Published in final edited form as:
Dig Dis Sci. 2013 March ; 58(3): 641–649. doi:10.1007/s10620-012-2388-9.

Modulation of function of sodium-dependent vitamin C transporter 1 (SVCT1) by Rab8a in intestinal epithelial cells: Studies utilizing Caco-2 cells and Rab8a knockout mice

Veedamali S. Subramanian[1,2,*], **Sandeep B. Subramanya**[1,2], **Abhisek Ghosal**[1,2], **Jonathan S. Marchant**[3], **Akihiro Harada**[4], and **Hamid M. Said**[1,2]

[1]Departments of Medicine, Physiology and Biophysics, University of California, Irvine, CA 92697

[2]Department of Veterans Affairs Medical Center, Long Beach, CA 90822

[3]Department of Pharmacology, University of Minnesota Medical School, MN 55455

[4]Osaka University, Osaka, 565-0871, Japan

Abstract

Background—Ascorbic Acid (AA) is required for normal human health and development. Human intestine expresses two sodium-dependent vitamin C transporters (hSVCT-1 and -2) that mediate cellular AA transport, with hSVCT1 targeting to the apical membrane of polarized epithelia. Studies have shown a role for the Rab8a in the apical membrane targeting of transporters in intestinal cells.

Aims—The purpose of this study was to determine whether Rab8a impacts the function and/or targeting of hSVCT1, and intestinal AA uptake.

Methods—We used human intestinal cells and cells from a Rab8a knockout mouse. ^{14}C-AA uptake was performed to determine functionality. PCR and Western blot were performed to determine RNA and protein expression, respectively. Confocal imaging was performed to determine co-localization.

Results—We show that hSVCT1 co-localized with Rab8a in intestinal cells. Knockdown of Rab8a lead to a significant inhibition in AA uptake and cell surface biotinylation studies revealed a lower cell surface expression of hSVCT1 in Rab8a siRNA-treated cells. Similarly, in the small intestine of a Rab8a knockout mouse, AA uptake was significantly inhibited. This effect again resulted from a decreased expression level of mSVCT1 protein, even though mRNA expression of SVCT1 was similar in intestinal cells from Rab8a knockout and wild-type litter-mates. The latter data are suggestive of enhanced lysosomal degradation of hSVCT1 protein in Rab8a deficient cells; indeed confocal imaging of Rab8a siRNA-treated intestinal cells revealed a strong overlap between hSVCT1-YFP and LAMP1-RFP.

Conclusions—These findings extend a role for Rab8a in the physiological function of hSVCT1 in intestinal epithelia.

Keywords

Transport; vitamin C; uptake; ascorbic acid; GTPase; trafficking; intestine

[*]To whom correspondence may be addressed: vsubrama@uci.edu, Phone: 562-826-5803; Fax: 562-826-5018 .

Introduction

Vitamin C (Ascorbic Acid, AA) is an essential water-soluble micronutrient that is required for normal cellular function, growth and development. Vitamin C deficiency leads to a variety of abnormalities including scurvy, delayed wound healing, bone and connective tissue damage as well as vasomotor instability [1]. Optimal vitamin C body homeostasis has been reported to have a protect effect against gallbladder diseases, cardiovascular disease, cancer, and cataract formation [2-4].

Humans have lost the capability of synthesizing vitamin C and therefore obtain the vitamin from dietary sources. Significant progress has been made in understanding the molecular basis of vitamin C absorption across intestinal epithelia, a process which involves two sodium-dependent vitamin C transporters (hSVCT1 and hSVCT2). hSVCT1 is expressed at the apical surface, and hSVCT2 at the basolateral surface of polarized enterocytes, distributions suggestive of a route for vectorial transport of vitamin C from the gut to the bloodstream [5-8]. Less well understood are aspects of the cell biology of these transporters, for example the cellular determinants that control membrane targeting and intracellular trafficking in intestinal epithelia.

Recent studies have shown that specific Rab proteins regulate the localization, trafficking and function of membrane proteins in intestinal epithelial cells [9-11]. Notably, Rab8 has been shown to be necessary for the apical localization of a number of proteins in enterocytes [10, 11]. However, nothing is known about whether the function of this small GTPase impacts hSVCT1 functionality, or its functional impact extends to only a subset of apically targeted transporters. Therefore, we used human-derived cultured intestinal epithelial cell lines and tissue from a conditional, intestine-specific Rab8a knockout (KO) mouse model to investigate the role of Rab8a in hSVCT1 cell biology and physiology. Our results demonstrate that *in vitro* knockdown or *in vivo* ablation of Rab8a impairs AA uptake. In both model systems, this effect correlated with decreased levels of hSVCT1 protein, but not mRNA levels. Our results support a model where (i) Rab8a is required for apical plasma membrane targeting of hSVCT1, and (ii) lysosomal accumulation and degradation of this transporter occurs in the absence of the Rab8a-sensitive cell surface trafficking pathway.

Materials and Methods

Materials

[^{14}C]-AA (specific activity ~13mCi/mmol) was from American Radiolabeled Chemical (ARC, St. Louis, MO). YFP-N1 and DsRed-C1 fluorescent proteins were from Clontech (Palo Alto, CA). LAMP1-RFP was obtained from Addgene Inc (Cambridge, MA). Caco-2, HuTu-80, and HT-29 cells were from ATCC (Manassas, VA) and NCM460 cells were from Incell (San Antonio, TX) [12]. DNA oligonucleotide primers were synthesized by Sigma Genosys (Woodlands, TX).

Generation of hSVCT1 and Rab8a fusion constructs

The full-length hSVCT1-YFP construct was used previously. The DsRed-Rab8a (Dr. David R. Sheff, University of Iowa, IA has generously provided GFP-Rab8a plasmid) construct was generated by PCR using the primer combinations shown in Table 1 and conditions as described before [8]. The Rab8a PCR products and the DsRed-C1 vector were digested with *SalI* and *Bam HI,* and products were gel separated and ligated to generate in-frame fusion proteins with the red fluorescent protein (DsRed-C1) fused to the NH_2-terminus of Rab8a full-length protein. The nucleotide sequence of the construct was verified by sequencing (Laragen, CA).

