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The Sodium/Multivitamin Transporter (SMVT): a Multipotent System With Therapeutic Implications

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Abstract

The Na⁺/multivitamin transporter (SMVT) is a member of the Solute:Sodium Symporter (SSS) family, that catalyzes the Na⁺-dependent uptake of the structurally diverse water-soluble vitamins pantothenic acid (vitamin B5) and biotin (vitamin H), α -lipoic acid – a vitamin-like substance with strong anti-oxidant properties – and iodide. The organic substrates of SMVT play central roles in the cellular metabolism and are, therefore, essential for normal human health and development. For example, biotin deficiency leads to growth retardation, dermatological disorders, and neurological disorders. Animal studies have shown that biotin deficiency during pregnancy is directly correlated to embryonic growth retardation, congenital malformation, and death of the embryo. This chapter focuses on the structural and functional features of the human isoform of SMVT (hSMVT) the discovery of which was greatly facilitated by the cloning and expression of hSMVT in tractable expression systems. Special emphasis will be given to mechanistic implications of the transport process of hSMVT that will inform our understanding of the molecular determinants of hSMVT-mediated transport in dynamic context to alleviate the development and optimization of hSMVT as a multipotent platform for drug delivery.

1. INTRODUCTION

The Na⁺/multivitamin transporter (SMVT) is a member of the Solute:Sodium Symporter (SSS) family, a gene family of membrane proteins that catalyze the coupled transport of their respective solute(s) to the flux of sodium ions (Na⁺) across the plasma membrane of all living organisms (Jung, 2002; Saier, 2000; Wright, Loo, Hirayama, & Turk, 2004). The human isoform of SMVT is one of 12 members of the solute carrier gene family 5 (SLC5A) (Hediger, Coady, Ikeda, & Wright, 1987) which includes Na⁺-coupled transporters for sugars, myo-inositol, iodide, short-chain fatty acids, and choline (Wright, Loo, & Hirayama, 2011; Wright & Turk, 2004). A breakthrough in the analysis of SMVT came in 1998 with

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the cloning of the rat placental cDNA of SMVT (rSMVT) by Prasad and colleagues (Prasad et al., 1998), shortly followed by the isolation of the cDNA of the human isoform of SMVT (hSMVT) from placenta (Wang et al., 1999). Following its cloning and expression in suitable expression systems, it was shown that SMVT mediates the Na^+ -dependent uptake of the structurally diverse water-soluble vitamins pantothenic acid (vitamin B5) and biotin (vitamin H) (Prasad et al., 1998; Wang et al., 1999). In addition, confirming previous indirect studies (Wang et al., 1999), recent radiotracer uptake measurements have revealed the Na^+ -coupled uptake of α -lipoic acid, a vitamin-like substance with strong anti-oxidant properties, as well as the uptake of iodide (I^-) (de Carvalho & Quick, 2011). The organic substrates of SMVT play central roles in the cellular metabolism and are, therefore, essential for normal human health and development. For example, biotin deficiency leads to growth retardation, dermatological disorders, and neurological disorders (Ahmad & Ahmad, 1991). Animal studies have shown that biotin deficiency during pregnancy is directly correlated to embryonic growth retardation, congenital malformation and death (Mock, Quirk, & Mock, 2002; T. Watanabe, 1983, 1996; Zemleni & Mock, 2000). Whereas there are several excellent reviews with scope on the physiology of the substrates of SMVT, in particular for biotin and pantothenic acid (Bender, 1999; Kelly, 2011; Said & Mohammed, 2006; Tahiliani & Beinlich, 1991; Trumbo, 2014; Zemleni & Kuroishi, 2012; Zemleni & Mock, 1999; Zemleni, Wijeratne, & Hassan, 2009), this chapter relates the advances in SMVT research in context with the recent progress in the field of Na^+ -coupled transport processes at the mechanistic and structural level.

2. 'ACTIVE' TRANSPORT

Active transport of solutes across biological membranes driven by electrochemical gradients (i.e., secondary active transport) plays a central role in fundamental cellular processes such as nutrient uptake, excretion of toxic compounds, and signal transduction (DeFelice, 2004; Saier & Ren, 2006). Actively excreted by the action of the Na^+/K^+ -ATPase located in membranes of animal cells, Na^+ is the predominant coupling cation in secondary active transport of a diverse range of molecules such as ions, nutrients, vitamins, and osmolytes in higher organisms (Jung, 2002; Wright et al., 2004; Wright et al., 1994; Wright, Loo, Turk, & Hirayama, 1996). Formulated in the 1960s by Robert Crane (Crane, 1962, 1965), Na^+ -driven substrate co-transport (symport) was hypothesized for the active transport of glucose across the brush border membrane of the enterocytes lining the intestine. The Na^+ /glucose symport hypothesis has been tested and confirmed by work performed in the labs of Ernest Wright and others (see (Wright et al., 2011) for a recent review), and in 1987, using expression cloning, the gene product that catalyzes Na^+ /glucose symport was identified and isolated from a rabbit intestinal cDNA library (Hediger et al., 1987). Two years later the human isoform of the sodium/glucose transporter (hSGLT1) was cloned using a similar approach (Hediger, Turk, & Wright, 1989), thus making possible the functional characterization of substrate flux and substrate-induced electrical currents (Hediger et al., 1987; Ikeda et al., 1989; Parent, Supplisson, Loo, & Wright, 1992a). For SGLT1 this approach has led to the formulation of an ordered kinetic model of transport (Parent, Supplisson, Loo, & Wright, 1992b) (Fig. 1) that follows the key principles of the so-called 'alternating access model', a concept that was introduced in the 1950s (Patlak, 1957). According to this model

transporters alternate between two key conformations, the 'inward-open' and 'outward-open' state, thereby making accessible, in reciprocal fashion, the Na^+ and substrate sites to one side of the membrane but never to both sides at the same time (Abramson & Wright, 2009; Jardetzky, 1966; Weyand et al., 2011). In recent years a number of X-ray crystallographic structures of Na^+ -coupled symporters have been reported and these static snapshots of proteins in a particular conformational state support the basic principle of the alternating access mechanism (Faham et al., 2008; Krishnamurthy, Piscitelli, & Gouaux, 2009; Ressler, Terwisscha van Scheltinga, Vonnrhein, Ott, & Ziegler, 2009; Weyand et al., 2008; Yamashita, Singh, Kawate, Jin, & Gouaux, 2005; Zhou et al., 2014).

3. IDENTIFICATION OF THE MULTIVITAMIN TRANSPORTER

Similar to the initial characterization of Na^+ /glucose transport in animal tissue prior to the cloning of the SGLT1 gene, key elements of the transport of biotin, pantothenic acid, and lipoic acid have been described in studies involving animal and human tissue (Baur & Baumgartner, 1993, 2000; Beinlich, Naumovitz, Song, & Neely, 1990; Chatterjee, Kumar, Ortiz, Rubin, & Said, 1999; Prasad, Ramamoorthy, Leibach, & Ganapathy, 1997; Said, Korchid, Horne, & Howard, 1990; Said et al., 1998; Said & Redah, 1988; Said, Redha, & Nylander, 1987, 1988a; Spector & Mock, 1987; Spencer & Brody, 1964). In particular, radiotracer uptake studies by Grassl (Grassl, 1992a, 1992b) in brush border membrane vesicles from human term placenta indicated that the transport of radiolabelled pantothenate into membrane vesicles was inhibited by biotin and lipoate; likewise, transport of radiolabelled biotin was inhibited by pantothenate and lipoate. The uptake of radiolabeled biotin was inhibited by non-radioactive pantothenate in concentration-dependent manner, and the concentration of pantothenate to yield half-maximum inhibition (K_i) of biotin uptake was comparable to the concentration of radiolabeled pantothenate to yield the half-maximum velocity of transport (K_m). Likewise, the K_i of biotin for radiolabeled pantothenate transport was similar to the K_m of radiolabeled biotin transport, thus, leading to the hypothesis of the involvement of a single transport system that mediates the uptake of these diverse substrates.

