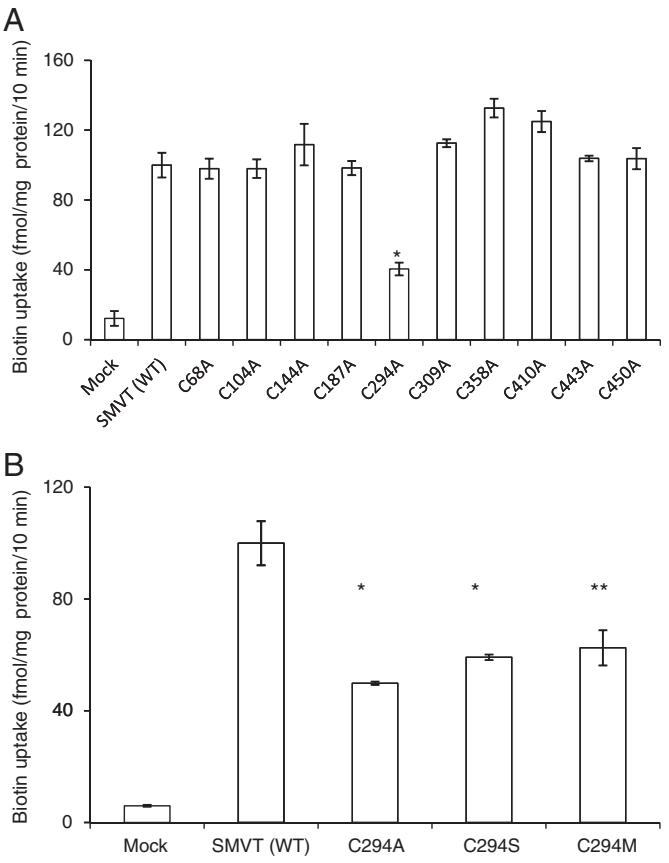

Fig. 2. Effect of mutating the conserved cysteine residues of hSMVT on biotin uptake in transiently transfected ARPE19 cells. (A) Each of the ten conserved cysteine residues was mutated to alanine and mutants were transiently transfected (individually) into ARPE19 cells. Biotin uptake was measured 48 h following transfection in KR buffer at pH 7.4. Control (Mock) was transiently transfected with empty vector [pcDNA 3.1(−)]. (B) Effect of polar status of the replacing residue. The Cys^{294} residue was substituted with non-polar alanine, polar serine, and methionine respectively. Uptake data shown in (A) and (B) are mean ± SE of at least three independent experiments. $^{*}p<0.01$, $^{**}p<0.05$.