Cell culture, transient transfection and uptake assay

The human-derived intestinal epithelial Caco-2 and HuTu-80 cells were grown in MEM (ATCC, Manassas, VA), HT-29 cells were grown in McCoy's medium (Sigma) and NCM460 cells were grown in Ham's F-12 medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS), glutamine (0.29 g/l), sodium bicarbonate (2.2 g/l), penicillin (100,000 U/l), and streptomycin (10 mg/l) in 75-cm^2 plastic flasks at 37 C in a 5% CO_2-95% air atmosphere with media changes every 2-3 days. For imaging studies, cells were grown on sterile glass-bottomed petri-dishes (MatTek, MA) and transfected at 90% confluency with 2-4μg plasmid DNA using Lipofectamine 2000 (Invitrogen, CA). After 48 h, live cells were imaged using confocal microscopy. For uptake, mRNA, and western blot analysis, Caco-2 cells were grown on 12 well plats (Corning Inc., NY) and Rab8a siRNA [pool of three different siRNA duplexes (duplex-1, sense: GAACUGGAUUCGCAACAUUtt, antisense: AAUGUUGCGAAUCCAGUUCtt; duplex-2, sense: GAACAAGUGUGAUGUGAAUtt, antisense: AUUCACAUCACACUUGUUCtt; duplex-3, sense: CCAGAAUGCAAUUGAGAAAtt, antisense: UUUCUCAAUUGCAUUCUGGtt; specific for human Rab8a (Santa Cruz, CA)] was transfected at 80% confluence. Studies were performed after 48 h of transfection. [^{14}C]-AA uptake assays were performed on transfected Caco-2 cells following established procedures [13]. Protein contents were estimated on parallel wells using a protein assay kit (Bio Rad, CA).

Ascorbic acid uptake assay in Rab8a knockout (KO) mice

Small intestine-specific Rab8a KO mice were generated before [11] and were housed at the Long Beach VA Medical Center facility. The animal use committee of Long Beach VA Medical Center approved the experimental protocols. To determine the AA uptake in small intestinal tissue, Rab8a KO and wild-type (litter-mate) mice were euthanized and jejunum portion of small intestinal segments were incubated in Kreb's Ringer's buffer containing ^{14}C-AA (32 μM) at pH 7.4 for 5 min as described [14, 15]. Total protein for each sample was measured using a protein assay kit (Bio-Rad). A portion of scraped small intestinal mucosa was stored in RIPA buffer (Sigma) for protein isolation and a portion of small intestine was stored in Trizol (Invitrogen) for RNA isolation.

Real-time PCR

Total RNA was isolated from Caco-2 cells or whole mouse small intestine. Conditions used for the real-time PCR to determine the level of SVCT1, -2, Rab8a and β-actin mRNA were as described previously [16]. The data were normalized to β-actin and then quantified using a relative relationship method as described previously [17]. Gene specific primers are listed in Table 1.

Western blot analysis

Western blot analysis was performed on cellular extracts prepared from control and Rab8a siRNAs treated Caco-2 cells or on Rab8a KO mice and wild-type (litter-mate) intestinal mucosal scrapping using established procedures [18]. Proteins (60μg) were resolved onto 4-12% Bis-Tris mini-gel (Invitrogen) as described [18]. After electrophoresis, proteins were electroblotted onto polyvinylidene fluoride membrane (LI-COR, Lincoln, NB) and blocked with a LI-COR blocking buffer (LI-COR) for 1 h at room temperature (RT). Membranes were then incubated with either anti-rabbit hSVCT1/mSVCT1 (Santa Cruz, CA) or human Rab8a (Sigma)/mouse Rab8a (Santa Cruz) along with mouse monoclonal β-actin antibodies (Santa Cruz). Immunodetection was performed by incubating the membrane with secondary antibodies [IRDye 800 labeled goat anti-rabbit or IRDye 680 labeled goat anti-mouse (1:30000 dilutions for both secondary antibodies), LI-COR] for 45 min at RT. Signals were

detected and quantified with the Odyssey Infrared Imaging Systems (LI-COR) and accompanying LI-COR software (LI-COR).

Cell surface biotinylation

Control and Rab8a siRNAs (Santa Cruz) were transiently transfected into Caco-2 cells (90% confluent). Samples were biotinylated after 48 h of transfection using an EZ-Link Sulfo-NHS-Biotinylation Kit (Thermo Fisher Scientific Inc., Rockford, IL) and then lysed according to the manufacturer's instructions. An equal concentration of total soluble protein from Rab8a or control siRNAs treated cells was then incubated with avidin-conjugated agarose. The biotinylated protein bound to the agarose-avidin beads was washed three times and eluted in NuPAGE LDS sample loading dye (Invitrogen) and subjected to western blot analysis in a NuPAGE 4-12% Bis-Tris gradient minigel (Invitrogen). For normalization, an equal protein concentration of total cell lysate was loaded simultaneously and the level of surface expression of hSVCT1 was determined using specific hSVCT1 antibody followed by IRDye 800 labeled goat-anti rabbit (1:30000) secondary antibody. Signals were detected and quantified as described above using the Odyssey Infrared Imaging Systems (LI-COR).

Live cell confocal imaging

Cells cultured on petri-dishes were imaged using a Nikon C-1 confocal scanner head attached to a Nikon inverted phase-contrast microscope. Fluorophores were excited using the 488 nm/543 nm line from an argon/HeNe ion-laser, and emitted fluorescence was monitored with a 515 30 nm short band pass filter (YFP) and 570±50 nm long pass filter (DsRed).

Statistical analysis

Uptake data are presented as mean ± SE from multiple different experiments and are expressed in percentage relative to controls or fmol/mg protein/5 min. Western blot, mRNA, and imaging analysis data presented are the result of at least three separate experiments. The differences between the means of control and knockout/siRNA-treated cells for various outcome parameters were tested for significance at $p < 0.05$ using the Student's t-test.