In 1998, Prasad et al. (Prasad et al., 1998) reported the isolation of the first mammalian gene that codes for a single polypeptide that, when expressed in HeLa cells, exhibits the key characteristics of this long-sought system – which was termed the Na^+ /multivitamin transporter (SMVT) – from a rat placental cDNA library. This breakthrough at the molecular level was followed by the cloning and expression of the human isoform of SMVT, which was isolated from a placental choriocarcinoma cell line (JAR) (Wang et al., 1999), and the isolation of a rabbit intestinal SMVT (riSMVT) (Prasad et al., 1999). In addition, the mouse isoform of SMVT was cloned in the Said lab (unpublished, GenBank® Accession number AY572835). The sequence and kinetic properties of the cloned SMVTs are very similar and, thus, for simplicity, the following description will focus on the human isoform of SMVT (hSMVT).

4. THE hSMVT GENE

The hSMVT gene is about 14.3 kilo base pairs in length and contains 17 exons separated by 16 introns (GenBank® accession number AF116241). All exon-intron boundaries are consistent with consensus donor-acceptor sequences (gt/ag) for RNA splicing (Wang et al., 1999). Using chromosomal assignment studies with somatic cell hybrid analysis and fluorescence *in situ* hybridization (FISH) of metaphase chromosomes revealed that the hSMVT gene is located on the p23 band on the short arm of the human chromosome 2 (Wang et al., 1999).

4.1. Expression of hSMVT in various tissues

Analysis of the expression of hSMVT in human tissues was analyzed using Northern blots on commercial mRNA samples when the hSMVT gene was cloned (Wang et al., 1999). According to this seminal study hSMVT is ubiquitously distributed in the human body as positive signals were detected in all eight tested tissue samples (heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas). Since the strongest signals were detected in absorptive tissues such as the liver, kidney, and placenta it seems justified to conclude that hSMVT plays a central role in the homeostasis of the essential vitamins H and B5 in the human body. Those studies involving cloned hSMVT (Wang et al., 1999) are consistent with observations regarding biotin and pantothenate uptake studies performed with human or animal tissues (Baur & Baumgartner, 1993, 2000; Beinlich et al., 1990; Said et al., 1990; Said et al., 1998; Said & Redah, 1988; Said et al., 1987, 1988a; Spector & Mock, 1987). Biotin uptake studies performed in brush border membrane vesicles isolated from different regions in the rabbit or rat intestine further demonstrated that uptake of this vitamin is higher in the duodenum and jejunum than in the ileum (Said & Redah, 1988). However, studies at the mRNA level reveal no significant difference in the expression of hSMVT along the rabbit intestinal tract (Prasad et al., 1999), thus suggesting that additional regulatory elements may influence protein expression, trafficking, and/or insertion of SMVT in the plasma membrane. Regulation of the hSMVT gene expression at the transcriptional level has been shown (Reidling, Nabokina, & Said, 2007; Reidling & Said, 2007) and was suggested to counteract conditions of biotin deficiencies (Sweetman & Nyhan, 1986; Wolf, Heard, McVoy, & Raetz, 1984). Guided by experiments that showed adaptive changes of biotin uptake in rats in response to the availability of extracellular biotin with respect to the maximal velocity (V_{max}) of biotin transport (Said, Mock, & Collins, 1989), the hSMVT activity was tested under biotin-deficient and biotin-sufficient states of the cells. The results of this study revealed that the upregulation of hSMVT-mediated biotin transport was accompanied by an increase of the mRNA and protein levels of hSMVT (Reidling et al., 2007). Further *in vitro* studies showed that hSMVT expression in human intestinal epithelial cells is regulated at the transcriptional level via the specific *cis*-regulatory elements Kruppel-like factor 4 (KLF-4) and activator protein-2 (AP2) in the minimal promoter region of hSMVT. *In vivo* studies that utilized a transgenic mouse model supported the *in vitro* findings, and although corresponding data are not available for the transcriptional regulation of hSMVT expression in the human intestine, the authors suggest a similar regulation pattern in humans as the promoters for mouse and human SMVT share a high degree of sequence homology (Reidling & Said, 2007).

4.2. An additional high-affinity hSMVT-like uptake system?

Several studies involving animal and human tissues indicated the presence of a single saturable transport system that mediates the Na^+ -dependent uptake of biotin and/or pantothenate in the intestine (Chatterjee et al., 1999; Said et al., 1987), kidney (Baur & Baumgartner, 1993), liver (Said et al., 1990), brain microvessel endothelial cells of the blood-brain barrier (Baur & Baumgartner, 2000; Spector & Mock, 1987), placenta (Grassl, 1992a, 1992b), heart (Beinlich et al., 1990), as well as in keratinocytes (Grafe, Wohlrab, Neubert, & Brandsch, 2003). Whereas the majority of these studies point to the involvement of SMVT in the transport process due to the kinetic properties and specificity/inhibition profile of the transport features, few studies suggest the presence of an additional uptake system with SMVT-like properties. For example, Grafe et al. (Grafe et al., 2003) reported that biotin uptake in keratinocytes shows features that are reminiscent of the kinetic properties of hSMVT (i.e., μM affinity for ^3H -biotin uptake that was inhibited by pantothenate and lipoate with K_s in the μM range); however, the authors also identified an additional component (or transport system) with preference for pantothenate and high affinity biotin uptake kinetics (with a K_m in the nM range). Likewise, Zempleni and Mock (Zempleni & Mock, 1998) described a high-affinity biotin transport in human peripheral blood mononuclear cells (PBMC) with a K_m for biotin transport of 2.6 nM. Furthermore, *RS*-lipoic acid failed to inhibit transport of biotin. A study that targeted the high-affinity biotin transport phenomenon in Caco-2 and HepG2 cells concluded that such a high-affinity system is not present/functional in human intestinal or liver epithelial cells (Balamurugan, Ortiz, & Said, 2003). Using siRNA silencing techniques to repress the expression of hSMVT showed that under these conditions biotin uptake (tested at 2.6 nM ^3H -biotin concentration) was inhibited by more than 85 %. The authors, thus, concluded that hSMVT is the major (and probably the only) biotin uptake system in intestinal and hepatic cells. It remains to be seen whether the reported high-affinity transport of biotin reflects a rare component in the hSMVT transport reaction that was elicited by particular experimental conditions or whether it stems from an additional, hitherto unknown transport system present in keratinocytes or PBMCs.

5. FROM GENE TO PROTEIN

The hSMVT gene product is 635 amino acids in length and has a calculated molecular weight of 68696.97 in un-modified form. On the basis of hydrophobicity analysis (Kyte-Doolittle plot (Kyte & Doolittle, 1982)) Wang and colleagues (Wang et al., 1999) proposed a secondary structure model according to which hSMVT consists of 12 hydrophobic transmembrane segments (TMs) connected by hydrophilic loops with the amino (N) and carboxy (C) termini located in the cytoplasm (Fig. 2). An elegant study in the Said lab involving the introduction of a green fluorescent protein (GFP)-based assay concluded that the C terminus of hSMVT is indeed located in the cytoplasm and that several domains within the C-terminal region are important for the trafficking of hSMVT to the apical cell membrane (Subramanian, Marchant, Boulware, Ma, & Said, 2009).

