BASIC RESEARCH

Cloning and expression of *SLC10A4*, a putative organic anion transport protein

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Abstract

AIM: To determine if novel bile acid transporters may be expressed in human tissues.

METHODS: *SLC10A1* (NTCP) was used as a probe to search the NCBI database for homology to previously uncharacterized ESTs. The homology search identified an EST (termed *SLC10A4*) that shares sequence identity with *SLC10A1* and *SLC10A2* (ASBT). We performed Northern blot analysis and RT-PCR to determine the tissue distribution of *SLC10A4*. *SLC10A4* was cloned in frame with an epitope tag and overexpressed in CHO cells to determine cellular localization and functional analysis of bile acid uptake.

RESULTS: Northern analysis revealed that *SLC10A4* mRNA is ubiquitously expressed in human tissues with the highest levels of mRNA expression in brain, placenta, and liver. In *SLC10A4*-transfected CHO cells, immunoblotting analysis and immunofluorescence staining demonstrated a 49-kDa protein that is expressed at the plasma membrane and intracellular compartments. Functional analysis of *SLC10A4* showed no significant taurocholate uptake in the presence of sodium when compared to untransfected CHO cells.

CONCLUSION: To date, we have shown that this protein has no capacity to transport taurocholate relative to *SLC10A1*; however, given its ubiquitous tissue distribution, it may play a more active role in transporting other endogenous organic anions.

INTRODUCTION

Bile acids, the major solute in bile, are physiologically important for promoting bile flow and facilitating the absorption of dietary lipids[1,2]. Bile acids are also involved in cholesterol homeostasis, xenobiotic excretion, as well as apoptotic and cell signal transduction pathways[1-6]. Unlike most other biliary solutes, bile acids are efficiently conserved and cycle in the enterohepatic circulation (EHC). This EHC is maintained by the vectorial transport of bile acids *via* specific bile transport proteins located on the plasma membranes of liver and intestinal epithelia[1].

Within the liver, hepatocytes express the sodium dependent taurocholate transporting protein (*SLC10A1*) and several sodium independent organic anion-transporting proteins at the sinusoidal membrane[6-9]. On their canalicular membrane, hepatocytes express the bile salt export pump (*ABCB11*)[10]. Cholangiocytes, the epithelial cells that line the bile ducts, express the apical sodium dependent bile acid transporter (*SLC10A2*) on lumenal surface[11]. Rat cholangiocytes express a truncated form of ASBT, that may export bile acids across the basolateral membrane[12]. To complete the EHC of bile acids, the enterocytes of the distal ileum express *SLC10A2*[13]. After *SLC10A2*-mediated uptake, bile acids are directed across the ileal enterocyte and secreted into the portal circulation, allowing for efficient recycling of the bile acids from the intestine to the liver[13].

Given the redundancy of biological systems, we hypothesize that additional bile acid transporters may exist within the human genome. In this study, we identified a novel member termed *SLC10A4* of the SLC10A family of sodium bile acid symporter related proteins. We also examined the expression of *SLC10A4* mRNA presence in human tissues and have over expressed this protein in CHO cells.

MATERIALS AND METHODS

Materials

All chemicals were of highest commercially available purity and were purchased from Sigma, St. Louis, MO Chemical C. (St. Louis, MO) unless otherwise indicated.

Cell culture

Cells were maintained at 37℃ in a humidity-controlled incubator with CO_2. The cholangiocarcinoma cell-lines (Witt, KMC, and KMCH) were grown in Dulbecco's modified Eagle's medium containing F12 (Sigma, St. Louis, MO) supplemented with 5% (v/v) FBS (Mediatech, Herndon, VA), Penicillin (100 U/mL)/Streptomycin 100 μg/mL (Sigma, St. Louis, MO), and 2 mmol/L-glutamine (Sigma, St. Louis, MO). H69 cells are SV40 transformed normal human biliary epithelial cells (a gift from Dr. Douglas Jefferson, Tufts University) and were grown as previously described. T84 cells, derived from a human adenocarcinoma, were grown in Dulbecco's modified Eagle's medium containing F12 (Sigma, St. Louis, MO) supplemented with 5% (v/v) FBS (Mediatech, Herndon, VA), Penicillin (100 U/mL)/Streptomycin 100 μg/mL (Sigma, St. Louis, MO). Chinese hamster ovary (CHO) cells were grown in Dulbecco's modified Eagle's medium containing F12 (Sigma, St. Louis, MO) supplemented with 10% (v/v) FBS (Mediatech, Herndon, VA), Penicillin (100 U/mL)/Streptomycin 100 μg/mL (Sigma, St. Louis, MO), 1 × MEM vitamin solution (Sigma, St. Louis, MO), and 2 mmol/L L-glutamine (Sigma, St. Louis, MO).

Identification of the SLC10A4 EST

To initially identify novel members of the SLC10 family of proteins, a bioinformatic approach was used. Amino acids 1-200 of *SLC10A1* (GenBank accession no. AAA36381) were used to perform a tBlastN search of the NCBI database. The candidate EST (Htm1-361F, GenBank accession no. BE39690) sequence was then used for BlastN and BlastX database searches to confirm that it had not been previously identified and as a template to design PCR primers.

RNA isolation

Total RNA was extracted from the tissue culture cell-lines using Tri-Reagent (Sigma, St. Louis, MO). The cells were lysed using 1 mL of Tri-Reagent/$1.0 × 10^6$ cells and stored for 5 min at room temperature. Next, 0.1 mL of 1-bromo-3-chloropropane was added, the samples were vortexed, incubated at room temperature for 15 min, and centrifuged at $12\,000 × g$ for 15 min at 4℃. RNA was subsequently precipitated with isopropanol. The RNA pellet was resuspended with RNA Secure (Ambion, Austin, TX), and the concentration and purity was assessed by spectroscopy.

Reverse transcription-polymerase chain reaction and pCRII cloning

5 μg of total RNA was reverse transcribed using a SuperScript™ II reverse transcritpase (In Vitrogen, Carlsbad, CA). The reaction mixture contained total RNA, 10 mmol/L deoxynucleotide triphosphates and random hexamers in a final volume of 10 μL. This mixture was incubated 5 min at 65℃. Reverse transcription buffer, 25 mmol/L $MgCl_2$, 0.1 mmol/L dithiothreitol (DTT), and RNase inhibitor was then added to the reaction mixture and incubated for 2 min at 25℃. Finally, the reverse transcriptase was added to the reaction mixture and incubated for 50 min at 42℃. The reverse transcriptase reaction was terminated at 70℃ for 15 min and chilled on ice. The *SLC10A4* cDNA was PCR amplified using *SLC10A4* specific primers; sense (5'-GGCAATCTCTC CAATCTTATGTC-3') and antisense (5'-CAGTTGGTG GAGATGAAGAGAGT-3'). The 441 bp PCR amplicon was electrophoresed on a 1% agarose gel and the bands were visualized by ethidium bromide staining. The PCR amplicons were then cloned into the pCRII dual promoter vector (In Vitrogen, Carlsbad, CA) according to the manufacturers protocol. Briefly, 2 μL of the PCR product was mixed with 10 × ligation buffer, 50 ng of pCRII vector, water, and T4 DNA ligase. The ligation reaction was incubated overnight at 14℃ and transformed into competent *Escherichia coli* (*E. coli*). Following the isolation of plasmid DNA, the pCRII (*SLC10A4*) was sequenced to confirm identity of the DNA insert (Mayo Molecular Core Facility, Rochester, MN).