Results

hSVCT1 and Rab8a co-localize in human intestinal epithelial cells

We investigated whether hSVCT1 and Rab8a co-localize in human intestinal epithelial cells. For that, we used several human-derived intestinal epithelial cells (Caco-2, HT-29, NCM460, and HuTu-80) and performed live cell confocal imaging. Both hSVCT1-YFP and DsRed-Rab8a constructs were co-transfected into Caco-2, HT-29, NCM460, and HuTu-80 cells. After 48 h, hSVCT1 showed its expected pattern of localization [5, 8, 19] at the cell surface and within intracellular vesicular compartments while DsRed-Rab8a was found to be expressed throughout the cytoplasm in each intestinal cell line examined (Fig.1). The overlapped confocal images of the Caco-2, HT-29, NCM460, and HuTu-80 cells showed strong co-localization of the two constructs in vesicular structures that were located throughout the cytoplasm (Fig. 1).

Effect of knockdown and knockout (KO) of Rab8a gene on AA uptake in vitro and in vivo systems

Our aim in these experiments was to examine the effect of selective knockdown of endogenous Rab8a on ^{14}C-AA uptake. First, we examined the effect of silencing endogenous Rab8a on ^{14}C-AA (32 μM) uptake by Caco-2 cells transiently expressing hSVCT1; data was compared to uptake by control (scrambled siRNA-treated) hSVCT1

expressing Caco-2 cells. The results showed a significant ($p < 0.02$) inhibition in the AA uptake by Rab8a siRNA-treated hSVCT1 expressing cells compared to control cells (Fig. 2A). We also examined the effect of Rab8a siRNA-treatment on uptake of AA by untransfected Caco-2 cells, i.e., examined the effect of Rab8a siRNA treatment on endogenous AA uptake. The results again showed a significant ($p < 0.03$) inhibition in AA uptake when compared to control (data not shown). To verify that Rab8a mRNA expression level was reduced in these assays, we assessed mRNA and protein levels in the Caco-2 samples. Treating Caco-2 cells with Rab8a siRNA significantly ($p < 0.01$) decreased the Rab8a mRNA expression level compared to control siRNA-treated Caco-2 cells (Fig. 2B). Finally, western blot analysis with human Rab8a specific antibodies showed that the Rab8a protein expression level was also significantly ($p < 0.01$) decreased compared to control siRNA-treated Caco-2 cells (Fig. 2C).

To complement the results of the *in vitro* studies, we used Rab8a KO mice previously generated by Sato et al [11] as an *in vivo* model system. Results showed significant ($p < 0.01$) inhibition in carrier-mediated AA uptake in the jejunum of Rab8a KO mice compared to uptake in the jejunum of their wild-type litter-mate (Fig. 2D). Rab8a mRNA expression was severely ($p < 0.01$) ablated in Rab8a KO mice compared to control litter-mates (Fig. 2E), [with the residual expression being explained by co-isolation of non-intestinal cells from the tissue (intestinal)-specific KO animals]. As expected, western blot analysis of mucosal scrapping showed that the Rab8a protein expression was absent in Rab8a KO mice as expected compared to their wild-type litter-mates (Fig. 2F).

Effect of Rab8a impairment on SVCT1 mRNA and protein expression levels

Next, we examined hSVCT1 (and hSVCT2) mRNA expression levels after treatment with gene specific Rab8a siRNA. Results showed that no significant change in hSVCT1 (or hSVCT2) mRNA expression levels in Rab8a siRNA-treated cells compared to control siRNA-treated cells (Fig. 3A). We also compared hSVCT1 protein expression in Rab8a siRNA-treated Caco-2 cells as compared to control cells by western blot analysis. Results showed that the hSVCT1 (Fig. 3B) protein expression level was significantly ($p < 0.01$) decreased in Rab8a siRNA-treated cells compared to control cells. Similarly, in Rab8a KO mice, mRNA levels for mSVCT1 (and mSVCT2) were similar to those in wild-type litter-mates (Fig. 3C). However, mSVCT1 protein expression level was significantly ($p < 0.02$) reduced in Rab8a KO mice jejunal mucosa compared to levels observed with wild-type litter-mates (Fig. 3D).

Effect of silencing Rab8a on cell surface expression of hSVCT1 in Caco-2 cells

To understand the reasons for the observed inhibition in AA uptake after Rab8a siRNA-treatment of Caco-2 cells, the cell surface expression levels of the hSVCT1 protein was examined. To do this, we compared biotinylation of cell surface proteins in Rab8a siRNA-treated with control cells, having normalized the cell surface expression level of hSVCT1 with total cellular hSVCT1 protein in the same samples. The cell surface expression of hSVCT1 was significantly ($p < 0.01$) decreased in Rab8a siRNA treated cells compared with cells treated with control siRNA (Fig. 4).

Effect of silencing Rab8a gene on hSVCT1 trafficking

Finally, human-derived intestinal epithelial HuTu-80 cells were co-transfected with hSVCT1-YFP, LAMP1-RFP (a lysosomal marker) and control or Rab8a siRNAs. Live cell imaging was performed 48 h after transfection. Results showed that hSVCT1 was expressed at the cell membrane in control cells, whereas cell surface expression was less evident in Rab8a siRNA-treated cells. In Rab8a siRNA-treated cells, there was significant overlap between hSVCT1-YFP and LAMP1-RFP (Fig. 5). These results may suggest that Rab8a

activity is needed for physiological targeting/trafficking of hSVCT1 to the cell surface in intestinal epithelial cells.

Discussion

Recent studies have shown the importance of Rab8a in apical localization of several brush border membrane proteins in polarized intestinal epithelial cells [10, 11]. Specifically, dipeptidyl peptidease IV, alkaline phosphatase, sucrose-isomaltase and the oligopeptide transporter 1 showed impaired localization at the brush border membrane domains of intestinal epithelial cells of Rab8 KO mice [11]. These markers were found to be mislocalized to late endosomes/lysosomes, where the proteins were degraded. Phenotypically, this impairment in polarized targeting of important proteins lead to a reduction in various nutrient digestion and absorption in the small intestine (shortened microvilli), that resulted in malnutrition and death of the KO mice [11]. This outcome parallels what is believed to be happening in the clinical condition known as microvillus inclusion disease [20, 21].