On the basis of the 12-TM model Wang et al. (Wang et al., 1999) further predicted four *N*-linked glycosylation sites in hSMVT. *N*-linked glycosylation is a common protein

modification of Asn residues in the sequence Asn-X-Ser/Thr-X (X defines any amino acid except Pro) and is catalyzed in the endoplasmatic reticulum (ER) of virtually all eukaryotic cells. The predicted *N*-glycosylation sites in hSMVT are Asn138, Asn489, Asn498, and Asn534, compare Fig. 2). While the first putative site (Asp138) is located between TM 3 and 4, the other three putative sites are located between transmembrane domains 11 and 12. Furthermore, the deduced hSMVT amino acid sequence features two sites with consensus sequence for protein kinase C (PKC)-dependent phosphorylation. These two putative sites are Ser283 and Thr286 and both are located in the cytoplasmic loop between transmembrane domains 6 and 7. A recent study by Ghosal et al. (Ghosal, Subramanian, & Said, 2011) targeted the putative *N*-glycosylation and PKC sites and analyzed the effect of mutating these putative modification sites on biotin uptake kinetics. Supporting previous immunological studies that demonstrated that hSMVT has a higher molecular mass than the 68.7 kDa predicted from its molecular mass (Nabokina, Subramanian, & Said, 2010; Subramanian et al., 2009), the authors show that hSMVT is indeed subjected to post-translational modifications. The post-translational modification of hSMVT has been confirmed by studies in one of the authors' laboratory (de Carvalho & Quick, 2011); however, in contrast to the aforementioned studies performed in the Said lab (Nabokina et al., 2010; Subramanian et al., 2009), hSMVT is detected as fully glycosylated protein at the expected molecular weight of about 70 kDa and as core-glycosylated/unglycosylated species at ~ 55 kDa (Fig. 3). Although the predicted molecular mass of hSMVT is 68.7 kDa, the unglycosylated polypeptide migrates faster (55 kDa) than its predicted molecular mass. This electrophoretic behavior has been observed in many other membrane proteins (Jung, Tebbe, Schmid, & Jung, 1998; Levy et al., 1998; Turk, Kerner, Lostao, & Wright, 1996) and is probably due to differential (and incomplete) solvation of the highly hydrophobic membrane-embedded polytopic polypeptides by SDS (Rath, Glibowicka, Nadeau, Chen, & Deber, 2009). A similar observation with respect to the presence of 70 kDa and 55 kDa protein bands has been reported for the rabbit isoform of SMVT by Griffin et al. (Griffin, Stanley, & Zemleni, 2002). This discrepancy in the size of the glycosylated form(s) is probably due to the variations in the glycosylation patterns in different expression host systems. Nevertheless, these separate studies emphasize consistently the modification of hSMVT by *N*-glycosylation. Using site-directed mutagenesis, Ghosal et al. targeted the individual putative glycosylation sites and found that positions Asn138 and Asn489 are important for the function of hSMVT as replacement of these sites significantly reduces the V_{max} of biotin uptake (Ghosal et al., 2011), thus highlighting the importance of glycosylation on the function of hSMVT. Since previous studies identified a significant effect on the level of intestinal biotin uptake by modulators of the PKC-mediated regulatory pathway without affecting the affinity of the uptake process (Said, 1999; Said et al., 1998), Ghosal and colleagues tested the effect of mutating the proposed PKC sites by mutating them in individual fashion. Whereas replacing Thr286 led to a significant decrease in the PKC-mediated inhibition of biotin uptake (note that this effect was not associated to changes in the level of expression or insertion of hSMVT in the cell membrane), the replacement of Ser283 did not show any significant effect when compared to the wild-type (WT) protein, thus emphasizing the modulation of the hSMVT-mediated transport process by protein regulatory elements.

6. FAMILY TIES

The amino acid sequences of hSMVT (635 amino acids) shares 84 % identity and 89 % similarity with its rat counterpart (634 amino acids) and 87 % and 92% identity and similarity, respectively, with riSMVT (636 amino acids). With the mouse SMVT (634 amino acids) hSMVT shares 82 % identity and 88 % similarity. Based on homology studies, the newly identified proteins are encompassed in the sodium/solute symporter (SSS) family (Saier, 1999). Together with other human members of the SSS family hSMVT is grouped in the solute carrier family series 5 (SLC5) (Hediger et al., 2004). hSMVT (SLC5A6) shares the highest sequence identity and similarity with the Na⁺/iodide symporter (NIS; SLC5A5) (Dai, Levy, & Carrasco, 1996) (40% and 64%, respectively). Surprisingly, the functional homologous bacterial SSS member PanF (the pantothenate transporter) (Vallari & Rock, 1985) shares only 15% identity and 18% similarity with hSMVT, similar to values obtained for sequence homology studies between hSMVT and PutP, the well characterized bacterial Na⁺/proline transporter (Jung, 2002) (Fig. 4).

7. THE PREDICTED STRUCTURE OF hSMVT

Unlike soluble proteins membrane proteins are generally notoriously resistant to the determination of their high-resolution structure by 3D crystallographic methods most likely due to their hydrophobic and dynamic nature – the key to their biological role as mediator of a ‘system in flux’ that is situated in the membrane. To circumvent this bottleneck in their structural elucidation a new trend emerged in the late 1980s that encompassed the developed of molecular biological techniques to engineer membrane proteins for site-directed biochemical and biophysical assays. For example, substituted-cysteine scanning mutagenesis (Karlin & Akabas, 1998) in combination with chemical cross-linking and spectroscopic methods has become an important tool that has provided – and still provides - invaluable information about the structure and dynamic of membrane transport proteins (Kazmier et al., 2014; Raba et al., 2014; Y. Zhao et al., 2011). The work performed in the lab of Ron Kaback and others on the lactose permease (LacY) of *Escherichia coli* is probably the best example for the extensive use of these techniques to collect structural information in lieu of a crystal structure of a particular membrane protein (Guan & Kaback, 2006; Kaback, Sahin-Toth, & Weinglass, 2001; Kaback, Smirnova, Kasho, Nie, & Zhou, 2011; Kaback & Wu, 1997). Moreover, this information provides a comprehensive framework for the interpretation of data collected from crystallographic X-ray analyses to amount to a coherent mechanistic picture (Abramson et al., 2003; Chaptal et al., 2011; Guan, Mirza, Verner, Iwata, & Kaback, 2007; X. Jiang, Driessen, Feringa, & Kaback, 2013; Kumar et al., 2014; Serdiuk et al., 2014; Smirnova et al., 2007).

While proteins of eukaryotic origin are well-suited to a variety of functional studies that allow the detailed collection of functional data (i.e. tracer flux and electrophysiology; see below), their low abundance in native tissue or mammalian expression systems has, with a few serendipitous exceptions (Bibi, Gros, & Kaback, 1993; Quick & Wright, 2002), precluded their large-scale purification - a prerequisite for their crystallization. On the other hand, to date only crystallization yields detailed high-resolution information about 3D protein structures. Whereas bacterial homologs of a particular gene family have been used

successfully as model systems for crystallization (Abramson & Wright, 2009; Krishnamurthy et al., 2009), their expression in bacterial systems precludes the application of many, if not most of the commonly used functional characterization techniques applicable to membrane proteins expressed in eukaryotic systems.

Direct (crystallographic) or indirect (biochemical/biophysical) structural information is missing for hSMVT. On the basis of hydrophobicity analysis (Kyte-Doolittle plot (Kyte & Doolittle, 1982) a secondary structure model according to which hSMVT consists of 12 hydrophobic TMs was proposed (Wang et al., 1999). This secondary structure model was consistent with the common transmembrane protein model on the basis of the available structural information gathered for LacY (Kaback & Wu, 1997). However, at about the time when hSMVT was cloned this 12-TM model was implicitly challenged by several studies involving members of the SSS family. Most notably, the N termini of hSGLT1 (Turk et al., 1996) and NIS (Levy et al., 1998) were shown to be located on the extracellular face of the membrane, a result that had been confirmed in a study in which PutP was subjected to reporter-protein fusion analyses and site-directed cysteine-accessibility studies (Jung, Rubenhagen, et al., 1998). The study also showed that the C terminus of PutP is located in the cytoplasm, yielding a topological model of this SSS member of 13 TMs with an extracellularly located N terminus. The experimental elucidation of the extracellular location of the N terminus, and the consequential re-arrangement of the N-terminal region in the PutP model, revealed that several residues that were identified to be critical for Na⁺ and substrate binding and transport (Quick & Jung, 1997; Quick, Stolting, & Jung, 1999; Quick, Tebbe, & Jung, 1996) were, in fact, located in TM2 and not in extracellular loops as suggested by the 12-TM model (Nakao, Yamato, & Anraku, 1987). Wright and colleagues proposed a general structural motif for all members of the SSS family, featuring an extracellular N terminus and a common core of 13 TMs, but suggested that some SSS (including all SGLT1s and their bacterial homolog vSGLT of *Vibrio parahaemolyticus* have one additional TM appended to the C terminus (Turk & Wright, 1997).