Random priming and Northern blot analysis

The pCRII (*SLC10A4*) plasmid was linearized and random primed using radiolabeled [α-^{32}P] dCTP. The random primer protocol was followed per manufactures (In Vitrogen, Carlsbad, CA) directions. Briefly, 25 ng of pCRII (*SLC10A4*) template DNA was denatured by boiling for 5 min and cooled on ice. To the template DNA, the following was added; 2 μL of dATP, 2 μL of dGTP, 2 μL of dTTP, 15 μL of random primer buffer, 5 μL of [α-^{32}P] dCTP (10 Ci/L). This reaction mixture was brought up to 49 μL. The 1 μL of Klenow fragment was added, mixed, and centrifuged briefly. This reaction mixture was incubated for 1 h at 25℃. Following the incubation, 5 μL of stop buffer was added and the ^{32}P-labeled *SLC10A4* cDNA probe was purified using a NAP-25 column. Next, the Human MTN Blot (Clontech, Palo Alto, CA) and the Human Digestive System MTN Blot (Clontech, Palo Alto, CA) was prehybridized in ExpressHyb solution (Clontech, Palo Alto, CA) for 30 min at 68℃ and then $1 × 10^6$ cpm/mL of the labeled probe was added and incubated for an additional 60 min at 68℃. Following hybridization, the blots were washed several times and exposed to x-ray film (Kodak, Rochester, NY) and developed. Subsequently, the membranes were stripped with 0.5% SDS at 95℃ for 10 min and reprobed with β-actin to normalize for mRNA loading.

Cloning the open reading frame (ORF) of SLC10A4

We obtained IMAGE clone 3502817 (ATCC, Manassas, VA) that contains the complete ORF of *SLC10A4* in the pOTB7 vector. The IMAGE clone was used as template DNA for PCR to delete the stop codon using specific PCR primers; sense (5'-GGATCCCCAAGTAACTATAACGG TCC-3') and antisense (5'-GAATTCGTATCTCCACAT TTGGAGAGAAGTCTG -3'). This fragment was cloned into pCRII (In Vitrogen, Carlsbad, CA) using the method

described above. The pCRII (*SLC10A4*) was digested with *Eco*RI and *Bam*HI restriction enzymes, the *SLC10A4* fragment was isolated by gel purification, and subcloned into the *Eco*RI and *Bam*HI sites of pcDNA4 V5-HisA (In Vitrogen, Carlsbad, CA), a mammalian expression vector which expresses the V5 epitope tag. The *SLC10A4* insert was sequenced to confirm identity of the DNA insert (Mayo Molecular Core Facility, Rochester, MN).

Transfection of CHO cells

Transfections were performed using Lipofectamine reagent (In Vitrogen, Carlsbad, CA) according to the manufactures directions. The day of transfection, the CHO cells were 50%-80% confluent. The media was replaced with Optimem (In Vitrogen, Carlsbad, CA) and the cells were transfected with 3 μg of pcDNA V5-HisA (*SLC10A4*) or empty vector pcDNA V5-HisA plasmid DNA for 4 h after which the transfection media was replaced with complete CHO media. After 48 h, Zeocin (500 μg/mL) was added to the complete media and the cells were selected. Individual stable clones were isolated using cloning cylinders and confirmed by RT-PCR and immunoblotting to confirm the expression of *SLC10A4*.

Immunofluorescent staining

CHO cells were grown on collagen-coated coverslips and fixed by 0.1 mol/L PIPES, pH 6.95, 1 mmol/L ethylene glycolbis (β-amino-ethylether-N, N, N', N'-tetra acetic acid (EGTA), and 2% paraformaldehyde in 1X phosphate buffered saline (PBS) for 20 min at room temperature. The cells were then permeabilized in 0.2% Triton for 2 min and incubated for 1 h with V5 epitope tag (1:500; In Vitrogen, Carlsbad, CA) monoclonal antibody. The cells were washed with PBS and subsequently incubated Texas Red-conjugated goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR). The coverslips were washed with PBS and then mounted using Prolong Antifade mounting medium (Molecular Probes, Eugene, OR) and analyzed using a confocal microscope.

Immunoblotting for the V5 epitope tag

For the immunoblotting assay, cell lysates and mixed plasma membranes were prepared as previously described[14]. Cell lysates and MPM were heated to 95℃ for 10 min in sample buffer containing 0.8 mol/L dithiothreitol (Sigma, St. Louis, MO) and 10% SDS (Sigma, St. Louis, MO) for protein denaturation and solubilization. These protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. After blocking, blots were incubated with the V5 epitope tag antibody (1:2000 dilution; In Vitrogen, Carlsbad, CA) and incubated over night at 4℃. The blots were washed and incubated for 1 h at room temperature with horseradish peroxidase conjugated secondary antibody (1:2000 dilution), and bands were detected using the enhanced chemiluminescent plus detection system (ECL Plus; Amersham, Arlington Heights, IL). Autoradiographs were obtained by exposing the nitrocellulose to Kodak XAR film (Rochester, NY).

Bile Acid Uptake Study

Uptake experiments were performed in triplicate using un-transfected, empty vector transfected or *SLC10A4*-transfected CHO cells. CHO cells transfected with *SLC10A1* was used as the positive control for the uptake experiments. [^{3}H]Taurocholate (specific activity 2.0 Ci/mmol) of > 95% purity was purchased from PerkinElmer (Boston, MA). The uptake experiments were performed as previously described[15].

RESULTS

Identification of SLC10A4 and sequence analysis

Using a bioinformatics approach, numerous expressed sequence tags (EST), including (Htm1-361f [GenBank accession no. BE439690] and Htm1-302f [GenBank accession no. BE439637]) were identified within the NCBI database. Upon further analysis it was determined that these ESTs represented a novel gene (*SLC10A4*) belonging to the sodium bile acid symporter family. The DNA and deduced amino acid sequence are shown in Figure 1. The human *SLC10A4* cDNA encodes a protein of 437 amino acids with a predicted molecular mass of 46.5 kDa. The cDNA encompasses a consensus methionine start codon[16] and an in-frame stop codon (Figure 1). The cDNA also encompasses a 5' untranslated region (UTR) of 183 bp and a 271 bp 3' UTR which contains a poly A tail. Kyte-Doolittle hydropathy analysis suggests that SLC10A4 contains 8 putative transmembrane domains (Figure 1). Furthermore, the amino terminus is predicted to contain three potential N-linked glycoslylation sites.