Does the role of Rab8 extend to regulation of the targeting of multivitamin transporters within the solute carrier (SLC) family? To investigate this issue, we tested whether hSVCT1, the apically localized vitamin C transporter also displays Rab8-dependent trafficking in a number of well-established human-derived intestinal epithelial cell models and native small intestine of intestinal-specific Rab8a KO mice. These preparations represented *in vitro* and *in vivo* experimental model systems for investigating the role of Rab8a in AA transport physiology, respectively. Co-expression studies revealed that there was significant overlap between hSVCT1 and Rab8a proteins in vesicular structures within the intestinal epithelial cell lines *in vitro*. Further, we found that knockdown of the endogenous Rab8a in Caco-2 cells resulted in a significant inhibition in AA uptake. This functional impairment in AA uptake appeared to be the result of the marked reduction in expression of the hSVCT1 protein at the cell surface of the Rab8a siRNA-treated cells. On the other hand, no change in the level of expression of hSVCT1 mRNA was observed in Rab8a siRNA-treated Caco-2 cells compared to control cells. In agreement with these observations, we found a significant inhibition in AA uptake in the jejunal portion of Rab8a KO mice compared to their control litter-mate animals. Again this functional impairment was associated with a marked reduction in the level of mSVCT1 protein expression at the cell membrane in the Rab8a KO mice compared to wild-type litter-mates with no change in the expression level of mSVCT1 (and mSVCT2) mRNA. One possible explanation for the reduction in cell surface expression of the SVCT1 protein in both of the above described systems is that the SVCT1 protein is degraded following mis-targeting to lysosomes, as suggested previously [11]. To assess this possibility we co-transfected with hSVCT1-YFP, LAMP1-RFP and control or Rab8a siRNAs in intestinal epithelial cells. The endogenous Rab8a knockdown in these cells yielded significant overlap between hSVCT1-YFP and the LAMP1-RFP. Therefore, our findings agree with the previously proposed explanation [11] that enhanced lysosomal degradation of hSVCT1 may follow impairment of Rab8a function, and extends previous findings that Rab8a supports the proper localization of a broad swathe of apical proteins in the intestine.

Acknowledgments

This study was supported by grants from the National Institute of Health (DK84094 to VSS, and DK 56061 to HMS, GM088790 to JSM) and the Department of Veterans Affairs.

References

1. Packer L, Fuchs J. Vitamin C in health and disease. Marcel Dekker Inc. 1997

2. Carr AC, Frei B. Toward a new recommended dietary allowance for vitamin C based on antioxidant and health effects in humans. Am J Clin Nutr. 1999; 69:1086–1107. [PubMed: 10357726]
3. Li Y, Schellhorn HE. New developments and novel therapeutic perspectives for vitamin C. J Nutr. 2007; 137:2171–2184. [PubMed: 17884994]
4. Simon JA, Hudes ES. Serum ascorbic acid and gallbladder disease prevalence among US adults: the Third National Health and Nutrition Examination Survey (NHANES III). Arch Intern Med. 2000; 160:931–936. [PubMed: 10761957]
5. Boyer JC, Campbell CE, Sigurdson WJ, Kuo SM. Polarized localization of vitamin C transporters, SVCT1 and SVCT2, in epithelial cells. Biochem Biophys Res Commun. 2005; 334:150–156. [PubMed: 15993839]
6. Maulen NP, Henriquez EA, Kempe S, et al. Up-regulation and polarized expression of the sodium-ascorbic acid transporter SVCT1 in post-confluent differentiated CaCo-2 cells. J Biol Chem. 2003; 278:9035–9041. [PubMed: 12381735]
7. Savini I, Rossi A, Pierro C, Avigliano L, Catani MV. SVCT1 and SVCT2: key proteins for vitamin C uptake. Amino Acids. 2008; 34:347–355. [PubMed: 17541511]
8. Subramanian VS, Marchant JS, Boulware MJ, Said HM. A C-terminal region dictates the apical plasma membrane targeting of the human sodium-dependent vitamin C transporter-1 in polarized epithelia. J Biol Chem. 2004; 279:27719–27728. [PubMed: 15084584]
9. Kato Y, Sugiura T, Nakadera Y, et al. Investigation of the role of oligopeptide transporter PEPT1 and sodium/glucose cotransporter SGLT1 in intestinal absorption of their substrates using small GTP-binding protein Rab8-null mice. Drug Metab Dispos. 2009; 37:602–607. [PubMed: 19074526]
10. Lakkaraju A, Rodriguez-Boulan E. Cell biology: caught in the traffic. Nature. 2007; 448:266–267. [PubMed: 17637651]
11. Sato T, Mushiake S, Kato Y, et al. The Rab8 GTPase regulates apical protein localization in intestinal cells. Nature. 2007; 448:366–369. [PubMed: 17597763]
12. Said HM, Ortiz A, Subramanian VS, Neufeld EJ, Moyer MP, Dudeja PK. Mechanism of thiamine uptake by human colonocytes: studies with cultured colonic epithelial cell line NCM460. Am J Physiol Gastrointest Liver Physiol. 2001; 281:G144–150. [PubMed: 11408266]
13. Said HM, Balamurugan K, Subramanian VS, Marchant JS. Expression and functional contribution of hTHTR-2 in thiamin absorption in human intestine. Am J Physiol Gastrointest Liver Physiol. 2004; 286:G491–498. [PubMed: 14615284]
14. Reidling JC, Subramanian VS, Dahhan T, Sadat M, Said HM. Mechanisms and regulation of vitamin C uptake: studies of the hSVCT systems in human liver epithelial cells. Am J Physiol Gastrointest Liver Physiol. 2008; 295:G1217–1227. [PubMed: 18845575]
15. Subramanya SB, Subramanian VS, Kumar JS, Hoiness R, Said HM. Inhibition of intestinal biotin absorption by chronic alcohol feeding:cellular and molecular mechanisms. Am J Physiol Gastrointest Liver Physiol. 2011; 300:G494–501. [PubMed: 21148397]
16. Reidling JC, Nabokina SM, Balamurugan K, Said HM. Developmental maturation of intestinal and renal thiamin uptake: studies in wild-type and transgenic mice carrying human THTR-1 and 2 promoters. J Cell Physiol. 2006; 206:371–377. [PubMed: 16206251]
17. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). Method Methods. 2001; 25:402–408.
18. Subramanya SB, Subramanian VS, Sekar VT, Said HM. Thiamin uptake by pancreatic acinar cells: effect of chronic alcohol feeding/exposure. Am J Physiol Gastrointest Liver Physiol. 2011; 301:G896–904. [PubMed: 21868632]
19. Subramanian VS, Marchant JS, Reidling JC, Said HM. N-Glycosylation is required for Na+-dependent vitamin C transporter functionality. Biochem Biophys Res Commun. 2008; 374:123–127. [PubMed: 18619416]
20. Davidson GP, Cutz E, Hamilton JR, Gall DG. Familial enteropathy: a syndrome of protracted diarrhea from birth, failure to thrive, and hypoplastic villus atrophy. Gastroenterology. 1978; 75:783–790. [PubMed: 100367]
21. Phillips AD, Jenkins P, Raafat F, Walker-Smith JA. Congenital microvillous atrophy: specific diagnostic features. Arch Dis Child. 1985; 60:135–140. [PubMed: 3977385]