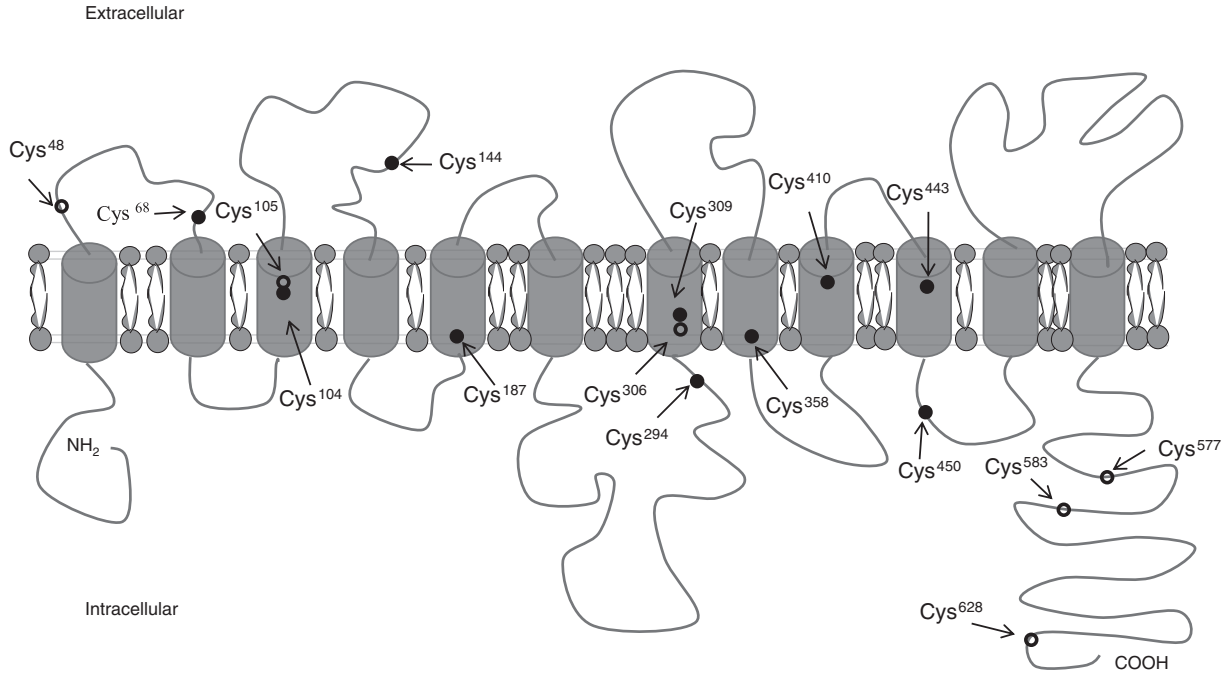

Fig. 1. Predicted membrane topology of hSMVT and location of the conserved cysteine residues. The hSMVT polypeptide is predicted to have 12 TMD [9]; filled circles depict the location of conserved cysteine residue whereas empty circles represent position of non- conserved cysteine residues.

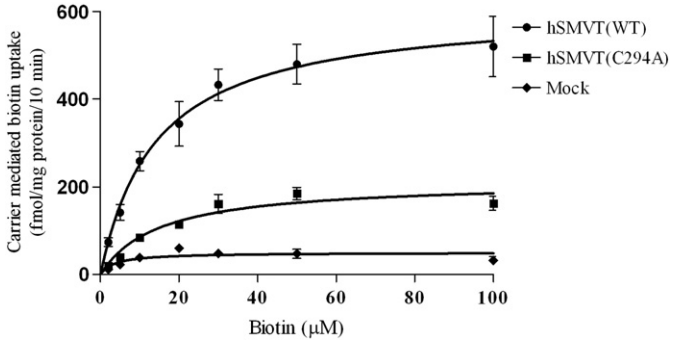

Fig. 3. Kinetic parameters of biotin uptake by Cys^{294}-hSMVT mutant. Cells were transfected with Cys^{294} mutant or wild-type hSMVT. Biotin uptake was measured in KR buffer at pH 7.4. Mock represents endogenous carrier-mediated uptake in ARPE19 cells.

polar status of cysteine, we also examined the effect of mutating Cys^{294} to the polar residue serine or to sulfur containing methionine on the function of hSMVT. Again, a significant inhibition in biotin uptake was observed suggesting that it is the cysteine residue itself, and neither its polar status nor the presence of sulfur residue, is important for the function of hSMVT (Fig. 2B).

3.2. Effect of mutating Cys^{294} on kinetic parameters of biotin uptake by hSMVT

To determine whether the effect of mutating Cys^{294} is mediated via an effect on the number/activity of the hSMVT carriers and/or on their affinity, we examined the effect of this mutation on kinetic parameters (V_{max} and apparent K_m) of the biotin uptake process following transient expression of the mutated protein in ARPE19 cells. Data were compared to kinetic parameters of biotin uptake by cells transiently expressing the wild-type hSMVT. The results (Fig. 3) showed that mutating Cys^{294} leads to a significant ($p<0.01$) inhibition in the V_{max} (608.6 ± 27.83 and 213.3 ± 14.54 fmol/mg protein/10 min for wild-type and the mutated hSMVT, respectively), but not the apparent K_m (14.28 ± 2.009, and 14.69 ± 3.08 μM, respectively) of the carrier-mediated biotin uptake process The findings suggest that mutating Cys^{294} leads to a decrease in the number (and/or activity), but not affinity, of hSMVT. This suggestion was further tested below.

3.3. Effect of mutating Cys^{294} on level of expression of hSMVT

To determine whether mutating Cys^{294} affects the level of expression of cellular hSMVT protein and mRNA, we transiently transfected ARPE19 cells with equal amounts of Cys^{294} mutant and wild-type hSMVT and performed real-time PCR and Western blotting, respectively (see Materials and methods). The results showed similar level of total hSMVT mRNA and hSMVT-GFP protein expression in cells transfected with the mutated and wild-type hSMVT (Fig. 4A and B).

3.4. Effect of mutating Cys^{294} on level of expression of hSMVT protein at the cell surface

To determine whether mutating Cys^{294} affects expression of the hSMVT protein at the cell membrane, we performed surface biotinylation assay. Equal amounts of hSMVT (WT)-GFP and hSMVT (C294A)-GFP constructs were transiently transfected into ARPE19 cells, followed by determination of the relative level of hSMVT-GFP expression at the cell membrane compared to total cellular expression (see Materials and methods). The results showed a significant ($p<0.01$; 60%) reduction in membrane expression of the mutated compared to the wild-type hSMVT (Fig. 5).

