Published in final edited form as:

Protein J. 2010 August ; 29(6): 417–426. doi:10.1007/s10930-010-9268-y.

Isoforms, Expression, Glycosylation, and Tissue Distribution of CTL2/SLC44A2

P. K. Kommareddi,

Kresge Hearing Research Institute, Department of Otolaryngology/Head and Neck Surgery, The University of Michigan, 1150 West Medical Center Drive, Room 5311 Medical Science I, Ann Arbor, MI 48109-5516, USA

T. S. Nair,

Kresge Hearing Research Institute, Department of Otolaryngology/Head and Neck Surgery, The University of Michigan, 1150 West Medical Center Drive, Room 5311 Medical Science I, Ann Arbor, MI 48109-5516, USA

L. V. Thang,

Kresge Hearing Research Institute, Department of Otolaryngology/Head and Neck Surgery, The University of Michigan, 1150 West Medical Center Drive, Room 5311 Medical Science I, Ann Arbor, MI 48109-5516, USA

M. M. Galano,

Kresge Hearing Research Institute, Department of Otolaryngology/Head and Neck Surgery, The University of Michigan, 1150 West Medical Center Drive, Room 5311 Medical Science I, Ann Arbor, MI 48109-5516, USA

E. Babu,

Department of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta, GA, USA

V. Ganapathy,

Department of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta, GA, USA

T. Kanazawa,

Kresge Hearing Research Institute, Department of Otolaryngology/Head and Neck Surgery, The University of Michigan, 1150 West Medical Center Drive, Room 5311 Medical Science I, Ann Arbor, MI 48109-5516, USA

J. B. McHugh, and

Department of Pathology, The University of Michigan, 2G332 University Hospital, Ann Arbor, MI 48109-0054, USA

T. E. Carey

Kresge Hearing Research Institute, Department of Otolaryngology/Head and Neck Surgery, The University of Michigan, 1150 West Medical Center Drive, Room 5311 Medical Science I, Ann Arbor, MI 48109-5516, USA

T. E. Carey: careyte@umich.edu

Abstract

Correspondence to: T. E. Carey, careyte@umich.edu.

Present Address: T. Kanazawa, Department of Otolaryngology, Jichi Medical University, Saitama Medical Center, 857-1 Amanuma, Omiya, Saitama City, Saitama 330-8503, Japan

Antibodies to the solute carrier protein, CTL2/SLC44A2, cause hearing loss in animals, are frequently found in autoimmune hearing loss patients, and are implicated in transfusion-related acute lung injury. We cloned a novel CTL2/SLC44A2 isoform (CTL2 P1) from inner ear and identified an alternate upstream promoter and exon 1a encoding a protein of 704 amino acids which differs in the first 10–12 amino acids from the known exon 1b isoform (CTL2 P2; 706 amino acids). The expression of these CTL2/SLC44A2 isoforms, their posttranslational modifications in tissues and their localization in HEK293 cells expressing rHuCTL2/SLC44A2 were assessed. P1 and P2 isoforms with differing glycosylation are variably expressed in cochlea, tongue, heart, colon, lung, kidney, liver and spleen suggesting tissue specific differences that may influence function in each tissue. Because antibodies to CTL2/SLC44A2 have serious pathologic consequences, it is important to understand its distribution and modifications. Heterologous expression in *X. laevis* oocytes shows that while human CTL2-P1 does not transport choline, human CTL2-P2 exhibits detectable choline transport activity.

Keywords

CTL2; Choline transporter-like protein 2; Autoimmune hearing loss; Solute carrier protein44A2; Isoforms; N-linked glycosylation; Deglycosylation; Core protein; Tissue differences; Alternate splicing; Inner ear; Hearing loss; Transfusion related acute lung injury; TRALI; Antibody-mediated tissue damage