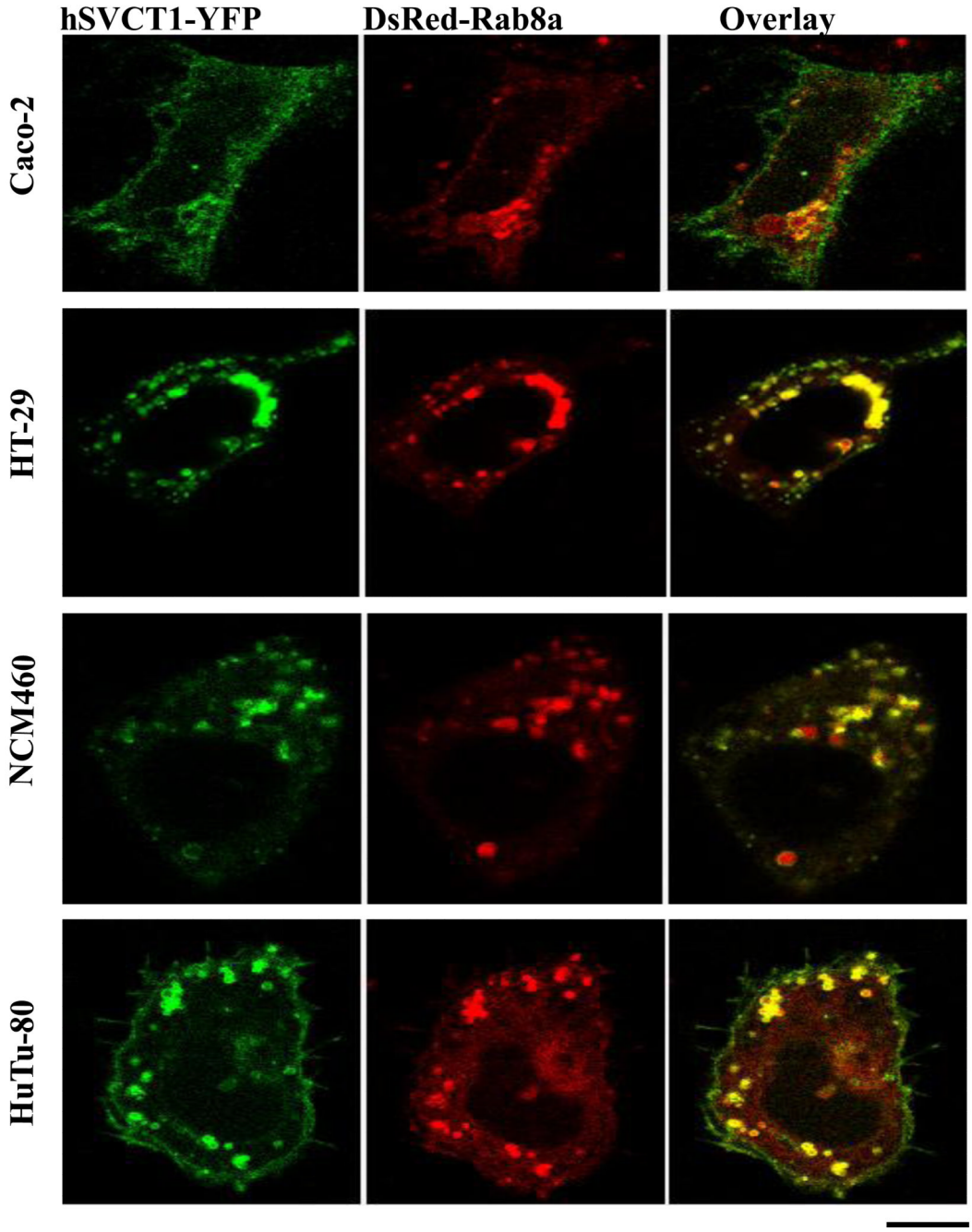

Figure 1. Distribution of hSVCT1 and Rab8a in intenstinal epithelial cells

Caco-2, HT-29, NCM460 and HuTU-80 cells were transiently co-transfected with hSVCT1-YEP and DsRed-Rab8a constructs. Data are from n > 6-10 transfected cells. Scale bar is 10 μm