3.5. Does Cys^{294} of hSMVT polypeptide form a disulphide bridge with other cysteine?

Cys residues of a polypeptide are known to form disulphide linkage with other cysteine residues. To determine if hSMVTCys^{294} forms a disulphide bridge with other cysteine residue of the protein (the hSMVT has six non-conserved cysteine residues in addition to the 10 conserved residues), we subjected the hSMVT to computational analysis (CYS_REC from Softberry. Inc, NY). Result of the analysis predicted a probable disulphide bond forming pattern between Cys^{294} and Cys^{628} (similarly, Cys^{294} residue of SMVT of other mammals was predicted to form similar bridge with non-conserved cysteine

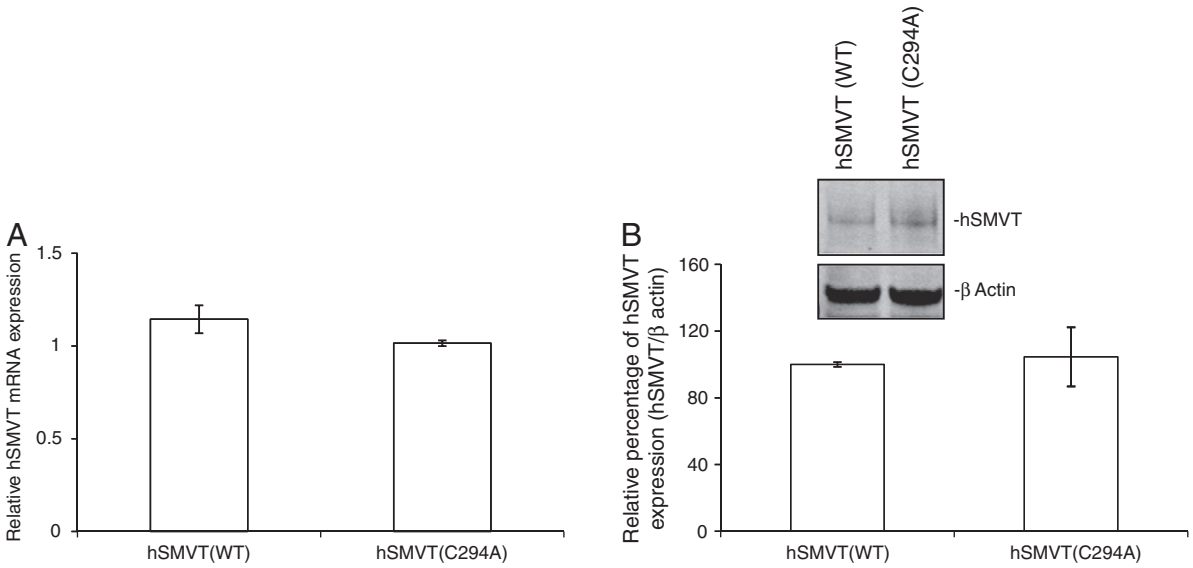

Fig. 4. Effect of mutating Cys^{294} on level of expression of hSMVT. Equal amounts of Cys^{294} and wild-type hSMVT (WT) constructs were transfected into ARPE19 cells and level of (A) hSMVT mRNA (real-time PCR; data normalized to β-actin), and (B) total cellular hSMVT protein (Western blot analysis) were determined 48 h after transfection (see Materials and methods). Anti-GFP monoclonal antibody was used in Western blot and data was normalized relative to β-actin. Data shown are mean ± SE of three independent experiments. Inset shows a representative gel.

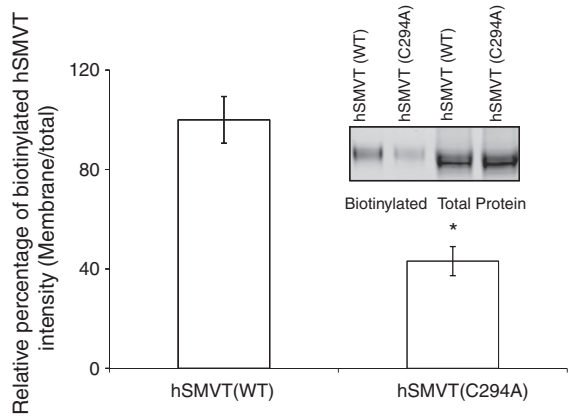

Fig. 5. Effect of mutating Cys^{294} on level of expression of hSMVT at cell surface. ARPE19 cells were transfected with Cys^{294} mutant and wild-type hSMVT. After 48 h of transfection, biotinylation assay was performed (see Materials and methods). Induced expression of hSMVT was identified using anti-GFP monoclonal antibody and level of membrane expression was quantified by normalization relative to total amount of cellular hSMVT. *$p<0.01$. Inset shows representative Western blot image.

according to the analysis). We mutated other two cysteine residues (Cys^{577} and Cys^{583}) in addition to Cys^{628} as they are also located on the cytoplasmic side of the membrane. Mutating Cys^{628} of the hSMVT to alanine or serine led to a significant inhibition in biotin uptake compared to wild-type hSMVT, whereas mutating Cys^{577} or Cys^{583} did not show any inhibition (Fig. 6).