1 Introduction

We developed a monoclonal antibody (KHRI-3) [28] to inner ear supporting cells that can induce hearing loss in mice and guinea pigs [17–19]. Many patients (75%) with the clinical diagnosis of autoimmune hearing loss AHL have similar antibodies to inner ear supporting cells and are 3 times more likely to respond to treatment with corticosteroids than those who lack such antibodies [29], suggesting that the antibodies affect hearing. We isolated and sequenced the guinea pig inner ear protein, which is recognized by KHRI-3, using tandem mass spectroscopy. The peptide sequences matched a human sequence for choline transporter-like protein 2 (CTL2), also called solute carrier protein 44A2 (SLC44A2) [16]. Studies using the KHRI-3 monoclonal antibody suggested a very restricted distribution of CTL2 expression, but in this paper we use a rabbit antiserum to a conserved peptide within CTL2 that shows a broader tissue distribution. Recently, it has been reported that a single nucleotide polymorphism in CTL2/SLC44A2 encodes the human neutrophil alloantigen-3a which is the target of antibody-mediated transfusion-related acute lung injury (TRALI) [2,7]. Thus, it is important to understand the range of expression of CTL2/SLC44A2 isoforms, their modifications and their tissue distribution.

Sequencing of the complete human inner ear CTL2 cDNA sequence revealed that the N-terminal sequence did not match sequences in the NCBI databases. In this paper we report the identification of a novel exon 1a and its predicted P1-promoter region, located 22 kb upstream of the known P2-promoter and exon 1b. We show that both CTL2 isoforms are expressed in the murine inner ear and we examine the tissue distribution and relative expression of the CTL2-P1 (exon 1a) and CTL2-P2 (exon 1b) isoforms in multiple other tissues of mice. We also show that the posttranslational carbohydrate modification varies among mice and guinea pig tissues suggesting that these modifications may confer different functions in different tissues.

2 Materials and Methods

2.1 Inner Ear and Other Tissues

All animal studies were reviewed and approved by the University of Michigan Committee on Use and Care of Animals and NIH NIDCD Division of Research Grants (R01 DC02272 and R01 DC03686) reviewed and approved all studies described in this report. Tissues were collected immediately after euthanasia.

2.2 Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

For RNA isolation tissues were dissected in RNAlater (Ambion, Austin, TX), placed in lysis buffer, homogenized with a pestle and sheared with 18-gauge needle 5 times. Total RNA was extracted with the RNeasy Mini and Midi kits (Qiagen, Valencia, CA), respectively. cDNA was synthesized from 2 µg of total RNA using oligo dT primers with Superscript II reverse transcriptase (Invitrogen Life Technologies (Carlsbad, CA). Primers were designed using PrimerSelect software from Lasergene (DNASTAR.com). CTL2 isoform specific forward primers based on the human CTL2 gene sequence (GenBank Accession numbers: NM_020428 (P2 sequence) and AK0275519 (P1 sequence) were used with a single reverse primer (Hu1AF (P2) GTGCCTCCCTCCAGACTCGG, P1 cDNA pri1f (P1) GCCATGGAGGACGAGCGGAAAAAC, Hu1AR AAC TGTTTCAGGTGTGGAGAAGG). Mouse CTL2 (GenBank accession numbers: NM_152808 (P2 sequence) and AK041533 (P1 sequence) specific primers included the following forward and reverse sequences: (Ms P1 CTL2 f TCGCGCTGGCTTCGGACTCA; Ms P2 CTL2 f GCGG GTGGCGGCTGTGTC; Ms P1 CTL2 r GCACAGGGCT GGGCATACAAG; Ms P2 CTL2 r GGATGGCCAAAGTA GGGGTGAGG). PCR was performed using the GC-RICH PCR System (Roche Diagnostics, Indianapolis, IN). PCR products were visualized on a 1.5% agarose gel using ethidium bromide fluorescence, purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA), and sequenced in the University of Michigan DNA Sequencing Core.

2.3 Immunoprecipitation and Western Blotting

Rabbit antiserum (CTL2-NT) raised to a phylogenetically conserved antigenic amino acid segment in the N-terminal domain of CTL2 corresponding to exon 2 and shared by all isoforms was used for immunoprecipitation and western blotting as described previously [16]. Fresh tissues in cold Tris buffer containing CaCl₂ and protease inhibitors were homogenized with a polytron (Brinkman, Switzerland). Extracts were centrifuged at 13,000 rpm for 5 min at 4 °C. The pellets were isolated, lysed in buffer containing 1% NP-40, and the protein concentration in the supernatant was assessed using the Bradford assay. CTL2-NT IgG antibody purified on a protein A column and immobilized on cyanogen bromide (CNBr)-activated Sepharose 4B beads (Sigma, MO) was used to immunoprecipitate the CTL2 protein from tissue extracts. Proteins from electrophoresis gels were transferred to nitrocellulose membranes and probed with primary and secondary antibody as described earlier [9,16].