This prediction has been confirmed by the available crystal structures of vSGLT; and it is indeed composed of 14 TMs (Faham et al., 2008; A. Watanabe et al., 2010). To date, vSGLT is the only member of the SSS family for which high-resolution structural information is available (Abramson & Wright, 2009). Surprisingly, however, although vSGLT and LeuT, a Na⁺-coupled amino acid transporter and member of the neurotransmitter:sodium symporter (NSS) family, share only insignificant sequence homology (Wong et al., 2012), they have a common so-called “LeuT-like” structural fold. The LeuT fold refers to a core structure of 10 TMs arranged in two inverted repeats of five TMs (in vSGLT these include TMs 2 to 6 and 7 to 11, which are structurally homologous to TMs 1 to 5 and 6 to 10 in LeuT) (Abramson & Wright, 2009; Krishnamurthy et al., 2009). This common protein architecture was first identified in the crystal structure of LeuT (Yamashita et al., 2005) and is shared among several Na⁺ or proton (H⁺)-coupled transporter families including – in addition to vSGLT of the SSS family - the nucleobase:cation symporter-1 (NCS1) (Shimamura et al., 2010; Weyand et al., 2008), the betaine/carnitine/choline transporter (BCCT) (Ressl et al., 2009; Schulze, Koster, Geldmacher, Terwisscha van Scheltinga, & Kuhlbrandt, 2010; Tang, Bai, Wang, & Jiang, 2010), and the amino acid/polyamine/organocation transporter (APC) (Shaffer, Goehring, Shankaranarayanan, & Gouaux, 2009) families.

Despite these advances in structural understanding of the related SSS members PutP and vSGLT, the 12-TM model of hSMVT (Fig. 2) is still commonly used as a working model for the interpretation of functional and regulatory data for hSMVT (Ghosal & Said, 2011; Said, 2009; Subramanian et al., 2009). For comparison, we present here a model of hSMVT which contains 13 TMs, with the N terminus located on the extracellular side of the membrane and the C terminus situated in the cytoplasm. This proposed model emerged from our sequence alignment of an exhaustive collection of the relevant pro- and eukaryotic SSS sequences and the topology revealed by the vSGLT crystal structure (Fig. 5) (Mazier, Quick, & Shi, 2011). Note that Asn138 in the original TM-12 model of hSMVT is located on the extracellular side of the membrane, whereas the 13-TM model of hSMVT predicts it to be in the cytoplasm. Previous studies have shown that mutating this residue to Ala leads to a dramatically reduced V_{max} of biotin uptake; however, this phenomenon could be explained by the ~20-fold reduced expression/insertion of the mutated gene product in the cytoplasm membrane as detected with Western blot analysis (Ghosal et al., 2011). So far, the observed effects could be attributed solely to the reduced amount of protein in the membrane caused by the presence of Ala at position 138 but the study did not unequivocally reveal whether Asn138 is indeed an *N*-glycosylation site and/or that glycosylation of this particular residue is critical for the function of hSMVT.

8. THE (CO)SUBSTRATES OF hSMVT

As mentioned above hSMVT transports the water-soluble vitamins pantothenic acid (vitamin B5) and biotin (vitamin H) (Wang et al., 1999), as well as α -lipoic acid, a vitamin-like substance with strong anti-oxidant properties (de Carvalho & Quick, 2011). In addition, the transport of I^- by hSMVT expressed in *Xenopus* oocytes has been reported (de Carvalho & Quick, 2011). Biotin and pantothenate are essential cofactors in key metabolic reactions that are vital for normal growth and development (Said, 1999; Trumbo, 2014; Zempleni & Kuroishi, 2012). Since they are not synthesized in the body, humans (and mammals in general) depend on external sources from which these vitamins can be ingested. In the human intestine there are two sources of these vitamins: a) the dietary source and b) a bacterial source, the latter of which stems from the microflora predominantly found in the large intestine (Said & Mohammed, 2006; Said et al., 1998; Said, Redha, & Nylander, 1988b; Tahiliani & Beinlich, 1991). Found in virtually all dietary sources, these vitamins exist in protein-bound and free form. Only the free forms of the vitamins can be transported by hSMVT (Said, 2004). Prior to the hSMVT-mediated absorption in the small intestine, protein-bound biotin and pantothenate are digested by gastrointestinal proteases and peptidases, leading to the generation of biocytin (and biotin-short peptides) and 4'-phosphopantetheine, respectively (Wolf et al., 1984). The enzyme biotinidase then liberates free biotin (Said, 2012), whereas pantetheinase dephosphorylates 4'-phosphopantetheine to pantothenate (Trumbo, 2014). Lipoic acid is readily absorbed from the diet and can be removed from protein complexes in the intestine by lipoamidase (Y. Jiang & Cronan, 2005; Yoshikawa et al., 1996) (note that it has been reported that lipoamidase and biotinidase are identical enzymes (Garganta & Wolf, 1990; Nilsson & Kagedal, 1993; Nilsson & Ronge, 1992)).

There is compelling evidence that hSMVT couples the transport of its substrates exclusively to the electrochemical gradient of Na^+ across the plasma membrane. In contrast to other SSS members (Eskandari et al., 1997; Hirayama, Loo, & Wright, 1994, 1997; Quick & Jung, 1998; Quick, Loo, & Wright, 2001) hSMVT does not perform H^+ and/or Li^+ -dependent substrate transport (de Carvalho & Quick, 2011; Grassl, 1992a, 1992b; Wang et al., 1999). In analogy to the SSS family member SGLT1 (Mackenzie, Loo, & Wright, 1998), hSMVT is proposed to couple the flux of two Na^+ to the flux of one substrate molecule per transport cycle (Wang et al., 1999). Thus, substrate transport by hSMVT is rheogenic (see 9.1).

Among the members of the SSS family hSMVT exhibits a relatively broad substrate specificity encompassing unrelated organic compounds and an inorganic anion. Whereas the SGLT group of SSS members features highly specific uptake of sugars (see Wright et al. for a recent review (Wright et al., 2011)), NIS, on the other hand, transports a variety of inorganic anions such as I^- , ClO_3^- , SCN^- , SeCN^- , NO_3^- , Br^- , BF_4^- , IO_4^- , BrO_3^- (Eskandari et al., 1997), as well as perchlorate (ClO_4^-) (Dohan et al., 2007). In this regard hSMVT seems to have an intermediate position in the SSS family as it transports the structurally diverse water-soluble vitamin B5, vitamin H, and α -lipoic acid, as well as the anion I^- . Although hSMVT and NIS share the highest sequence identity/similarity (40% and 64%, respectively) (Dai et al., 1996) among the SSS family, with the exception of I^- hSMVT does not transport any of the other monovalent anions transported by NIS (M. Quick, unpublished). Chloride (Cl^-), an anion that is involved in the Na^+ -coupled transport – either in a Cl^- co-transport (Keynan & Kanner, 1988) or Cl^-/Cl^- exchange mechanism (Loo, Eskandari, Boorer, Sarkar, & Wright, 2000) – of neurotransmitters by most, if not all mammalian NSS proteins (Rudnick, 2002; Sonders, Quick, & Javitch, 2005; Zomot et al., 2007), seems to be not involved in the transport reaction catalyzed by NIS or by hSMVT (de Carvalho & Quick, 2011; Eskandari et al., 1997). Interestingly, I^- transport by hSMVT has been adumbrated by Wang and colleagues (Wang et al., 1999) when their team studied the effect of replacing Cl^- as the counter ion for Na^+ . However, the role of I^- transport by hSMVT in human physiology is not entirely understood. The central role of NIS in the physiology of the I^- -containing thyroid hormones thyroxine (T_4) and triiodothyronine (T_3) by mediating the Na^+ -coupled transport of I^- from the blood across the basolateral membrane of thyroid cells (Dohan et al., 2003) as well as in the intestine (Nicola et al., 2009). However, early reports (Acland & Illman, 1959; Pastan, 1957) indicate a rather complex I^- homeostasis in the gastrointestinal tract that may involve other proteins in addition to NIS. This notion is supported by the fact that the accumulation of pertechnetate (TcO_4^-), a commonly used NIS substrate, in the blood of rats that were administered $^{99\text{m}}\text{TcO}_4^-$ via a duodenal catheter, was only partially inhibited by the potent NIS blocker/substrate ClO_4^- (Nicola et al., 2009). Since hSMVT is expressed in virtually every tissue in the body (Said, 1999; Wang et al., 1999) it may play a hitherto unknown role in the homeostasis of I^- .