Comparison of SLC10A4 with other species and with other members of the sodium bile acid symporter family

The *SLC10A4* deduced amino acid sequence for human compared to rat (accession no. XP_579196) share the greatest evolutionary conservation of *SLC10A4* with 89% identity (Figure 2A). In contrast the amount of identity shared between human compared to mouse (XP_775579) or bovine (XP_591836) was equal, 73%.

To further our understanding between the relationships of *SLC10A4* and the other members of the SLC10 family, we performed multiple amino acid alignments and examined the evolutionary relationship between the SLC10 proteins. At the amino acid level, *SLC10A4* shares 30% identity with *SLC10A3* (P3; a SLC10A family member of unknown function), 27% identity with *SLC10A5*, 27% identity with *SLC10A2*, and 24% identity with *SLC10A1* (Figure 2B). We have also looked for relationships of this *SLC10A4* protein compared with anion transporters but no conservation was found (data not shown).

RT-PCR amplification of the SLC10A4 fragment

RT-PCR was performed using *SLC10A4*-specific primers and cDNA prepared from human colon, T84 cells, and cholangiocyte cell lines. This analysis identified *SLC10A4* mRNA in T84 cells, a normal human cholangiocyte cells (H69), and the cholangiocarcinoma cell-lines Witt, KMC and KMCH (Figure 3). In each case, the PCR amplicon was sequenced to confirm the identity of the PCR product.

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   1   ATTGCACGATCCACAAGTTTGTACAAAAAAGCAGGCTTGTAAACGCGGCCAAGTAACTAT
  61   AACGGTCCTAAGGTAGCGAGGCCTGGGTGGCGAATTCGGCACGAGGGCGGCGACTGCGGC
 121   GACCGCGGGACGGCGAGAGGCACGCGGCGGGAGGGGACCGGAATCCGCAGCTCCGGCCGC
 181   GCCATGGACGGCAACGACAACGTGACCCTGCTCTTCGCCCCTCTGCTGCGGGACAACTAC
         M  D  G  N  D  N  V  T  L  L  F  A  P  L  L  R  D  N  Y      19
 241   ACCCTGGCGCCCAATGCCAGCAGCCTGGGCCCCGGCACGGACCTCGCCCTCGCCCCTGCC
         T  L  A  P  N  A  S  S  L  G  P  G  T  D  L  A  L  A  P  A   39
 301   TCCAGCGCCGGCCCCGGCCCTGGGCTCAGCCTCGGGCCGGGTCCGAGCTTCGGCTTCAGC
         S  S  A  G  P  G  P  G  L  S  L  G  P  G  P  S  F  G  F  S   59
 361   CCCGGCCCCACTCCGACCCCGGAGCCCACGACCAGCGGCCTCGCGGGCGGCGCGGCGAGC
         P  G  P  T  P  T  P  E  P  T  T  S  G  L  A  G  G  A  A  S   79
 421   CACGGCCCTTCCCCGTTCCCTCGGCCCTGGGCGCCCCACGCGCTCCCGTTCTGGGACACG
         H  G  P  S  P  F  P  R  P  W  A  P  H  A  L  P  F  W  D  T   99
 481   CCGCTGAACCACGGGCTGAACGTGTTCGTGGGCGGCGCCCTGTGCATCACCATGCTGGGC
         P  L  N  H  G  L  N  V  F  V  G  A  A  L  C  I  T  M  L  G   119
 541   CTGGGCTGCACGGTGGACGTGAACCACTTCGGGGCGCACGTCCGTCGGCCCGTGGGCGCG
         L  G  C  T  V  D  V  N  H  F  G  A  H  V  R  R  P  V  G  A   139
 601   CTGCTGGCAGCGCTCTGCCAGTTCGGCCTCCTGCCGCTGCTGGCCTTCCTGCTGGCCCTC
         L  L  A  A  L  C  Q  F  G  L  L  P  L  L  A  F  L  L  A  L   159
 661   GCCTTCAAGCTGGACGAGGTGGCCGCCGTGGCGGTGCTCCTGTGTGGCTGCTGTCCCGGC
         A  F  K  L  D  E  V  A  A  V  A  V  L  L  C  G  C  C  P  G   179
 721   GGCAATCTCTCCAATCTTATGTCCCTGCTGGTTGACGGCGACATGAACCTCAGCATCATC
         G  N  L  S  N  L  M  S  L  L  V  D  G  D  M  N  L  S  I  I   199
 781   ATGACCATCTCCTCCACGCTTCTGGCCCTCGTCTTGATGCCCCTGTGCCTGTGGATCTAC
         M  T  I  S  S  T  L  L  A  L  V  L  M  P  L  C  L  W  I  Y   219
 841   AGCTGGGCTTGGATCAACACCCCTATCGTGCAGTTACTACCCCTAGGGACCGTGACCCTG
         S  W  A  W  I  N  T  P  I  V  Q  L  L  P  L  G  T  V  T  L   239
 901   ACTCTCTGCAGCACTCTCATACCTATCGGGTTGGGCGTCTTCATTCGCTACAAATACAGC
         T  L  C  S  T  L  I  P  I  G  L  G  V  F  I  R  Y  K  Y  S   259
 961   CGGGTGGCTGACTACATTGTGAAGGTTTCCCTGTGGTCTCTGCTAGTGACTCTGGTGGTC
         R  V  A  D  Y  I  V  K  V  S  L  W  S  L  L  V  T  L  V  V   279
1021   CTTTTCATAATGACCGGCACTATGTTAGGACCTGAACTGCTGGCAAGTATCCCTGCAGCT
         L  F  I  M  T  G  T  M  L  G  P  E  L  L  A  S  I  P  A  A   299
1081   GTTTATGTGATAGCAATTTTTATGCCTTTGGCAGGCTACGCTTCAGGTTATGGTTTAGCT
         V  Y  V  I  A  I  F  M  P  L  A  G  Y  A  S  G  Y  G  L  A   319
1141   ACTCTCTTCCATCTTCCACCCAACTGCAAGAGGACTGTATGTCTGGAAACAGGTAGTCAG
         T  L  F  H  L  P  P  N  C  K  R  Y  V  C  L  E  T  G  S  Q   339
1201   AATGTGCAGCTCTGTACAGCCATTCTAAAACTGGCCTTTCCACCGCAATTCATAGGAAGC
         N  V  Q  L  C  T  A  I  L  K  L  A  F  P  P  Q  F  I  G  S   359
1261   ATGTACATGTTTCCTTTGCTGTATGCACTTTTCCAGTCTGCAGAAGCGGGGATTTTTGTT
         M  Y  M  F  P  L  L  Y  A  L  F  Q  S  A  E  A  G  I  F  V   379
1321   TTAATCTATAAAATGTATGGAAGTGAAATGTTGCACAAAGCGAGATCCTCTAGATGAAGAT
         L  I  Y  K  M  Y  G  S  E  M  L  H  K  R  D  P  L  D  E  D   399
1381   GAAGATACAGATATTTCTTATAAAAAACTAAAAGAAGAGGAAATGGCAGACACTTCCTAT
         E  D  T  D  I  S  Y  K  K  L  K  E  E  E  M  A  D  T  S  Y   419
1441   GGCACAGTGAAAGCAGAAAATATAATAATGATGGAAACCGCTCAGACTTCTCCTAAATG
         G  T  V  K  A  E  N  I  I  M  M  E  T  A  Q  T  S  L  *
1501   TGGAGATACACAGGAGCTTCTATCTTGCTGAAATATTGCTTCATATTTATAGCCTGTGGT
1561   AGTGCACATGGTTAACATAAAAGATAACACTGGTTCACATCATACATGTGACAATTCTGA
1621   TCTTTTTTAAGGGTCACTGGGGTATTAACCAAACGTTGTCACAAATTCAAATCAATGCTGT
1681   TATATAATTTGCACCCGGAATGGCTTACGCGAAGACTGAATTCAAAGTGGGTTTTACGTT
1741   TTACCAGCCCAAAAAAAAAAAAAAAAAT
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Figure 1 Analysis of the *SLC10A4* DNA and protein sequence. Nucleotide and deduced amino acid sequences of *SLC10A4* cDNA. The nucleotide sequence is numbered on the left and the amino acid sequence is numbered on the right. The 5' UTR encompasses nucleotides (1 to 183) and the initiator methionine lies in an appropriate Kozak sequence (underline). The transmembrane domain is highlighted in bold font. The location of the stop codon is indicated by the asterisk.