2.4 Cell Culture and Transfection

HEK293 cells were grown to 90–95% confluency in DMEM with 10%FBS. The cells were washed with PBS containing calcium and magnesium to prevent cell detachment during transfection. CTL2 (P1 and P2) were amplified from a human inner ear cDNA library and cloned in frame with a 3× c-terminal flag tag in the pIRES hr-GFP1a vector (Invitrogen, Carlsbad, CA). In the CTL2-P1-3X-Flag vector the Flag tag was not expressed because we discovered that CTL2 retained its stop codon. For the CTL2-P2 3X-Flag construct we removed the stop codon, and the flag tag was in frame and expressed. The recombinant vectors were suspended in 1 mL of opti-MEM containing 35 µl lipofectamine 2000 at room temperature for 20 min to form complexes with the lipid that were then added to the cells and incubated at 37

°C for 6 h. After transfection the HEK293 cells were selected with hygromycin, the surviving cells were subcloned and selected for the highest GFP expression, and then for CTL2 expression by western blotting. Stably transfected cells were plated on cover slips, fixed in 2% paraformaldehyde and permeabilized with 0.1% triton $\times 100$ for immunofluorescence assays or plated in T25 flasks and harvested for protein.

2.5 Evaluation of Transport Function of Human CTL2-P1 and Human CTL2-P2

To determine the ability of the two isoforms of human CTL2 to transport choline, we subcloned the respective cDNAs representing the full-length sequence of the coding region into an oocyte expression vector (pGH19) and used the constructs for heterologous expression in *X. laevis* oocytes. For comparison, we also studied the choline transport function of the two known splice variants of human CTL1 (CTL1a and CTL1b). The respective cRNAs were injected into oocytes, and uptake measurements were made 4 days following injection. Water-injected oocytes served as controls. The transport function was monitored by measurement of [^3H]-choline uptake. The protocols for the preparation of oocytes, microinjection of cRNAs, and uptake measurements have been described previously [5,6].

2.6 Immunofluorescence

Fresh tissues were dissected in PBS, oriented in O.C.T. Tissue-Tek embedding medium (Miles Scientific, IL) and rapidly frozen in acetone and hexane 2:1 pre-chilled solution in liquid nitrogen. Ten μm thick sections were transferred to gelatin subbed slides, immersed in cold acetone/hexane, air-dried for about 10 min and fixed with 2% paraformaldehyde (Ted Pella, Inc., CA). The slides were washed in PBS, incubated in sodium borohydride (1 mg/mL; Sigma, MO) at pH 8.0 for 10 min to reduce auto fluorescence, washed, incubated in 5% goat serum (Jackson ImmunoResearch Laboratories, Inc. PA) with 0.1% Triton (Fisher Scientific, NJ) and washed three times. Tissue sections or cultured cells were incubated with either rabbit anti-CTL2 antibody or anti-flag M2 (Sigma, St. Louis, MO) for 1 h at room temperature, after which they were washed and incubated for 20 min at room temperature in the secondary antibody (Alexa Fluor 488 goat anti-rabbit or rat anti-mouse IgG conjugates, Molecular Probes, OR), diluted in PBS (pH 7.4). The slides were washed and mounted in Prolong Antifade Mounting Media (Molecular Probes, OR).