The other substrates of hSMVT, pantothenic acid (vitamin B₅), biotin (vitamin H) and *R*- α -lipoic acid play vital roles in the energy metabolism of all cells. All three substrates contain a carboxyl group attached to a side chain of different lengths (Fig. 6). Biotin has an imidazole- and thiazole-fused ring structure and pantothenic acid has a pseudo-ring structure in which the amino group of the β -alanine moiety and the hydroxyl group of the pantoic acid

moiety are connected via a hydrogen bond. The disulfite bond in lipoic acid (in its oxidized form) confers to the ring structure of the molecule, whereas the reduced form of lipoic acid (dihydrolipoic acid) does not exist as a ring structure.

Pantothenic acid (3-[(2,4-dihydroxy-3,3-dimethylbutanoyl)amino]propanoic acid) is synthesized *de novo* by plants and microorganisms and it is an essential vitamin for humans (C. M. Smith & Song, 1996). Pantothenate is the precursor of the 4'-phosphopantetheine moiety of coenzyme A (CoA) and the acyl-carrier protein (ACP) (Alberts & Vagelos, 1966), two essential cellular enzymes involved in acyl-group transfer reactions (e.g., in the synthesis of fatty acids, cholesterol, acetylcholine and heme, and in the tri-citric acid (TCA) cycle) and in the synthesis of lipids, respectively (Bender, 1999; Higdon, 2003; Leonardi, Zhang, Rock, & Jackowski, 2005; Tahiliani & Beinlich, 1991).

Biotin (5-[(3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]pentanoic acid) is an essential cofactor for several carboxylase-mediated CO₂ transfer reactions (Knowles, 1989). Synthesized only in bacteria, yeasts, molds, algae, and some plant species (Mock, 1999) biotin is a coenzyme for the following carboxylases in mammals: pyruvate carboxylase (EC 6.4.1.1), propionyl-CoA carboxylase (EC 6.4.1.3), methylcrotonyl-CoA carboxylase (EC 6.4.1.4), and acetyl-CoA carboxylase (EC 6.4.1.2; both isoforms). In addition, biotin was recently found to be central in epigenetic gene regulation and cell signaling via biotinylation of histones (Zempleni, 2005; Zempleni et al., 2009).

α -Lipoic acid ((*R*)-5-(1,2-dithiolan-3-yl)pentanoic acid; thioctic acid) is a naturally occurring substance that serves as a cofactor in dehydrogenase complexes (Milne et al., 2006), enzymes that catalyze central redox reactions of all cells (Bustamante et al., 1998). Lipoic acid is synthesized in small amounts in mitochondria of humans and other mammals and plants, as well as in bacteria (Carreau, 1979). The biological active form of α -lipoic acid, the *R*-enantiomer, is endogenously synthesized *de novo* from octanoic acid (Reed, 2001) being covalently bound to specific lipoate-dependent enzyme complexes (X. Zhao, Miller, Jiang, Marletta, & Cronan, 2003). Most notably, *R*- α -lipoic acid serves as cofactor in the pyruvate dehydrogenase (PDG) and α -ketoglutarate dehydrogenase (KGDG) complexes (Milne et al., 2006), enzymes that catalyze central redox reactions related to energy production in the cell as well as in oxidative decarboxylations of α -keto acids and amino acids (Bustamante et al., 1998). For example, the PDG complex is responsible for the transition of pyruvate to acetyl-coenzyme A (CoA), while the KGDG complex catalyzes the production of succinyl-CoA from α -ketoglutarate, both important intermediates in the tri-citric acid (TCA) cycle. *R*- α -lipoic acid also serves as cofactor in the catabolism of the branched-chain amino acids, leucine, isoleucine and valine (Harris, Joshi, Jeoung, & Obayashi, 2005). Whereas the *R*-enantiomer is the biologically active form of lipoic acid, there is evidence that the *S*-enantiomer assists in the reduction of *R*-lipoic acid to dihydrolipoic acid (DHLA) (Biewenga et al., 1997). DHLA is the form of lipoic acid that is most commonly found within cells (Fig. 6D).

9. THE CHARACTERIZATION OF THE CLONED hSMVT

The cloning and expression of several mammalian, and in particular human membrane transport proteins, in the 1980s and 1990s (Dai et al., 1996; Guastella et al., 1990; Hediger et al., 1987; Kilty, Lorang, & Amara, 1991; Kopito & Lodish, 1985; Mueckler et al., 1985) has permitted rapid progress in the identification of the sites of gene expression, the generation of target-specific antibodies, and the biophysical/biochemical analysis of transporter function (such as substrate specificity, substrate and inhibitor kinetics, transport-associated electric currents, and currents that are not associated with substrate transport). The cloning of hSMVT by Wang and colleagues (Wang et al., 1999) opened the door for the detailed analysis of the human isoform of the multivitamin transporter in heterologous expression systems. Expression of hSMVT in human retinal pigment epithelial (HRPE) cells was used by the authors to perform uptake studies with radiolabeled biotin and pantothenic acid and to test the effect of the ionic composition of the assay medium on hSMVT-mediated transport. Consistent with previous measurements in native tissue transport of pantothenic acid was strictly dependent on the presence of Na^+ but not on Li^+ or K^+ . hSMVT-mediated transport did not require Cl^- as anion; however, replacement of Cl^- with I^- reduced the uptake of pantothenic acid by about 50 %, an observation that led to the discovery of I^- transport by hSMVT (de Carvalho & Quick, 2011). Uptake of radiolabeled pantothenic acid was abolished by the three known physiological substrates at an 100-fold concentration excess. The related vitamins thiamine and niacinamide had no effect on the transport of radiolabeled pantothenic acid, and neither had diaminobiotin or iminobiotin. Desthiobiotin, at 100-fold molar excess, showed a similar inhibitory efficacy like that observed with biotin. These data highlighted that the interaction of biotin with hSMVT requires the keto group at the second position of the imidazole ring in biotin (missing in diaminobiotin and iminobiotin), whereas the tetrahydrothiophene ring of biotin (present in desthiobiotin) is dispensable for substrate recognition by hSMVT. Likewise, recent studies indicate that the β -alanyl fragment of pantothenic acid is crucial for the recognition of this substrate by hSMVT (Chirapu et al., 2013). A closer analysis of the concentration dependence of the three physiological substrates on the transport reaction revealed that uptake of radiolabeled biotin and pantothenic acid exhibits micromolar (μM) affinities (1.5 μM for pantothenate and 3.2 μM for biotin). Since radiolabeled lipoic acid was not commercially available until recently, the authors determined the apparent affinity for lipoic acid with competition assays in which the concentration of non-labeled lipoic acid required to obtain 50 % inhibition of radiolabeled pantothenic acid transport (termed K_i or IC_{50}) was found to be 2.7 μM for oxidized lipoate and 7.3 μM for reduced lipoate (i.e., DHLA). The unequivocal hSMVT-mediated uptake of lipoic acid was shown in hSMVT-expressing *Xenopus* oocytes using radiolabeled α -lipoic acid (as *RS*-racemic mixture) (de Carvalho & Quick, 2011) (the uptake of *R*- α -lipoic acid is depicted in Fig. 7A) and the kinetic details and specificity of α -lipoic acid uptake by hSMVT are currently under investigation in the Quick lab (Zehnpfennig et al., in preparation).