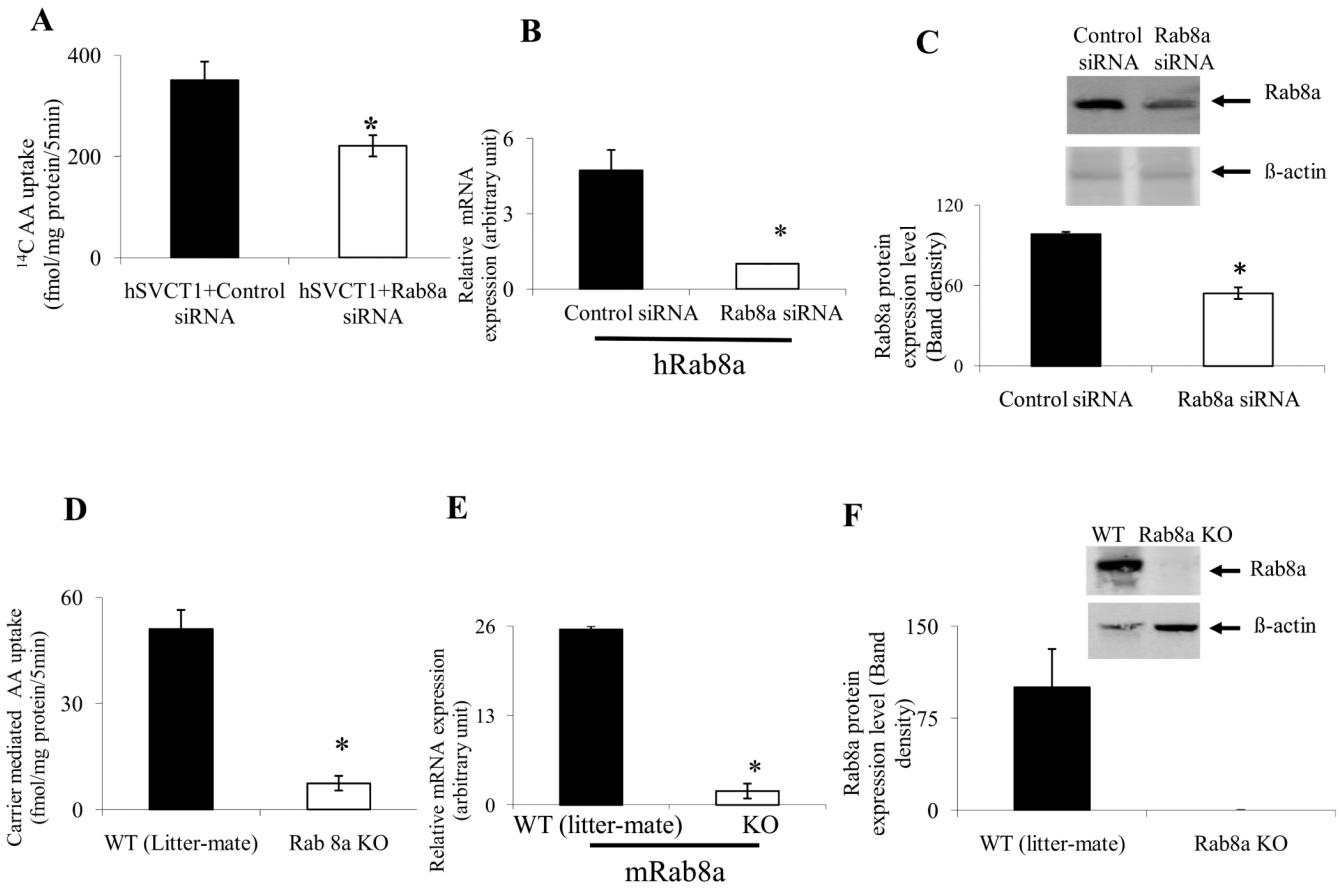

Figure 2. Effect of impairment of Rab8a expression on ^{14}C-AA uptake *in vitro* and *in vivo*

A) ^{14}C-AA uptake (32 μM) was performed on Rab8a siRNA treated hSVCT1 expressing Caco-2 cells. Data are mean ± SE of at least three separate experiments performed on different batches of cells on separate occasions. *$p < 0.02$. **B)** Real-time PCR was performed using gene specific primers for Rab8a and β-actin from total RNA isolated from Rab8a siRNA and control siRNA (scrambled) treated Caco-2 cells. Data are mean ± SE of at least three independent experiments. *$p < 0.01$. **C**, Top, Western blot analysis was performed on cell extract (60 μg) isolated from control (left) and Rab8a siRNAs treated Caco-2 cells (right). Blots were incubated with rabbit polyclonal anti-human Rab8a specific antibodies (top) along with β-actin antibodies (bottom). Bottom, densitometric quantification of the immunoreactive bands. Data are mean ± SE of at least three separate sample preparations. *$p < 0.01$. **D**) Carrier-mediated ^{14}C-AA uptake (32 μM) by Rab8a KO mice jejunal portion was performed as described in "Materials and Methods". Values are mean ± SE of at least three separate uptake determinations from multiple sets of mice. *$p < 0.01$. **E**) Real-time PCR was performed on total RNA isolated from Rab8a KO and wild-type (litter-mate) mice jejunal mucosa using mouse Rab8a and β-actin gene-specific primers. Data are mean ± SE of at least three separate samples from three different mice. *$p < 0.01$. **F**, Top) Western blot analysis was performed on Rab8a KO (right) and wild-type litter-mate (left) mouse jejunum mucosal (60 μg) proteins. Blots were incubated with rabbit polyclonal anti-mouse Rab8a antibodies along with β-actin antibodies. Bottom, densitometric values. Data are mean ± SE of at least three sets of samples from three different mice.