4. Discussion

The aim of the present study was to enhance our understanding of the structure–activity relationships of the hSMVT system, with special emphasis of the role of conserved cysteine residues in the function of this membrane carrier. Previous studies from our laboratory using group specific modifiers have suggested a role for cysteine residue (s) in the function of SMVT in biotin transport [19,20]. The hSMVT system has ten conserved (with other mammals) cysteine residues, and it is unclear which cysteine residue(s) is (are) involved in mediating this inhibition, i.e., which residues is important for SMVT function. This issue was addressed using the molecular approach of site-directed mutagenesis. Of the ten conserved cysteine residues, only Cys^{294} appeared to be important for the function of hSMVT as mutating this site led to a significant inhibition in biotin uptake. This inhibition occurred regardless of whether Cys^{294} was replaced by a non polar amino acid (alanine) or by another polar residue (serine), suggesting that it is the cysteine itself (and not its polar status) that is important for hSMVT function. We also examined the effect of mutating Cys^{294} to the sulfur containing amino acid methionine and observed comparable inhibition in biotin uptake confirming the importance of Cys residue itself.

The inhibition in biotin uptake upon mutating Cys^{294} of the hSMVT was mediated via a significant decrease in the V_{max}, but not apparent K_m, of the biotin uptake process. These findings suggest that the inhibition is mediated via a decrease in the number (and/or activity) but not affinity of the SMVT system. We next examined whether mutating Cys^{294} of hSMVT affects the steady state level of expression of hSMVT. Quantitative PCR data and Western blot analysis of total cell lysate showed similar level of mRNA and total hSMVT protein expression, respectively in cells transfected with the mutant and wild-type hSMVT; thus, we excluded the possibility of an effect(s) of the Cys^{294} mutation on hSMVT level of expression. However, since mutating a residue of a membrane protein could affect its level of expression at the cell surface (without affecting its total cellular level of expression) [18], we tested this possibility employing biotinylation assay. Our results indeed showed that the level of membrane expression of the mutated hSMVT is markedly lower than that of the wild-type protein. This finding clearly indicates that the inhibition in biotin uptake upon mutating Cys^{294} of hSMVT protein is due to a decrease in the number of the hSMVT molecule at the cell surface.

Cysteine residue in a given protein can form a disulphide bridge with other cysteine residues. To determine if Cys^{294} forms such bridge with other cysteine residues, we subjected the hSMVT to computational analysis. The result predicted Cys^{628} as probable bonding partner of Cys^{294}. It is interesting to mention here that Cys^{628} and Cys^{294} of hSMVT are oriented toward the cytoplasm. In addition, we examined the role of nearby cysteine residues (Cys^{577} and Cys^{583}), which are also located in the cytoplasmic tails of hSMVT. Mutating Cys^{628} (but not Cys^{577} or Cys^{583}) led to a significant inhibition in biotin uptake compared to wild-type hSMVT, thus, lending support to possible existence of a linkage between Cys^{294} and Cys^{628}. Of note, whether Cys^{294} and Cys^{628} also interact with accessory protein(s) (that is a component of the transport machinery) apart from interacting with each other is not known at this point and needs further research.

In summary, our study shows an important role for the conserved Cys^{294} residue in the function of hSMVT in biotin uptake, and that this role is mediated via affecting the expression of the hSMVT protein at the cell surface.

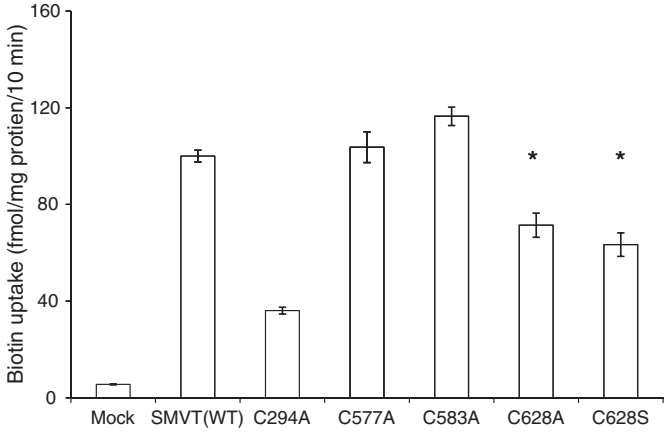

Fig. 6. Effect of mutating the non-conserved Cys residues for identifying disulfide bridge forming partner of Cys^{294}. Non-conserved Cys residues (Cys^{577}, cys^{583} and Cys^{628}) facing the cytoplasm were mutated to alanine and equal amount of constructs (3 μg) was transfected into ARPE19 cells. Biotin uptake was determined at 48 h after transfection. Data are mean ± SE of at least three independent experiments. *$p<0.01$.

Acknowledgements

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