9.1. Electrogenicity of hSMVT-mediated transport

Whereas previous reports claimed the uptake system for biotin and pantothenic acid in human and animal tissue to be Na^+ -dependent, the Hill coefficient - a measure of the Na^+ -to-

substrate coupling stoichiometry (Segel, 1975) - was calculated to be 1 for Na⁺-dependent biotin transport (Grassl, 1992a; Ma, Dyer, & Said, 1994) and 2 for Na⁺-coupled pantothenic transport (Prasad et al., 1997). Since biotin and pantothenic acid are negatively charged at physiological pH (the pK_a values of biotin and pantothenic acid are 4.51 (Said et al., 1988b) and 4.4 (Ball, 2005), respectively), this discrepancy indicates that biotin transport by hSMVT would be electroneutral whereas pantothenic acid would be electrogenic (i.e. 2 Na⁺: 1 pantothenic acid⁻).

Based on the Na⁺-dependence of radiolabeled biotin or pantothenic acid transport in HRPE cells, Wang and colleagues determined an apparent Na⁺ affinity for hSMVT of about 30 mM with a Hill coefficient of nearly 2 for the transport of biotin and pantothenic acid. Although the Hill coefficient does not provide an absolute measure for the Na⁺-to-substrate flux stoichiometry as the individual events may not affect each other in cooperative fashion, the results indicate that hSMVT might mediate electrogenic Na⁺-coupled substrate transport. Like the Na⁺-coupled transport of sugars by SGLT1 (Hirayama et al., 1997) or I⁻ by NIS (Eskandari et al., 1997), the addition the physiological substrates (biotin, pantothenic acid, and lipoic acid) to the Na⁺-containing bath solution of hSMVT-expressing oocytes elicited electrical currents (Wang et al., 1999), thus confirming the proposed rheogenic nature of hSMVT (Fig. 7B). These inward currents increased in magnitude with increasing concentrations of the physiological substrates and with hyperpolarization of the applied membrane potential following similar features observed for other SLC5 family members when expressed in oocytes (see (Wright et al., 2011) for review).

9.2. Mechanistic implications

Since hSMVT mediates the electrogenic transport of its physiological substrates in the presence of Na⁺, the expression of the protein in *Xenopus* oocytes in combination with the two-electrode voltage-clamp technique provided a unique opportunity to assess kinetic features of hSMVT with a combination of flux and electrophysiological experiments. Similar experiments have been successfully used for the analysis of the well-studied SLC5 members SGLT1 (Parent et al., 1992a; Wright et al., 1994) and NIS (Eskandari et al., 1997), and led to the formulation of a kinetic model (Parent et al., 1992b) (Fig.1). Figure 7B depicts the effect of the addition of the physiological substrates to oocytes injected with hSMVT-mRNA superfused in 100 mM NaCl-containing buffer under voltage-clamped conditions ($V_h = -50$ mV). The addition of pantothenic acid, biotin, lipoic acid, and I⁻ (at the same concentration) elicited the same magnitude of inward charge fluxes, which were reversible upon the removal of each compound from the bath solution. Analysis of the ion species that could contribute to the charge movement indicated that electrical currents were only observed in the presence of Na⁺, thus supporting previous observations that hSMVT-mediated transport is exclusively dependent on the presence of Na⁺ and that Na⁺ is most likely also the co-transported (charge-carrying) cation. A closer examination of the ionic conditions in one of the authors' laboratory showed that in striking contrast to several members of the SLC5 family (Wright et al., 2004), hSMVT exhibited no detectable Na⁺ leak currents under our experimental conditions (M. Quick, unpublished; Fig. 8A) but elicited substrate-induced currents only in the presence of Na⁺ (Fig. 8B–D). For example, these leak currents have been observed for NIS (Eskandari et al., 1997) and SGLT1 (Hirayama et al.,

1997) when 100 mM choline chloride was replaced with 100 mM NaCl in assay buffer at pH 7.4. For NIS and SGLT1, the Na⁺ leak currents reached ~35% and ~10% of the maximum Na⁺/substrate cotransport currents, respectively, and they are attributed to the uncoupled flux (uniport) of Na⁺ in the absence of substrate as described in the kinetic model of SGLT1 from TNAo→TNAi in Fig. 1 (Panayotova-Heiermann, Loo, & Wright, 1995; Quick et al., 2001; Vandenberg, Arriza, Amara, & Kavanaugh, 1995; Wright et al., 1996). However, a recent publication from the Wright, Abramson, and Grabe labs (A. Watanabe et al., 2010) suggests, based on new structural information and molecular dynamics simulations, that - in contrast to the original kinetic model for SGLT1 shown in Fig. 1 - the sequence of Na⁺ and substrate release on the cytoplasmic side of the membrane follows that proposed for the NSS member LeuT (Shi, Quick, Zhao, Weinstein, & Javitch, 2008). This modification of the 'classic' ordered binding model for SGLT1 inevitably affects the proposed mechanism of Na⁺ uniport as the conformation [TNA₂]I can only exist after the release of substrate. Recent evidence, originating from studies on mouse SGLT3b, suggests that Na⁺ is indeed released into the cytoplasm before sugar (Diez-Sampedro & Barcelona, 2011).

A key feature of SGLT1 that led in major part to the formulation of the kinetic model depicted in Fig. 1, is the appearance of relaxation currents upon stepping the test voltage (V_t) from a holding potential (V_h). Figure 9A shows a side-by-side comparison of current traces recorded in oocytes injected with hSMVT- or hSGLT1-mRNA in the presence of 100 mM NaCl upon stepping the voltage from -50 mV for 100 ms to V_t from +50 mV to -150 mV in 20 mV increments. As described (Quick et al., 2001), hSGLT1-expressing oocytes exhibited fast membrane capacitive currents (that are also observed in water-injected oocytes (Hazama, Loo, & Wright, 1997; Loo, Hazama, Supplisson, Turk, & Wright, 1993)) followed by transient currents that relaxed to steady-state currents. Integration of the transporter-mediated relaxations (termed pre-steady-state currents) revealed the hSGLT1-specific charge movement within the membrane electric field. Plotting the total charge as a function of membrane potential (V_t) yielded a sigmoidal charge-voltage ($Q-V$) curve that followed a Boltzmann equation,

$$\frac{Q - Q_{hyp}}{Q_{max}} = (1 + \exp[z(V_t - V_{0.5})F/RT])^{-1}$$

where Q is the displaced charge, Q_{max} and Q_{hyp} are Q at the maximal amplitude and at the hyperpolarizing limit, respectively; $V_{0.5}$ is the V_t at which 50 % of the charge has moved in the membrane field, z is the apparent valence of the moveable charge (1), F is the Faraday constant, R is the gas constant, and T is the temperature in K. The maximum charge (Q_{max}) and $V_{0.5}$ were calculated to be ~20 nC and -50.6 mV, respectively, for hSGLT1 (Fig. 9C). In contrast, hSMVT-elicited currents revealed only marginal relaxations that were at the border of resolution (Fig. 9C).