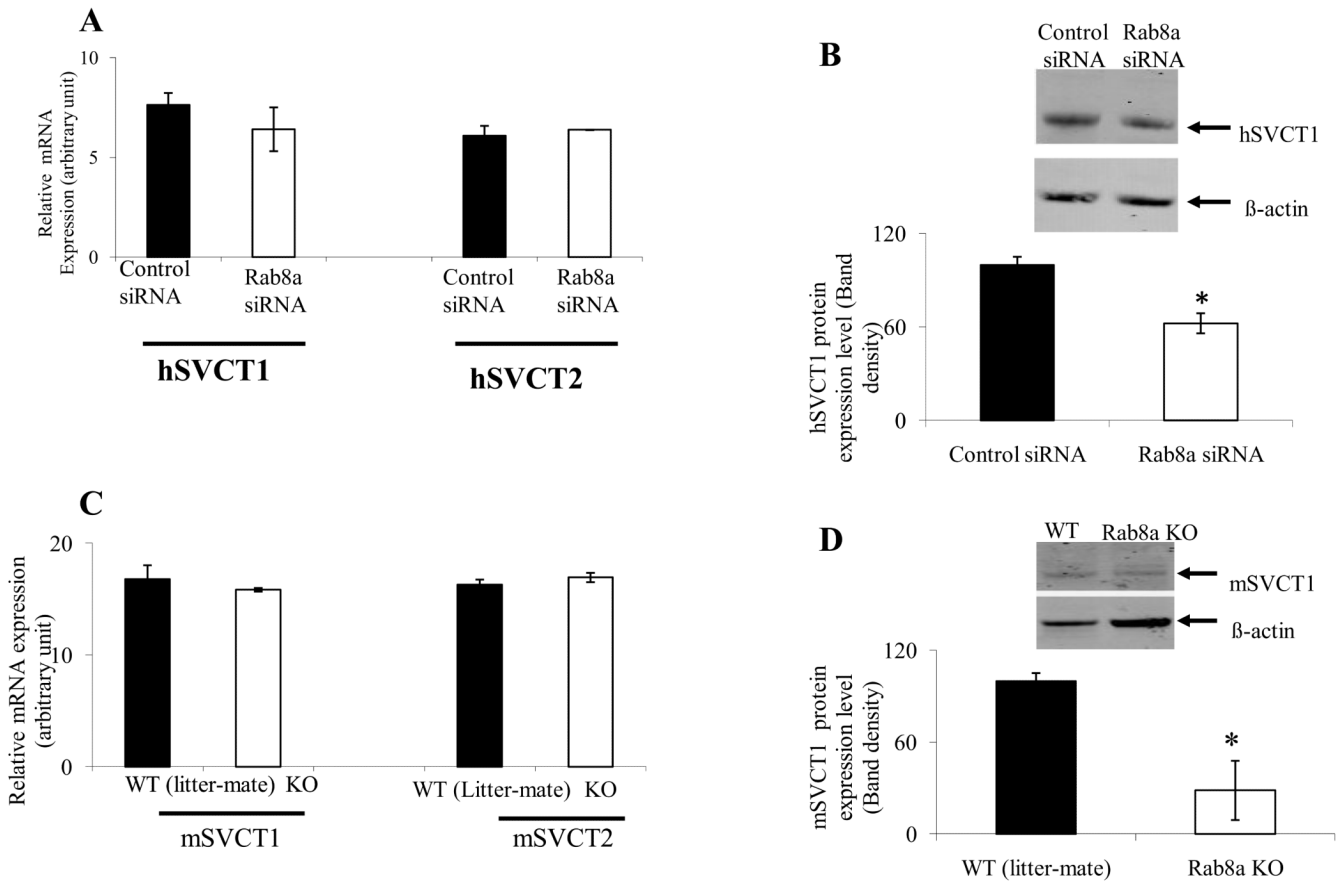

Figure 3. Effect of Rab8a knockdown and knockout on the level of expression of hSVCT1 mRNA and protein in Caco -2 cells and intestinal specific Rab8a KO mice jejunum

A) Real-time PCR was performed using primers for hSVCT1, hSVCT2 and β-actin on total RNA isolated from control (scrambled) and Rab8a siRNAs treated Caco-2 cells. Data are mean ± SE of multiple experiments performed on different batches of cells. **B**, Top, western blot analysis was performed on cell extract (60 μg) isolated from control and Rab8a siRNAs treated Caco-2 cells. Blots were incubated with rabbit anti-human hSVCT1 specific antibodies along with β-actin antibodies. Bottom, densitometric values. Data are mean ± SE of multiple experiments performed on separately isolated samples.*$p < 0.01$. **C**) Real-time PCR was performed on total RNA isolated from Rab8a KO and wild-type litter-mate mice jejunal mucosa using mouse SVCT1, SVCT2 and β-actin primers. Data are mean ± SE of at least three separate samples from three different mice. **D**, Top, western blot analysis was performed on Rab8a KO and wild-type (litter-mate) mouse jejunum mucosal (60 μg) proteins. Blots were incubated with rabbit anti-mouse SVCT1 antibodies along with β-actin antibodies. Bottom, densitometric values. Data are mean ± SE of at least three separate samples from three mice. *$p < 0.02$.

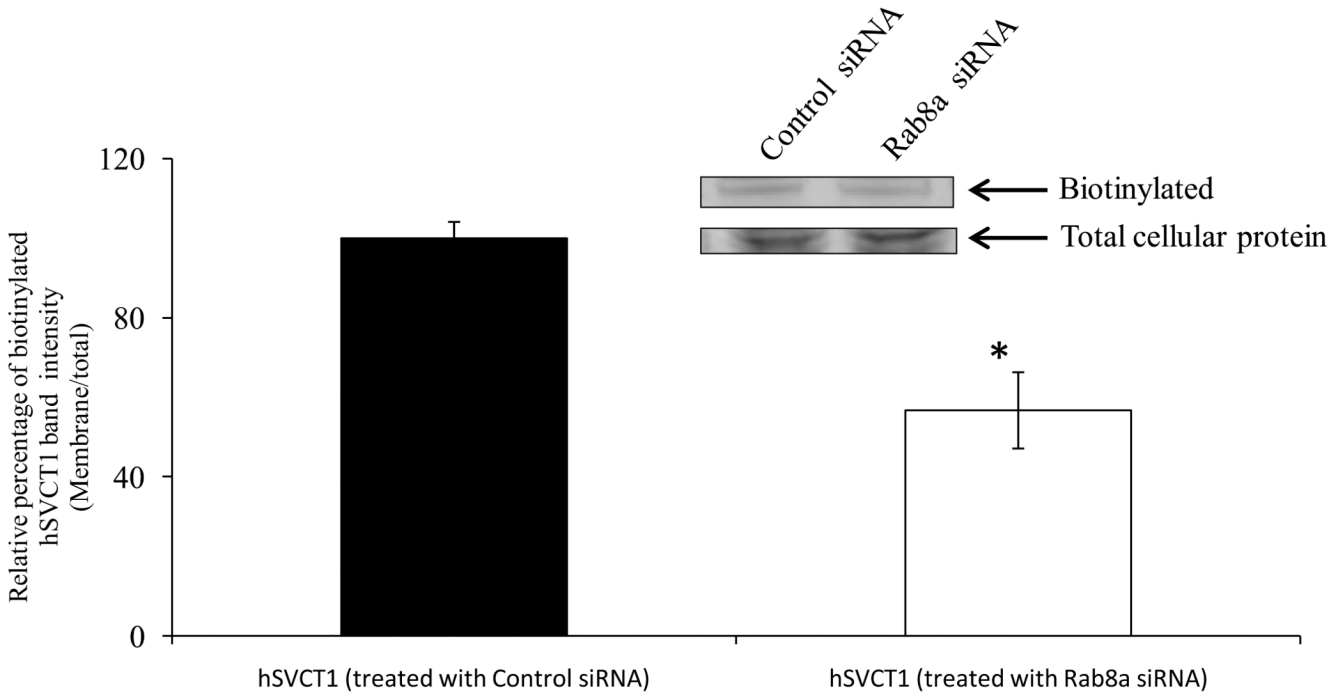

Figure 4. Effect of Rab8a siRNA on the cell surface expression level of hSVCT1 in Caco-2 cells