3 Results

3.1 In silico Identification [25] of CTL2 Promoters and Isoforms

The guinea pig CTL2 sequence we reported [16] (NCBI database [AY233002]) had a 5' sequence that was significantly different from the previously reported human and mouse sequences in the database. Translation of the guinea pig mRNA to protein revealed that the first ten amino acids were different than the first twelve amino acids of the known reference sequences in the databases. To determine the origin of this peptide, we searched the NCBI database, and identified a sequence [AK027519] from a human teratocarcinoma cell line NT2 that after translation encoded a protein isoform homologous to the guinea pig sequence. We aligned this human cDNA sequence to the CTL2 gene sequence on human chromosome 19p13.1, and revealed the presence of a novel exon 1a and its promoter region 22 kb upstream from the known CTL2 promoter (Fig. 1, P1 isoform). The genomic organization of the human CTL2-P1 and CTL2-P2 isoforms spans 42 kb and contains 23 possible exons (including exon 1a and 1b) as shown in Fig. 1. The full length transcript produced from promoter one was designated CTL2-P1 (Fig. 1a). This corresponds to the cDNA sequence we originally detected in the guinea pig inner ear mRNA [16]. The known downstream promoter (P2) drives expression of a full-length protein containing exon 1b. We designated this CTL2-P2 or the P2 isoform (Fig. 1b). Exon 1a encodes ten amino acids whereas exon 1b encodes 12 with multiple differences in those amino acids. The full length polypeptides specified by P1 and P2 in humans

contain 704 and 706 amino acids, respectively. A third isoform (Fig. 1c) was found in the NCBI database and in cDNA from one of our human squamous carcinoma cell lines. This C-terminal isoform designated C-ter AS (Carboxy-terminal alternate splice form), splices into exon 22 differently than the other isoforms to include part of intron 21 which changes the reading frame of the C-terminal portion. This may correspond to CTL2b (711 amino acids) reported by Traiffort et al. [23].

3.2 Distribution of CTL2 P1 and P2 mRNA Expression in the Inner Ear and Other Tissues

The entire mouse genome is known. Thus we could readily assess the relative expression of the P1 and P2 isoforms in cDNA from multiple tissues using reverse transcription-PCR amplification with primers specific for the murine P1 and P2 isoforms, respectively. The relative levels of expression varied in different tissues. Amplification of CTL2-P2 was more robust than P1 in cDNA from adult murine colon, heart, kidney, lung, cochlea, tongue, and muscle (Fig. 2). In contrast, in murine liver, brain, and spleen the P1 isoform predominated. In these experiments equal amounts of template were added to each reaction mixture and the P1 and P2 primer sets serve as controls for one another. Actin amplification controls were also run as controls for the cDNA template loading. Because the level of amplification from cochlear cDNA in this experiment was low an example of other experiments with more RNA is also shown to demonstrate that low level expression of the CTL2 P1 isoform is detectable in the murine inner ear although at a lower level than the P2 isoform (Fig. 2b). In contrast to the murine inner ear result, only the P1 isoform could be amplified using cDNA from human inner ear vestibular tissue (Fig. 2c).

3.3 CTL2 Protein Expression and Glycosylation in the Inner Ear and Other Tissues

There are no antibodies that will distinguish between the CTL2 P1 and P2 protein isoforms which differ in only the first 10 (P1) or 12 (P2) amino acids. Thus, CTL2 protein expression in various tissues can only be assessed by antibody that binds to both isoforms. CTL2 protein was immunoprecipitated from various tissues using anti-CTL2-NT beads and then the immunoprecipitated proteins were washed and western blotted with rabbit anti CTL2-NT antibody [16]. As shown in Fig. 3, CTL2 protein is broadly expressed in multiple tissues, although the amount of protein that could be immunoprecipitated and detected on western blots varied from tissue to tissue. Its molecular mass also varies in different tissues from guinea pig (Fig. 3a, c) and mouse (Fig. 3b, c) because of tissue specific differences in glycosylation. For comparison, CTL2 from inner ear in the guinea pig (Fig. 3a, right panel) and mouse (Fig. 3b, right panel; d left lane) migrates as a 68 and 72 kDa doublet. When the immunoprecipitated proteins are deglycosylated with PNGase F, the core CTL2 protein migrates at 62–64 kDa (Fig. 3a–c, PNGaseF +). Note that the IgG which is also N-glycosylated is also reduced in molecular mass in the samples treated with PNGase F (all Lanes marked with +). In other tissues of both the guinea pig (Fig. 3a), and mouse (Fig. 3b), the relative amount and mass of CTL2 protein vary, with the most robust expression in lung, colon, inner ear and spleen with progressively lower levels of expression in brain, tongue, liver, and kidney. To better appreciate the presence of CTL2 in liver a longer exposure from a separate experiment is shown in Fig. 3c. CTL2 protein expression in murine tongue (70 kDa) in (Fig. 3c) and spleen (80 kDa; Fig. 3d) are also shown. As we previously observed with the inner ear, the degree of glycosylation of the CTL2 protein also differs from tissue to tissue. The molecular mass of the mature glycosylated CTL2 protein is highest in kidney (84 kDa), followed by lung, colon and spleen (80 kDa), then brain and tongue (70 kDa). After enzymatic deglycosylation all of the core proteins migrate at 62–64 kDa (Fig. 3a–c, PNGase F + lanes).