Tissue distribution of SLC10A4 mRNA

The *SLC10A4* transcript was detected by Northern blot analysis in a wide variety of human tissues (Figure 4A). The highest *SLC10A4* mRNA expression was observed in brain, placenta, and pancreas. Lower levels of expression were also observed in liver and kidney. While the major *SLC10A4* transcript is approximately 2.4 kb, an additional prominent 0.7 kb transcript was observed in pancreas, and a minor 1.4 kb transcript was observed in liver and kidney. β-actin expression appears to be comparable in each tissue suggesting equal loading of mRNA (Figure 4B).

Analysis of SLC10A4-transfected CHO cells

CHO cells lacking endogenous expression of *SLC10A4* were stably transfected with the human *SLC10A4* cDNA. Figure 5A shows that the pcDNA V5-HisA (*SLC10A4*) transcript is expressed in the stably transfected CHO cells. In contrast, the *SLC10A4* mRNA was not detected in non-transfected CHO cells or in CHO cells transfected with the expression vector (pcDNA4 V5-HisA) alone.

Subcellular localization of SLC10A4-V5 in transfected CHO cells by immunofluorescent staining

To address the cellular localization of this novel protein, transfected cells were stained using an affinity-purified mouse monoclonal antibody raised against the V5 epitope tag. We observed strong staining for *SLC10A4* in the transfected CHO cells, confirming its expression (Figure 5B). The transfected V5-epitope tagged *SLC10A4* protein was expressed primarily in intracellular compartments and to a lesser degree on the plasma membrane.

Immunoblot analysis of the SLC10A4-V5 protein

Protein extracts from untransfected CHO cells, empty vector transfected CHO cells, and *SLC10A4*-transfected CHO cells were fractionated into mixed plasma membranes and an intracellular fraction and used for immunoblotting analysis. This analysis detected a 49-kDa protein and larger apparent aggregate, possibly a glycosylated form of this protein, in the *SLC10A4*-transfected cells, whereas no protein was detected in the non-transfected or empty

A

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Rat     ----------MDGLDNTTLLLAPSSLLPDNLTLSPNAS--STSASTLSPLPVTSSPSPGLS
Mouse   ----------MDSLDNTTLLLAPSSLLPDNLTLSPNAG--SPSASTLSPLAVTSSPGPGLS
Human   ----------MDGNDNVTLLFAPLLRDNYTLAPNASSL--GPGTDLALAPASSAGPGPGLS
Bovine  MDSTDNATLLFGTDNGTLLFGQSTLPPDNYTLSPTASSLSPGPDAPLAPASSAGPGPVLS
          :  .  **  ***:.        .  :  .:  ....  .  . . ::.*.* **

Rat     LAPTPSIGFSPDLTPTPEPTSSSLAGGVAGQDSSTFPRPWIPHEPPFWDTPLNHGLNVFV
Mouse   LAPSPSIGFSPEATPTPEPTSSSLTVGVAGQGSSAFPRPWIPHEPPFWDTPLNHGLNVFV
Human   LGPGPSFGFSPGPTPTPEPTTSGLAGGAASHGPSPFPRPWAPHALPFWDTPLNHGLNVFV
Bovine  VAPGPGVSFSPGPTPTPAPTAGSFAGGAGGPSPTLFARPEAAHEPPFWDTPLNHGLNVLV
          :.* *...*** **** **:.:::.*...  .:.** .*  ***********.*

Rat     GAALCITMLGLGCTVDVNHFGAHVRRPVGALLAALCQFGFLPLLAFLLALAFKLDEVAAV
Mouse   GAALCITMLGLGCTVDVNHFGAHVRRPVGALLAALCQFGFLPLLAFLLALIFKLDEVAAV
Human   GAALCITMLGLGCTVDVNHFGAHVRRPVGALLAALCQFGLLPLLAFLLALAFKLDEVAAV
Bovine  GAALCITMLGLGCTVDGNHFGAHVRRPVGALLAALCQFGFLPLLAFLLALAFSLDGSAAV
        ****************  ***************************** .*  .***

Rat     AVLLCGCCPGGNLSNLMSLLVDGDMNLSIIMTISSTLLALVLMPLCLWIYSRAWINTPLV
Mouse   AVLLCGCCPGGNLSNLMSLLVDGDMNLSIIMTISSTLLALVLMPLCLWIYSRAWINTPLV
Human   AVLLCGCCPGGNLSNLMSLLVDGDMNLSIIMTISSTLLALVLMPLCLWIYSRAWINTPLV
Bovine  AVLLCGCCPGGNLSNLMSLLVDGDMNLSIIMTISSTLLALVLMPLCLWIYSRPWIDTPLV
        ***************************************************.**.**:*