Several other secondary active transporters exhibit a comparable electrophysiological profile (Coady, Wallendorff, Bourgeois, & Lapointe, 2010; Eskandari et al., 1997; Forster, Hernando, Biber, & Murer, 1998) and it was assumed that their kinetic model was similar to that proposed for SGLT1 (Parent et al., 1992a, 1992b)(Fig. 1). However, given the limitation

in the classical characterization of hSMVT – either due to the low expression level of hSMVT in the oocyte membrane or due to its different kinetic properties – using electrophysiological techniques that were so helpful for other members of the SLC5 family, the formulation of a mechanistic model for hSMVT-mediated transport requires alternative approaches that will hopefully lead to a coherent picture of the molecular determinants that govern the transport of biotin, pantothenic acid and lipoic acid. A few studies employing site-directed mutagenesis have been performed to identify functional important amino acid residues in hSMVT (Ghosal & Said, 2011, 2012) but the interpretation of the data in functional context has been hampered by the effect caused by the mutations on the level of surface expression.

10. MEDICAL IMPLICATIONS

As outlined above the organic substrates of hSMVT play central roles in key reactions in the body. For example, biotin is essential for normal growth and development (Bender, 1999; Sweetman & Nyhan, 1986) and severe biotin deficiency is directly associated with clinical symptoms (Whitehead, 1985). In particular, biotin deficiency during pregnancy has been shown to cause growth retardation of the embryo, congenital malformation, and even death (T. Watanabe, 1983, 1996). Deficiency of biotin has been observed in patients with inborn errors of biotin metabolism (Sweetman & Nyhan, 1986; Wolf, 2001) and in patients on long-term therapy with anticonvulsant drugs (Krause et al., 1988), or in those on long-term parenteral nutrition (Forbes & Forbes, 1997), as well as in patients with seboric dermatitis and Leiner's disease (Nisenson, 1957). It has been shown that biotin therapy rapidly improves skin and other congenital abnormalities (Miller, 1989; Mock, 1991; Tsao & Kien, 2002). To date, most cases of biotin deficiency can be attributed to two major genetic disorders in the biotin metabolism of humans involving either a) the holocarboxylase synthetase (HCLS) deficiency (leading to a defect in the biotinylation) or b) the biotinidase deficiency (preventing the release of free biotin from conjugated forms) (McMahon, 2002; Sweetman & Nyhan, 1986). There are also a few reports that suggest that hSMVT may be defective in patients in whom the HCLS and biotinidase function normal (Baumgartner, Suormala, Wick, Geisert, & Lehnert, 1982; Munnich et al., 1981; Thoene, Lemons, & Baker, 1983). However, to date there is no confirmed evidence that mutations in the hSMVT gene are directly correlated with biotin deficiency.

Like biotin, pantothenic acid is ubiquitously available in foods and, thus, dietary deficiency is rare. Pantothenic acid deficiency symptoms include mood changes, sleep, neurological, cardiac and gastrointestinal disturbances, and skin abnormalities (C. M. Smith & Song, 1996; Trumbo, 2014). Also, malnourished prisoners of World War II in Asia reported numbness and burning sensation in their feet, and these symptoms were remedied by the administration of pantothenic acid supplementation (Davenport, Spaide, & Hodges, 1971). Also, the beneficial effect of pantothenic acid has been shown in the maintenance of healthy skin and in wound healing processes (Ebner, Heller, Rippke, & Tausch, 2002; Kelly, 2011; Shibata et al., 2013; Weimann & Hermann, 1999; Yang et al., 2014). Again, the direct involvement of hSMVT in pantothenate deficiency is not confirmed.

In contrast to the two essential vitamins that are transported by hSMVT, α -lipoic acid is synthesized in the body but is also readily absorbed in free form from the diet via the action of hSMVT (de Carvalho & Quick, 2011; Wang et al., 1999) after enzymatic cleavage of protein complexes by lipoamidase (Y. Jiang & Cronan, 2005). Besides its role in the energy metabolism of the cell, there is emerging evidence that free lipoic acid is a potent biological antioxidant and anti-inflammatory agent that has been used in the treatment of several human diseases (for recent reviews see (Goraca et al., 2011; Maczurek et al., 2008). The therapeutic effects of lipoic acid have been attributed to the free radical scavenger properties of the lipoic acid (oxidized)/DHLA redox couple, which – due to the high redox potential of -320 mV – is also a very potent reducing agent. Both the oxidized and reduced forms of lipoic acid are antioxidants that possess anti-inflammatory and neuroprotective properties (Packer, Witt, & Tritschler, 1995; A. R. Smith, Shenvi, Widlansky, Suh, & Hagen, 2004). Several chemical features have been identified that make this redox couple an outstanding ‘metabolic antioxidant’ (Roy & Packer, 1998): it can *i*) scavenge reactive oxygen and nitrogen oxide species (ROS and RNOS, respectively), *ii*) chelate toxic metals, *iii*) regenerate endogenous antioxidants, *iv*) is prone to destruction when quenching free radicals, *v*) can pass the blood-brain-barrier, and *vi*) is amphipathic and may thus exert its antioxidant properties in lipophilic as well as in hydrophobic environments. ROS and RNOS are byproducts of oxidative metabolism reactions as partially reduced molecular oxygen, such as superoxide anion and hydrogen peroxide, or nitric oxide (NO), e.g. peroxynitrite, nitroxyl and nitrogen dioxide. Whereas ROS and/or RNOS may function as signaling molecules in various signal transduction pathways (Lander, 1997), high levels of ROS and RNOS have been considered the key players in oxidative stress to potentially damage macromolecules, such as lipids, DNA, and proteins (Finkel & Holbrook, 2000). Thus, ROS and RNOS have been implicated in the pathogenesis and progression of chronic inflammatory diseases, atherosclerosis, diabetes, cancer, as well as in aging (Halliwell, Gutteridge, & Cross, 1992). The harmful effect of free radicals in the cell can be counteracted by the action of the antioxidant defense system, including enzymatic scavengers, e.g. superoxide dismutase, catalase, and glutathione peroxidase, as well as non-enzymatic antioxidants such as glutathione (GSH), thioredoxin, ascorbic acid (vitamin C), and lipoic acid (Nordberg & Arner, 2001). The unique chemical properties of the lipoic acid/DHLA redox couple make lipoic acid a potent and versatile ‘natural antioxidant drug’ in the non-enzymatic antioxidant defense system as it can also regenerate other antioxidants, e.g., the glutathione/glutathione disulfide pair. Being able to cross the blood-brain-barrier (Moini, Packer, & Saris, 2002; Packer, Tritschler, & Wessel, 1997), it is suggested to be used for detoxification of heavy metals (e.g. Hg^{2+}) attached to brain cells. There, it can mobilize bound heavy metals into the blood stream where another chelator, such as dimercaptosuccinic acid (DMSA) or methylsulfonylmethane (MSM), both of which cannot cross the blood-brain barrier in humans, is used to transfer mercury safely into the urine for excretion (Gregus, Stein, Varga, & Klaassen, 1992).

These diverse actions highlight that lipoic acid acts by multiple mechanisms, both physiologically and pharmacologically as safe and multipotent ‘natural drug’ with strong antioxidant and anti-inflammatory properties useful in the treatment and prevention of human diseases attributed to free radical damage, and, thus, highlight the potential of

hSMVT for the delivery of therapeutic compounds. In particular, the elucidation of key mechanistic features of the cloned hSMVT, together with its ubiquitous distribution in the human body, presents an excellent opportunity for its use as molecular target for drug delivery. While the therapeutic benefits of lipoic acid have been addressed above, new branches of research focus on the development of conjugates of the physiological substrates of hSMVT to increase the efficacy and bioavailability of drugs. For example, work in the Mitra lab has focused on the delivery of (pro)drugs conjugated to biotin (Vadlapudi, Vadlapatla, Earla, et al., 2013; Vadlapudi, Vadlapatla, Kwatra, et al., 2012; Vadlapudi, Vadlapatla, & Mitra, 2012), e.g., in the treatment against breast cancer (Vadlapudi, Vadlapatla, Pal, & Mitra, 2013).