3.4 CTL2 Expression in Tissues

A survey of CTL2 tissue expression was carried out by immunofluorescence in mouse and guinea pig tissues. In tongue CTL2/SLC44A2 is strongly expressed as distinct membrane staining of epithelial cells (Fig. 4a), and in nerves within the tongue musculature (Fig. 4c). Within the nerves, the expression of CTL2 protein can be appreciated in the perineurial cells of the nerve sheath (long arrows) and in the Schwann cell membrane and cytoplasm of individual myelinated nerve fibers (short arrow; Fig. 4c inset 100×). The staining of both is completely blocked by the immunizing peptide used to raise the antiserum (Fig. 4b, d, inset 100×). Second antibody alone gave no staining Fig. 4e (tongue epithelium) and Fig. 4f (tongue musculature). CTL2 expression is prominent in renal glomeruli in the lining of Bowman's capsule in both species as shown in mouse kidney (arrowhead Fig. 4g) and on the mesangial cells adjacent to the vessels within the glomerulus (Fig. 4g, Arrow). CTL2 expression was also particularly strong in the cells of the murine lung parenchyma (Fig. 4h). In both tissues absorption of the antiserum with the immunizing peptide removed the antibody binding (Fig. 4i (kidney) and 4j Lung). CTL2 is also strongly expressed on the membrane of splenocytes in both species. Examples of CTL2 expression in murine spleen are shown in Fig. 4k (100×), and Fig. 4l (40×). Secondary antibody alone on murine spleen at 100× is shown in Fig. 4m.

3.5 Exogenous Expression of CTL2 Isoforms in HEK293 Cells

CTL2 protein could be detected by immunoprecipitation and western blotting of lysates from HEK293 cells transfected with full length cDNAs of the CTL2-P1 or CTL2 P2 3× flag tag constructs (Fig. 5). The 3X-Flag tag increases the relative mass of the CTL2-P2 protein. The glycosylation of the two isoforms in the HEK293 cells, resulted in similar upward shifts in molecular mass recognized by the rabbit anti-CTL2 NT antibody. In the CTL2-P1 transfected cells (Fig. 5a lane 1) the glycosylated form migrates as a broad band centered on 80 kDa. In the CTL2-P2-3X-Flag transfected cells there were two prominent bands, one of 77 and a broad band of 88 kDa identified by both the anti-CTL2-NT antibody (Fig. 5a Lane 2) and by the anti-flag antibody (Fig. 5a Lane 4). No endogenous CTL2 could be detected in the HEK293 cells transfected with the empty vector (Fig. 5a lane 3). Treatment of the proteins with PNGase F, an enzyme that removes N-linked carbohydrate, reduced the size of the CTL2-P1 band to a single core protein band of 62 kDa as expected (Fig. 5b compare Lane 1 control, and lane 2, PNGase F treated). In the CTL2 P2 3X-flag the 77 and 88 kDa bands were both reduced to a core protein of 70 kDa (which includes the 3X-flag tag; Fig. 5c, lanes 5 and 6).

The transfected HEK293 cells were subcloned to select for high CTL2 expression. The CTL2 P1 mass culture exhibited relatively low CTL2 expression (as evidenced in the western blots in Fig. 5). Subcloning did not reveal any clones that express CTL2-P1 strongly enough to be detected by immunofluorescence. However, it was possible to select clones from the CTL2 P2 3X-Flag transfectants that have strong stable expression of CTL2. As shown in Fig. 6a, the CTL2 P2 3X-flag transfected HEK293 cells strongly expressed both GFP (green) and flag-tagged CTL2 (anti-flag = red; Fig. 6a). The anti Flag antibody (Fig. 6b red channel only) and the rabbit anti CTL2 (red; Fig. 6c) both detected the CTL2 protein at the membrane and in intracytoplasmic vesicles. No endogenous CTL2 was detectable in the empty vector transfected cells stained with anti CTL2-NT (red; Fig. 6e), and as expected there was no flag (red; Fig. 6d) staining in the empty vector-transfected HEK cells.