Caco-2 cells were transiently transfected with Rab8a siRNA or control siRNA. Forty eight hours after transfection cells were processed for biotinylation. Equal amount of protein was loaded onto pre-made 4-12% mini-gel and western blot was performed using anti-hSVCT1 antibody. The level of cell surface expression was normalized relative to the total amount of cellular hSVCT1 protein. Densitometric values are from mean ± SE of four independent experiments. Inset shows representative western blot images. *$p < 0.01$.

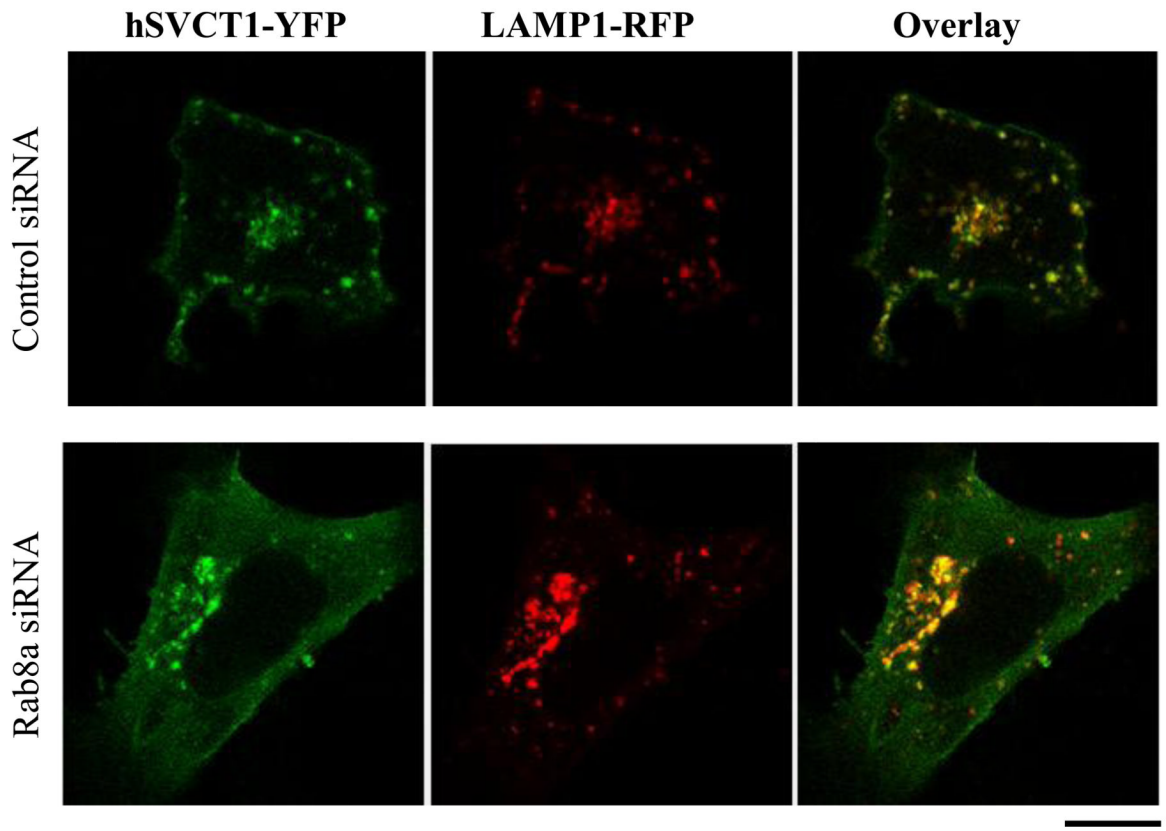

Figure 5. Effect of silencing Rab8a gene on hSVCT1 intracellular trafficking in intestinal cells

Hu-Tu-80 cells were co-transfected with hSVCT1-YEP, LAMP1-RFP, control or Rab8a siRNAs. Live cell confocal imaging (laterl sections-xy) was performed after 48 h of transfection. Data are from n > 6-10 transfected cells and representative images were shown. Scale bar is 10 μm.

Table 1
Primer sequences to clone hSVCT1 and Rab8a and real-time PCR primers

Construct	Forward and reverse primers (5′-3′)
hSVCT1-YFP	CCCAAGCTTATGAGGGCCCAGGAGGAC;
	TCCCCCGCGGGACCTTGGTGCACACAGATG
DsRed-Rab8a	GCGTCGACATGGCGAAGACCTACGATTAC
	CG*GGATCC*TCACAGAAGAACACATCGGAA
Realtime PCR primers	
hSVCT1	TCATCCTCTTCTCCCAGTACCT;AGAGCAGCCACACGGTCAT
hSVCT2	TCTTTGTGCTTGGATTTTCGAT;ACGTTCAACACTTGATCGATTC
mSVCT1	CAGCAGGGACTTCCACCA; CCACACAGGTGAAGATGGTA
mSVCT2	AACGGCAGAGCTGTTGGA; GAAAATCGTCAGCATGGCAA
mRab8a	GGACCATAGAGCTCGATG;CATGGCACCCCTGTAG
h β-actin	CATCCTGCGTCTGGACCT; TAATGTCACGCACGATTTCC
m β-actin	ATCCTCTTCCTCCCTGGA; TTCATGGATGCCACAGGA

Table 1 shows the underlined text (*HindIII*), bold text (*SacII*) enzyme sites introduced to subcloned to YFP-N1 vector and italics (*SalI*) underlined italics (*BamHI*) to DsRed-C1 vector.