Rat     QLLPLGAVTLTLCSTLIPIGLGVFIRYKYNRVADYIVKVSLCSLLVTLVVLFIMTGTMLG
Mouse   QLLPLGAVTLTLCSTLIPIGLGVFIRYKYNRVADYIVKVSLWSLLVTLVVLFIMTGTMLG
Human   QLLPLGTVTLTLCSTLIPIGLGVFIRYKYSRVADYIVKMSLWSLLVTLVVLFIMTGTMLG
Bovine  QLLPIGTVILTLCSTLIPIGLGVFIRYRYSRVADYIVKISLWSLLMTLVVLFILTGTMLG
        ****:*.* ***************** :*.*********:** ***.******:.******

Rat     PELLASIPAAVYVVAIFMPLAGYASGYGLATLFHLPPNCKRTVCLETGSQNVQLCTAILK
Mouse   PELLASIPATVYVVAIFMPLAGYASGYGLATLFHLPPNCKRTVCLETGSQNVQLCTAILK
Human   PELLASIPAAVYVIAIFMPLAGYASGYGLATLFHLPPNCKRTVCLETGSQNVQLCTAILK
Bovine  PELLASIPAAVYVVAIFMPLAGYASGYGLATLFHLPPNCKRTVSLETGSQNVQLCTAILK
        *********.***:***********************.*******.*.*************

Rat     LAFPPRFIGSMYMFPLLYALFQSAEAGVFVLIYKMYGSEILHKREALDEDDDTDISYKKL
Mouse   LAFPPRFIGSMYMFPLLYALFQSAEAGVFVLIYKMYGSEILHKREALDEDEDTDISYKKL
Human   LAFPPQFIGSMYMFPLLYALFQSAEAGIFVLIYKMYGSEMLHKRDPLDEDEDTDISYKKL
Bovine  LAFPPQNIGSMYMFPLLYALFQSAEAGIFVLIYKMYGSGVLHKQDPLDEDEDTDISYKKL
        *****: ********************:*********** :.**.:.:.***.********

Rat     KEEELADTSYGTVGTDDLVLMETTQTSL
Mouse   KEEEMADTSYGTGGTDDLVMMETTQTAL
Human   KEEEMADTSYGTVKAENIIMMETAQTSL
Bovine  KEEEMADTSYGTVKADNLIMMETTQTSL
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B

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SLC10A3  MVLMQDKGSSQQWPGLGGEGGGTGPLSMLRAALLLISLPWGAQGTASTSLSTAGGHTVPP
SLC10A5  ------------------------------------------------------------
SLC10A2  ------------------------------------------------------------
SLC10A1  ------------------------------------------------------------
SLC10A4  ------------------------------------------------------------

SLC10A3  TGGRYLSIGDGSVMEFEFPEDSEGIIVISSQYPGQANRTAPGPMLRVTSLDTEVLTIKNV
SLC10A5  --MSSLSFLNIEKTEILFFTKTEETILVSSSYE---NKRPNSSHLFVKIEDPKILQMVNV
SLC10A2  ------------------------------------------------------------
SLC10A1  ------------------------------------------------------------
SLC10A4  ---------------------------MDGNDNVTLLFAPLLRDNYTLAPNASSLGPGTD

SLC10A3  SAITWGGGGGFVVSIHSGLAGLAPLHIQLVDAHEAPPTLIEERRDFCIKVSPAEDTPATL
SLC10A5  AKKISSDATNFTINLVTDEEGETNVTIQLWDSEGRQERLIEEIKNVKVKVLKQKDS---L
SLC10A2  --------------------------------MNDPNSCVDNATVCSGASCVVP
SLC10A1  ----------------------------------MEAHNASAPFNFTLP
SLC10A4  LALAPASSAGPGPGLSLGPGPSFGFSPGPTPTPEPTTSGLAGGAASHGPSPFPRPWAPHA

SLC10A3  SADLAHFSENPILYLLLPLIFVNKCSFGCKVELEVLKGLMQSPQPMLLGLLGQFLVMPLY
SLC10A5  LQAPMHIDRN-ILMLILPLILLNKCAFGCKIELQLFQTVWKRLPVILGAVTQFLMPFC
SLC10A2  ESNFNNILSVVLSTVLTILLALVMFSMGCNVEIKKFLGHIKRPWGICVGFLCQFGIMPLT
SLC10A1  PNFGKRPTDLALSVILVFMLFFIMLSLGCTMEFSKIKAHLWKPKGLAIALVAQYGIMPLT
SLC10A4  LPFWDTPLNHGLNVFVGAALCITMLGLGCTVDVNHFGAHVRRPVGALLAALCQFGLLPLL
                      .*.  .::.:  : .** *  .::* :  **.  *:  ::*:

SLC10A3  AFLMAKVFMLPKALALGLIITCSSPGGGGSYLFSLLLGGDVTLAISMTFLSTVAATGFLP
SLC10A5  GFLLSQIVALPEAQAFGVVMTCTCPGGGGGYLFALLLDGDFTLAILMTCTSTLLALIMMP
SLC10A2  GFILSVAFDILPLQAVVVLIIGCCPGGTASNILAYWVDGDMDLSVSMTTCSTLLALGMMP
SLC10A1  AFVLGKVFRLKNIEALAILVCGCSPGGNLSNVFSLAMKGDMNLSIVMTTCSTFCALGMMP
SLC10A4  AFLLALAFKLDEVAAVAVLLCGCCPGGNLSNLMSLLVDGDMNLSIIMTISSTLLALVLMP
         .*::. .:   : .    *    .: ::  .*** . :::  : **.  *:: ** **. *  ::*

SLC10A3  LSSAIYSR-LLSIHETLHVPISKILGTLLFIAIPIAVGVLIKSKLPKFSQLLLQVVKPFS
SLC10A5  VNSYIYSR-ILGLSGTFHIPVSKIVSTLLFILVPVSIGIVIKHRIPEKASFLERIIRPLS
SLC10A2  LCLLIYTK-MWVDSGSIVIPYDNIGTSLVALVVPVSIGMFVNHKWPQKAKIILKIG-SIA
SLC10A1  LLLYIYSRGIYDGDLKDKVPYKGIVISLVLVLIPCTIGIVLKSKRPQYMRYVIKGGMIII
SLC10A4  LCLWIYSWAWINTPIVQLLPLGTVTLTLCSTLIPIGLGVFIRYKSRVADYIVKVSLWSL
         : . **:.   :          *  .:*   .:*  *:*::.:    .     : :

SLC10A3  FVLLLGGGLFLAYR-MGVFILAGIRLPIVLVGITVPLVGLLVGYCLATCLKLPVAQRRTVS
SLC10A5  FILMFVGIYLTFT-VGLVFLKTDNLEVILLGLLVPALGLLFGYSFAKVCTLPLPVCKTVA
SLC10A2  GAILIVLIAVVGG-ILYQSAWIIAPKLWIIGTIFPVAGYSLGFLLARIAGLPWYRCRTVA
SLC10A1  LLCSVAVTVLSAINVGKSIMFAMTPLLIATSSLMPFIGFLLGYVLSALFCLNGRCRRTVS
SLC10A4  LVTLVVLFIMTGTMLGPELLASIPAAVYVIAIFMPLAGYASGYGLATLFHLPPNCKRTVC
         :  .     *   *    ::  :      **  * ::    ::   . :        :.**.