3.6 Transport Function of CTL2

CTL1 has been shown to possess the ability to transport choline [3,4,8,10,¹²,14,15,22,23]. This was the basis for the naming of the gene family as "Choline Transporter-Like" [22]. Recent studies have shown that CTL2 may also transport choline [20]. Since we have identified two different isoforms of this protein (CTL2-P1 and CTL2-P2), we monitored the abilities of these two forms (human CTL2-P1 and human CTL2-P2) to transport choline using the *X. laevis*

oocyte expression system. For comparison, we used the two known isoforms of human CTL1 (CTL1a and CTL1b). As shown previously by other investigators, CTL1a and CTL1b were able to transport choline (~3-fold compared to water-injected controls; Fig. 7a). With CTL2 clones, only the CTL2-P2 isoform showed detectable choline transport activity while CTL2-P1 did not (Fig. 7b). Even with CTL2-P2, the magnitude of choline transport was significantly less than the activity observed with CTL1 isoforms.

4 Discussion

Our prior work focused on an antibody that binds to supporting cells in vivo and results in hearing loss in guinea pigs [17–19]. We identified the inner ear protein as CTL2 (Choline transporter-like protein 2) [16] by homology to CTL1 (choline transporter-like protein 1 [22]. The guinea pig inner ear cDNA had a 5' sequence that did not match the known reference sequences [16]. Herein we show that the 5' sequence defines CTL2 P1, an alternate transcript that arises from a promoter and a novel exon 1a located 22 kb upstream from the known promoter (CTL2-P2). CTL2-P1 is unique from anything previously reported for CTL2 or other CTL/SLC44A family members and it is expressed in human inner ear [16]. The CTL2-P1 and CTL2-P2 are full length isoforms that differ only by the amino acids encoded by their respective first exon. CTL1/SLC44A1 is also expressed in several isoforms—CTL1a, CTL1b and CTL1c [23] but these differ from CTL2. CTL1a and CTL1b [27], are c-terminal splice isoforms that swap exons 16 and 17. Two C-terminal variants of CTL2; CTL2a and CTL2b, which arise from alternate splicing of the C-terminal exon 22 described by Traiffort et al. [23]. Using primers for the C-terminal region we did not find expression of these CTL2 C-terminal isoforms in either the guinea pig or human inner ear. However, in a human squamous cell carcinoma cell line we did find an expressed CTL2 C-ter AS isoform (Fig. 1c) that appears to be equivalent to the CTL2b isoform described by Traiffort et al. [23]. This isoform may be an epithelial cell specific isoform. The existence of isoforms and splice variants suggests that transcriptional modifications of the CTL2 gene might regulate the function of the CTL2 protein in different cells and tissues [23]. Our results with choline transport indicate that CTL2-P1 and -P2 isoforms have different functions, thus it is useful to know the distribution and patterns of expression of the P1 and P2 isoforms in different tissues.

In the mouse, CTL2 P1 and P2 isoforms are both expressed in cochlea, tongue, heart, colon, lung, and kidney with the P2 isoform as the predominant message. In contrast, the P1 isoform is much more abundant than P2 in spleen, brain and liver. Both isoforms are used in many tissues, but CTL2 P1 and P2 are likely to have separate functions in some tissues. Just as it is likely that protein isoforms may have different functions in different tissues, SLC protein splice variants may have different functions in different regions of the same cell. For example, one isoform of SLC25A23 is restricted to mitochondria whereas another is not [1,11].

Differences in molecular mass of the CTL2 protein in different tissues are mostly due to variations in N-linked carbohydrate modification since in each case enzymatic deglycosylation reduced the protein to 62 kDa. This is less than the predicted size of 80 kDa for a core protein of 704–706 amino acids. However, as this protein has 10 or 11 transmembrane domains which are hydrophobic, CTL2 is most likely globular in solution, and therefore migrates with a lower than predicted relative molecular mass. Differences in isoform expression and tissue specific glycosylation strongly suggest that CTL2 function could be modified by either isoform selection or posttranslational modification that is specific to individual tissues.