11. CONCLUSION AND FUTURE DIRECTIONS

The cloning of hSMVT has led to a dramatic improvement in our understanding of the biology associated with the uptake of the two essential vitamins, vitamin B5 and vitamin H, α -lipoic acid, and I^- by this SLC5 member. Connecting the knowledge gathered in studies involving human and animal tissues prior to the cloning of the hSMVT gene with the detailed characterization of hSMVT expressed in suitable expression systems presents an unique opportunity to draw a coherent picture of the action of this protein in health and disease. Of course, further work is required to elucidate the interaction of Na^+ and the multiple substrates with the protein as this information will shine light on mechanistic details associated with the molecular determinants of hSMVT-mediated transport. Together with an improved understanding of the structure of hSMVT this knowledge will inform us about the action of this promising drug delivery tool in determining key functional characteristics in a structure-dynamic context.

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Figure 1. Kinetic model for the SSS member SGLT1

A six-state kinetic model of the hSGLT1 transport cycle was proposed (Parent et al., 1992b; Wright et al., 1994) according to which 2 external Na^+ bind to the empty, outward-facing transporter $[\text{T}]_o$. The Na^+ -bound transporter $[\text{TNa}_2]_o$ is poised to bind sugar (S), yielding the Na^+ and sugar-bound intermediate $[\text{TNa}_2\text{S}]_o$. Under physiological conditions this conformational state changes its accessibility to the inward-facing conformation $[\text{TNa}_2\text{S}]_i$ which is followed by the internal sequential release of sugar ($[\text{TNa}_2]_i$) and Na^+ ($[\text{T}]_i$). However, all state transitions are reversible. Since the transient charge relaxations are eliminated by transported sugars (compare Fig. 9B, left panel for hSGLT1), only the three shaded conformational states participate effectively in the observed charge transfer. Membrane voltage affects Na^+ binding and the transition between $[\text{T}]_o \leftrightarrow [\text{T}]_i$ (Loo, Hirayama, Cha, Bezanilla, & Wright, 2005). In the absence of substrate, the uncoupled flux of Na^+ (' Na^+ -leak') is observed, which is ~ 10 % of the maximum sugar-induced currents (Loo et al., 1993). ^{14}C -sugar and $^{22}\text{Na}^+$ flux studies show the direct correlation between the fluxes of substrates and charge (Mackenzie, Harper, Taylor, & Rennie, 1994).

Figure 2. Deduced topology of hSMVT

The secondary structure of hSMVT is based on hydrophobicity calculations (Kyte & Doolittle, 1982) and contains 12 transmembrane domains connected by hydrophilic extracellular and intracellular loops. N and C termini are facing in the cytoplasm. Putative *N*-glycosylation sites are highlighted (bold) and the two putative PKC phosphorylation sites are shown as rectangles(Wang et al., 1999)s.

Figure 3. Characterization of the hSMVT protein

A) Immunological detection of native hSMVT using a polyclonal antibody against SMVT (taken from de Carvalho & Quick (de Carvalho & Quick, 2011)). Non-injected oocytes (co) served as control. **B)** hSMVT was subjected to peptidyl *N*-glycosidase F treatment (indicated by “+”) to show the glycosylation of hSMVT (M.Q. unpublished).

Figure 4. The phylogenetic tree of the SSS family

The tree was generated by the workflow integrated in the phylogeny.fr server (Dereeper et al., 2008). Briefly, the sequences of selected SSS members were aligned by the MUSCLE program (Edgar, 2004) and curated by the Gblock program (Castresana, 2000); the tree was constructed with the maximum likelihood method using PhyML (Guindon & Gascuel, 2003) and rendered by TreeDyn (Chevenet, Brun, Banuls, Jacq, & Christen, 2006). The SSS members are indicated by their Swissprot codes and commonly known abbreviations. For simplicity, the lengths of the branches were not indicated.

Figure 5. Predicted topology of hSMVT

A) Pair-wise sequence alignment between hSMVT and vSGLT extracted from a structure-based and manually-adjusted multiple sequence alignment of all the available non-redundant prokaryotic and eukaryotic SSS sequences from refseq database (>1000 sequences). Identical residues in vSGLT and hSMVT are highlighted and the transmembrane domains in vSGLT (Faham et al., 2008) are indicated. **B)** The diagram of hSMVT secondary structure generated by the RbDe program (Skrabaneck, Campagne, & Weinstein, 2003), based on the pair-wise sequence alignment in **A**.

Figure 6. The structures of the organic substrates of hSMVT

A) biotin, **B)** pantothenic acid, **C)** (oxidized) *R*-α-lipoic acid, and **D)** *R*-dihydrolipoic acid.

Figure 7. Substrate transport by hSMVT in oocytes

A) Uptake of $1.4\ \mu\text{M}$ ^{14}C -pantothenic acid (PA), $2.2\ \mu\text{M}$ ^{14}C -biotin (BIO), $1.6\ \mu\text{M}$ ^3H - *R*- α -lipoic acid (LA) or $500\ \mu\text{M}$ $\text{K}[^{125}\text{I}]$ was measured for 15 min in hSMVT-expressing or control (co) oocytes in 100 mM choline chloride (open) or NaCl (solid). **B**) Substrate-elicited inward currents in a hSMVT-expressing oocyte at a holding potential of $-50\ \text{mV}$ in 100 mM NaCl. $25\ \mu\text{M}$ BIO, PA, LA, or NaI (I^-) were added as indicated.

Figure 8. hSMVT-mediated ionic currents

A) Current-voltage (I/V) relationship of total steady-state currents in hSMVT-expressing oocytes upon stepping the holding membrane potential of -50 mV to a series of test voltages (V_t) from $+50$ to -150 mV in the presence of 100 mM choline chloride, pH 7.4 (●) or pH 5.5 (▼), 100 mM NaCl=Na-gluconate (○) or 100 mM LiCl (□), at pH 7.4. hSMVT exhibited no detectable Na^+ leak currents under our experimental conditions (de Carvalho & Quick, 2011). For example, these currents have been observed for NIS (Eskandari et al., 1997) and SGLT1 (Hirayama et al., 1997) when 100 mM choline chloride was replaced with 100 mM NaCl in assay buffer at pH 7.4. Also note that substrate-elicited currents were only observed in the presence of Na^+ . **B)** Current-voltage (I/V) relationship of substrate-induced steady-state currents elicited in hSMVT-expressing oocytes upon stepping the holding membrane potential of -50 mV to a series of test voltages (V_t) from $+50$ to -150 mV. 25 μ M PA, BIO, LA, or KI (same abbreviations as in Figure 7) were used were added to buffer containing 100 mM NaCl. **C)** I/V relationship of currents induced by varying concentrations of PA in 100 mM NaCl. PA-induced currents were plotted as a function of [PA] for each V_t (except -10 and -30 mV), yielding **D)** the maximum current (I_{max}) and **E)** the apparent affinity (EC_{50}) of PA-induced electrical currents.

Figure 9. Current recordings in *Xenopus laevis* oocytes injected with hSGLT1- or hSMVT-cRNA in response to step changes in the test voltage

The test membrane potential (V_t) was stepped from a holding potential (V_h) of -50 mV to V_t of $+50$ to -150 mV in 20 mV decrements for 100 ms (*on* currents) before returning to V_h (*off* currents). **A**) Currents were recorded in the presence of 100 mM external NaCl in individual hSGLT-, hSMVT-, or water-injected control (co) oocytes. **B**) Currents were recorded the same oocytes shown in **A**) after the addition of 5 mM α -methyl-D-glucopyranoside (α MDG; hSGLT1, left panel) or 10 μ M LA (hSMVT, right panel). The addition of 5 mM α MDG or 10 μ M LA did not affect the current response in control oocytes (not shown). **C**) Charge transfer. The charge-voltage (Q - V) relationship for hSGLT1 (top panel) and hSMVT (lower panel) in the presence of 100 mM NaCl (corresponding to the left and center panel in **A**) were calculated according to the Boltzmann equation.