SLC10A3  IEVGVQNSLLALAMLQLSLRRLQADYASQAPFIVALSGTSEMLALVIGHFIYSSLFPVP-
SLC10A5  IESGMLNSFLALAVIQLSFPQSKANLASVAPFTVAMCSGCEMLLIILVYKAKKRCIFFLQ
SLC10A2  FETGMQNTQLCSTIVQLSFTPEELNVVFTFPLIYSIFQLAFAAIFLGFYVAYKKCHGKNK
SLC10A1  METGCQNVQLCSTILNVAFPPEVIGPLFFFPLLYMIFQLGEGLLLIAIFWCYEKFKTPKD
SLC10A4  LETGSQNVQLCTAILKLAFPPQFIGSMYMFPLLYALFQSAEAGIFVLIYKMYGSEMLHKR
         :* *  * *   *  *. .:::::::  .       *:  .      ::  .

SLC10A3  ------------------------------------------------------------
SLC10A5  DKRKRNFLI----------------------------------
SLC10A2  AEIPESKENGT-----EPESSFYKANGGFQPDEK-----------
SLC10A1  KTKMIYTAATT-----EETIPGALGNGTYKGEDCSPCTA-----
SLC10A4  DPLDEDEDTDISYKKLKEEEMADTSYGTVKAENIIMMETAQTSL
```
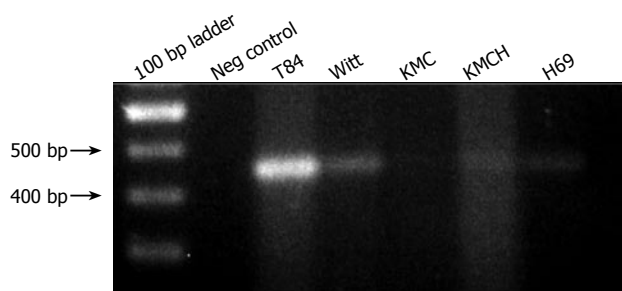
Figure 3 Expression of *SLC10A4* mRNA in cultured cholangiocyte. Positive bands were detected in normal human cholangiocytes (H69) cells, in cholangiocarcinoma cell-lines (Witt, KMC, and KMCH) and the positive control (T84 cells). No bands were detected in the negative control (i.e., no template cDNA).
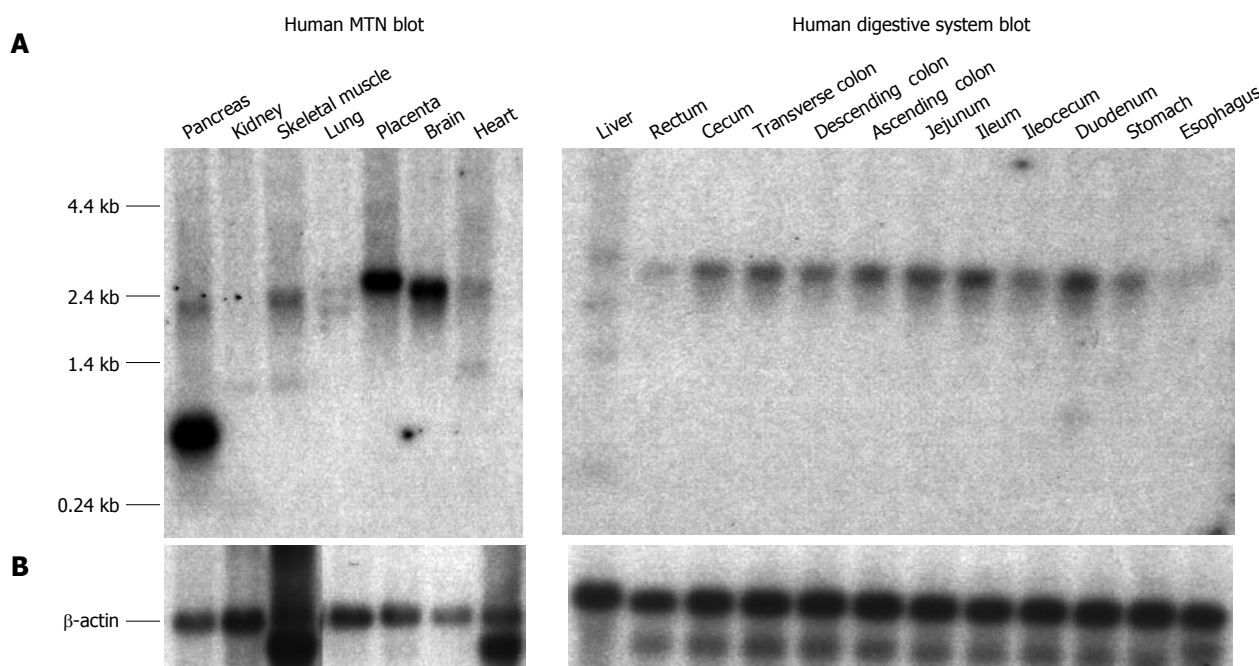

Figure 4 Northern blot analysis of *SLC10A4* mRNA expression in human tissues. (**A**) The Human MTN and Human Digestive System blots contain 2 μg and 1 μg of poly A+ mRNA per lane, respectively. Each blot was hybridized with a ³²P-labeled *SLC10A4* specific probe. (**B**) Hybridization using a ³²P-labeled β-actin specific probe to confirm equal loading of mRNA.

vector-transfected CHO cells (Figure 5C). The 49-kDa band was detected in the lysate, plasma membrane and vesicles of the *SLC10A4*-transfected CHO cells.

Taurocholate functional uptake study

To examine the possible functional capability of *SLC10A4*, we performed taurocholate uptake studies using stably transfected CHO cells (Figure 6). In functional uptake the positive control, namely, CHO cells stably transfected with *SLC10A1*, exhibited an approximate seven fold increase in taurocholate uptake compared to untransfected and empty vector transfected cells in the presence of sodium (Figure 6). In the same experiment, *SLC10A4*-transfected cells showed no significant increase in taurocholate uptake when compared to the untransfected cells (Figure 6). In the absence of sodium, neither transporter (i.e. *SLC10A1* or *SLC10A4*) showed a significant bile acid uptake when compared to control cells (Figure 6).

DISCUSSION

The major findings described here are: (a) *SLC10A4* shares amino identity with other members of the sodium bile acid symporter family (*SLC10A2, SLC10A1, SLC10A3,* and

SLC10A5); (b) the *SLC10A4* mRNA is widely expressed in human tissues, including cultured human cholangiocytes; and (c) the *SLC10A4* protein is expressed in intracellular compartments as well as on plasma membrane in transfected CHO cells.

As a result of the recent sequencing of the human genome, many novel genes have been identified through the cloning of ESTs (i.e., G protein coupled receptors)[17-20]. We utilized a bioinformatic approach to identify a novel SLC10A family member, termed *SLC10A4*. Prior to the completion of this work, two cDNA clones (accession no. AAH 12048 and AAH19066) have been sequence and listed in the NCBI database as SLC10a4 derived from neuroblastoma brain tissue. To date, only minimal bioinformatics observations have been made regarding *SLC10A4*[21]; however to our knowledge this is the first report to have experimentally analyzed *SLC10A4* mRNA distribution in various tissues, including cholangiocytes. Furthermore we have expressed the *SLC10A4* protein in a CHO cell-line to determine its subcellular localization.