The CTL2/SLC44A2 protein expression in tissues is consistent with the mRNA expression. However, the subcellular localization of the protein varies from tissue to tissue. In tongue, which expresses P2 message more than P1, CTL2/SLC44A2 protein is expressed strongly at the cell membrane in differentiating squamous epithelial cells and in myelinated nerve fibers

in the tongue musculature. The latter confirmed an early observation of KHRI-3 staining of nerve fibers in the guinea pig tongue [28]. The perineurial cells of the nerve sheath, the Schwann cells and the cytoplasm of myelinated nerve cells express CTL2/SLC44A2 protein as confirmed by peptide blockade of the staining. In the kidney, which expresses both CTL2 P1 and P2 mRNA, the CTL2/SLC44A2 protein is strongly expressed and highly glycosylated. The CTL2/SLC44A2 protein expression is localized to the lining of Bowman's capsule and the mesangial cells within the glomerulus. This localization could be associated with a specific transport function in these structures. Lung, which also expresses both isoforms strongly with a high level of N-linked glycosylation, contains brightly stained small cells, and a mixture of linear membrane staining and punctate cytoplasmic staining of most but not all cells. Spleen, which expresses almost exclusively the P1 isoform, expresses strongly glycosylated CTL2/SLC44A2 and exhibits mostly membrane staining of nearly all spleen cells. Complete determination of the primary transport function of CTL2/SLC44A2 and its isoforms will be critical to understanding how tissue expression patterns are linked to function.

Transfected HEK293 human kidney epithelial cells expressing CTL2 P1 and P2 exhibited a high level of carbohydrate modification of both CTL2 P1 and CTL2-P2 isoforms which is reminiscent of the high degree of glycosylation in guinea pig and mouse kidney tissue. This is similar to the level of glycosylation observed in murine kidney and is consistent with the idea that this modification may be tissue specific. The distribution of CTL2/SLC44A2 protein expressed in HEK293 cells is prominent along the membrane (Fig. 6a) and in cytoplasmic vesicles (Fig. 6b, c). The cytoplasmic distribution is in either the endoplasmic reticulum and the Golgi complex or in endosomal vesicles or both. Mitochondrial localization of CTL1 and CTL2 also has been reported [14,26] but our studies (unpublished data) have not yet unequivocally confirmed or rejected this observation for CTL2. It is possible that the CTL2 molecules traffic through the endocytic pathway to transport their solute, or that it is recycled through lysosomal vesicles. However, as the CTL2-P2 protein is over expressed in the HEK cells, it cannot be ruled out that some of the cytoplasmic expression is an artifact of buildup in the endoplasmic reticulum.

Several lines of evidence support CTL1 as a Na⁺ independent choline transporter [3,4,8,10, 12, 13, 15, 21, 22, 24], with a possible role in the spinal cord and brain to transport choline for membrane biosynthesis [15, 21]. Thus far the function of CTL2 has not been demonstrated unequivocally although recent studies with siRNA to silence CTL2 expression have indicated that CTL2 may possess choline transport activity [8, 20]. In the present study, we have examined directly the abilities of human CTL2-P1 and CTL2-P2 to transport choline using the *X. laevis* oocyte expression system. With this approach, we show that while CTL2-P2 possesses detectable choline transport activity, CTL2-P1 does not. Further work is needed to fully understand the transport features of the two CTL2 isoforms. Interestingly, we have demonstrated that CTL2 selectively binds specific phospholipids suggesting a possible role in phospholipid transport for membrane biosynthesis (Kommareddi et al. unpublished data). Two of these lipids (PI3P and PI4P) are involved in vesicular trafficking and the interaction with CTL2 might be involved in trafficking CTL2 to specific location in the cell or CTL2 may transport lipids to specific locations such as sites of membrane biosynthesis. The association of CTL2 with autoimmune hearing loss and the discovery of its broad distribution in multiple tissues suggest that it could be a target in other autoimmune diseases as well. We found antibodies to inner ear supporting cells in 6/6 sera with lupus (unpublished data), suggesting that CTL2 could be involved in multi-organ autoimmunity. Further investigations are needed to study these hypotheses.