The *SLC10A4* amino acid sequence is conserved amongst various mammalian species. This suggests that this protein may have an important role in substrate transport within various mammal species. *SLC10A4* protein
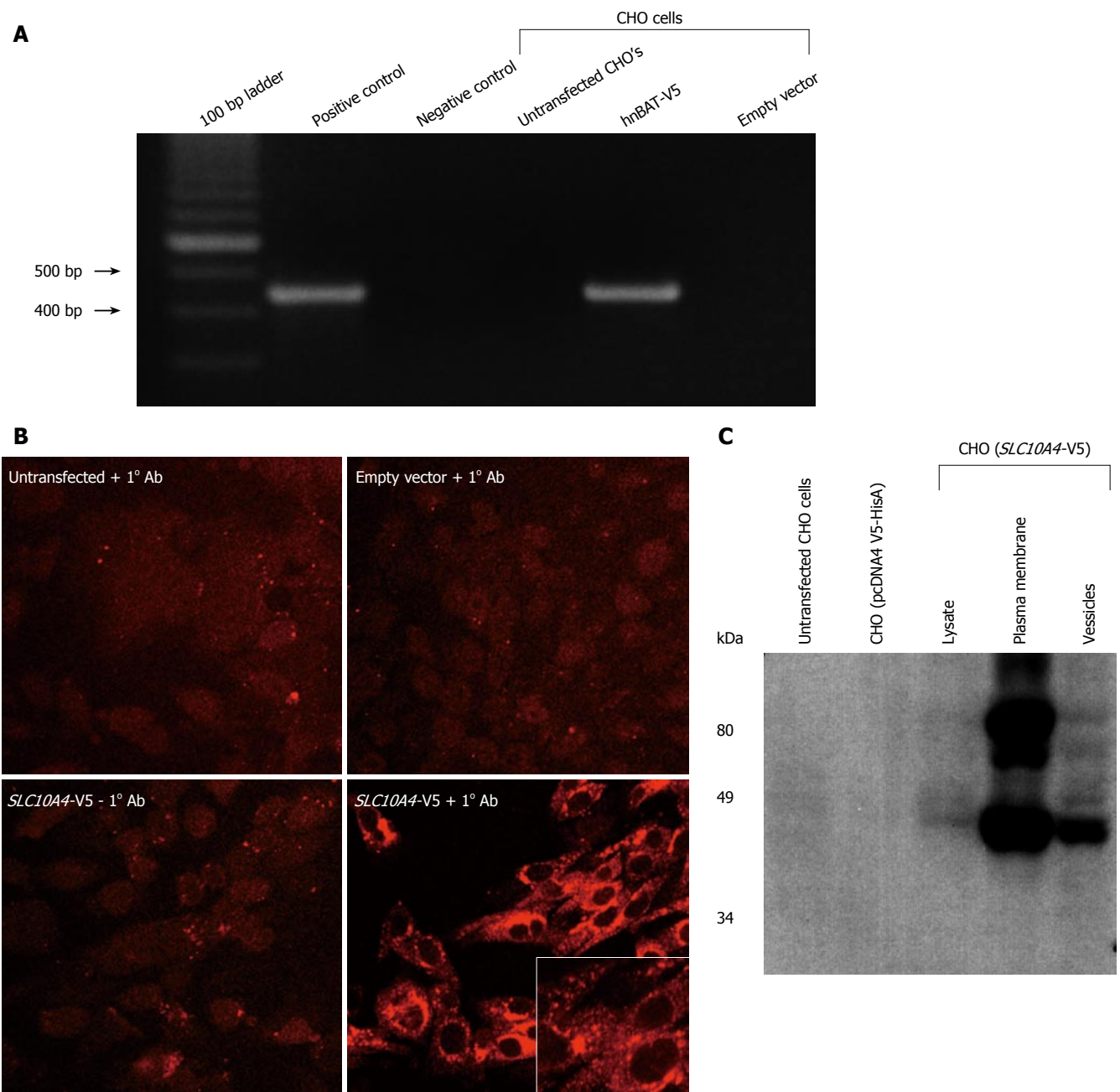
A

B

C

Figure 5 Analysis of the *SLC10A4* stably transfected CHO cells. (**A**) Expression of *SLC10A4* mRNA in transfected CHO cells. *SLC10A4* mRNA was detected by RT-PCR in the *SLC10A4* transfected cells but not in untransfected CHO cells or the negative control (i.e., no template cDNA). (**B**) Protein expression of *SLC10A4* in stably transfected CHO cells. No staining was found in untransfected CHO cells or CHO cells stably transfected with the empty vector (pcDNA4 V5-HisA) alone in the presence of the affinity-purified monoclonal V5 antibody, or in the CHO cells transfected with *SLC10A4* in the absence of the primary antibody. Robust staining was detected in CHO cells transfected with *SLC10A4* in the presence of the V5 antibody (× 40). (**C**) Immunoblotting analysis of *SLC10A4* in transfected CHO cells. *SLC10A4* is detected in the cell lysate (20 μg), mixed plasma membranes (40 μg) and vesicle (40 μg) fractions of the cells. No bands were seen in cell lysates (40 μg) from untransfected CHO cells or empty vector (pcDNA4 V5-HisA) stably transfected CHO cells.

has a long extracellular amino terminus (shared amongst species) relative to that of *SLC10A1* and *SLC10A2* which may suggest a unique physiological process and role that this protein may have.

SLC10A4 shares many characteristics of the human sodium dependent bile acid symporter family members. The SLC10 family is composed of five family members that show approximately 30% identity at the amino acid level. Within the SLC10 family, *SLC10A1* and *SLC10A2* share the greatest amino acid identity (about 36%) and are the only proteins shown to transport bile acids. From an evolutionary perspective, *SLC10A4* appears to be an intermediate between *SLC10A1* and the uncharacterized *SLC10A5* protein. To this end, the exon structure of the sodium dependent bile acid symporter family is of interest. *SLC10A5* has 1 predicted exon; *SLC10A4* and *SLC10A3* are composed of 3 predicted exons, *SLC10A1* is encoded by 5 exons, and *SLC10A2* is encoded by 6 exons. The simpler genomic organization of *SLC10A4*, *SLC10A5* and *SLC10A3* allows us to hypothesize that the genes may be ancestral in nature. SCL10A4 is composed of 437 amino acids compared to *SLC10A1* and *SLC10A2* that are made of 349 and 348 amino acids, respectively. Furthermore, *SLC10A1* and *SLC10A2* are glycosylated and *SLC10A4*
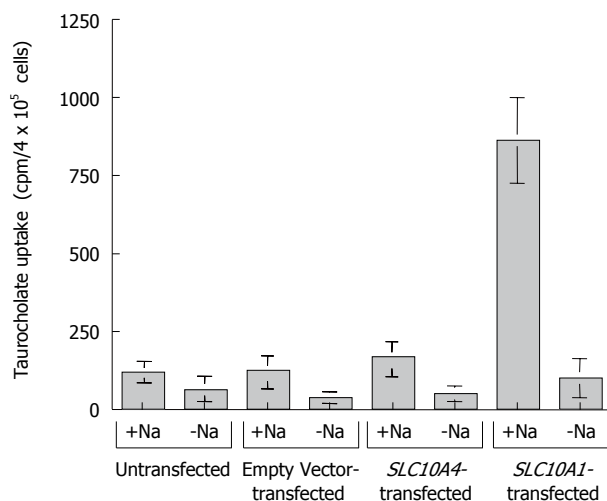
Figure 6 Taurocholate uptake studies by *SLC10A4*. The cells were exposed to 200 μmmol/L of taurocholate (a mixture of [³H]taurocholate and unlabeled taurocholate) in the presence or absence of Na⁺. After 5 min at 37℃, the cells were washed with ice-cold wash solution, lysed, and the amount of [³H] taurocholate was measured. The results are expressed as mean ± SE (n = 3).

is predicted to have numerous glycosylation sites on the amino terminus. The predicted size of *SLC10A4* is slightly smaller than the experimental molecular weight, which may be due to addition of the V5 epitope tag on its carboxyl terminus. Additionally, the immunoblot shows a larger band of about 80 kDa that is possibly due to the glycosylation of the native protein, which is expected to occur based on the bioinformatics predictions. In contrast to *SLC10A1* and *SLC10A2*, the hydropathy plot for *SLC10A4* suggests eight potential transmembrane-spanning domains whereas the more characterized models of *SLC10A1* and *SLC10A2* have only seven again suggesting that this protein may have diverged from the well studied *SLC10A1* and *SLC10A2* genes. This discrepancy will require further experimental analysis to determine the actual number of transmembrane domains that reside in *SLC10A4*. The major sodium bile acid symporters involved in the secretion and absorption of bile acids are *SLC10A1* and *SLC10A2*. *SLC10A1* is primarily found in the liver where it is localized in the hepatocytes[6]. Within the hepatocytes, the *SLC10A1* protein resides on the sinusoidal membrane[6]. In contrast, *SLC10A2* mRNA has been shown in the cholangiocytes, ileal enterocytes, and in the proximal tubular cells of the kidneys[11,15,22,23]. *SLC10A2* is an apical oriented protein in the afore mentioned cells. Both *SLC10A1* and *SLC10A2* have been shown to transport various bile acids in a sodium dependent manner[6,15,22,23]. Much less is known about the remaining members of the SLC10 family of sodium bile acid symporters. *SLC10A3* is ubiquitously expressed in tissues and has been localized to various ESTs including placenta[21] (GenBank accession no. BX377672, brain (GenBank accession no. BM559383), lung (GenBank accession No. BM981524), kidney (GenBank accession no. BG249893) and stomach (GenBank accession no. BM747336). Far less is known for *SLC10A5* mRNA tissue distribution that has been limited to fetal brain (GenBank accession no. XM_294493)[21]. *SLC10A4* is also ubiquitously expressed in all tissues we have tested. Interestingly, *SLC10A4* mRNA shows slight difference in mRNA size

amongst tissue by Northern blotting suggesting that this may be the result of tissue specific alternative transcription.

To our knowledge, the protein localization and substrate specificity have not been determined for either *SLC10A3* or *SLC10A5*, and these genes remain orphan transport proteins. In CHO cells expressing *SLC10A4*, we were not able to show transport of taurocholate, suggesting that *SLC10A4* is also orphan transport protein.

We have found that the *SLC10A4* mRNA is widely expressed in human tissues and is comparable to distribution pattern of *SLC10A3*. In fact, both *SLC10A3* and *SLC10A4* mRNA are expressed in similar tissues, suggesting both proteins may serve a ubiquitous function in the cell machinery. To define the localization of *SLC10A4* in CHO cells we created a chimeric protein in which the coding sequence of *SLC10A4* was cloned in frame with the V5-epitope tag. This construct was stably transfected in CHO cells to characterize the *SLC10A4* protein. We found that the novel protein localizes to both intracellular and plasma membrane of CHO cells. The intracellular localization infers that the protein is a regulated protein by some intracellular messenger. It has been shown the *SLC10A1*-GFP is regulated by cAMP when this construct was transfected into HepG2 cells[24]. It is possible that *SLC10A4* is regulated in a similar manner as *SLC10A1*. *SLC10A4* may be a regulated protein that requires an agonist to translocate the protein to the plasma membrane.

The physiological relevance of this novel transporter is currently unclear. Although the protein does not transport taurocholate and chenodeoxycholic acid, we cannot rule the transport capacity of other bile acids or other polar solutes. Nevertheless, *SLC10A4* protein shares homology with *SLC10A1* and *SLC10A2*. We have cloned and characterized the human *SLC10A4* in hoped that they data will improve our understanding on the phylogeny, origin and evolution of the SLC10A family of transport proteins which may provide important insight into which protein regions are involved in substrate specificity.

ACKNOWLEDGMENTS

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