Acknowledgments

This study was supported by NIH NIDCD (R01 DC03686), Autoimmune Sensorineural Hearing Loss Research fund, The Ruth and Lynn Townsend Family Fund, the NIDCD Research Center Core grant (P30 DC05188) and the NIH Rheumatic Core Diseases Center Grant (1P30 AR048310). PK was supported by NIDCD training grant T32 DC00011.

References

1. Bassi MT, Manzoni M, Bresciani R, Pizzo MT, Della Monica A, Barlati S, Monti E, Borsani G. *Gene* 2005;345:173–182. [PubMed: 15716113]
2. Curtis BR, Cox NJ, Sullivan MJ, Konkashbaev A, Bowens K, Hansen K, Aster RH. *Blood* 2010;115:2073–2076. [PubMed: 20040764]
3. Fujita T, Shimada A, Okada N, Yamamoto A. *Neurosci Lett* 2006;393:216–221. [PubMed: 16239069]
4. Fullerton MD, Wagner L, Yuan Z, Bakovic M. *Am J Physiol Cell Physiol* 2006;290:C1230–C1238. [PubMed: 16319125]
5. Ganapathy V, Fei YJ, Prasad PD. *Methods Mol Med* 2006;122:285–300. [PubMed: 16511988]
6. Gopal E, Miyauchi S, Martin PM, Ananth S, Roon P, Smith SB, Ganapathy V. *Pharm Res* 2007;24:575–584. [PubMed: 17245649]
7. Greinacher A, Wesche J, Hammer E, Furl R, Volker U, Reil A, Bux J. *Nat Med* 2010;16:45–48. [PubMed: 20037594]
8. Inazu M, Takeda H, Matsumiya T. *J Neurochem* 2005;94:1427–1437. [PubMed: 16000150]
9. Kommareddi PK, Nair TS, Vallurupalli M, Telian SA, Arts HA, El-Kashlan HK, Sataloff RT, Carey TE. *Laryngoscope* 2009;119:924–932. [PubMed: 19319905]
10. Kouji H, Inazu M, Yamada T, Tajima H, Aoki T, Matsumiya T. *Arch Biochem Biophys* 2009;483:90–98. [PubMed: 19135976]
11. Lam-Yuk-Tseung S, Gros P. *Biochemistry* 2006;45:2294–2301. [PubMed: 16475818]
12. Machova E, O'Regan S, Newcombe J, Meunier FM, Prentice J, Dove R, Lisa V, Dolezal V. *J Neurochem* 2009;110:1297–1309. [PubMed: 19519661]
13. Meunier FM, O'Regan S. *Neuroreport* 2002;13:1617–1620. [PubMed: 12352613]
14. Michel V, Bakovic M. *FASEB J* 2009;23:2749–2758. [PubMed: 19357133]
15. Michel V, Yuan Z, Ramsbair S, Bakovic M. *Exp Biol Med* (Maywood) 2006;231:490–504. [PubMed: 16636297]
16. Nair TS, Kozma KE, Hoefling NL, Kommareddi PK, Ueda Y, Gong TW, Lomax MI, Lansford CD, Telian SA, Satar B, Arts HA, El-Kashlan HK, Berryhill WE, Raphael Y, Carey TE. *J Neurosci* 2004;24:1772–1779. [PubMed: 14973250]
17. Nair TS, Prieskorn DM, Miller JM, Dolan DF, Raphael Y, Carey TE. *Hear Res* 1999;129:50–60. [PubMed: 10190751]
18. Nair TS, Prieskorn DM, Miller JM, Mori A, Gray J, Carey TE. *Hear Res* 1997;107:93–101. [PubMed: 9165350]
19. Nair TS, Raphael Y, Dolan DF, Parrett TJ, Perlman LS, Brahmabhatt VR, Wang Y, Hou X, Ganjei G, Nuttall AL, et al. *Hear Res* 1995;83:101–113. [PubMed: 7607976]
20. Nakamura T, Fujiwara R, Ishiguro N, Oyabu M, Nakanishi T, Shirasaka Y, Maeda T, Tamai I. *Biol Pharm Bull* 2010;33:691–696. [PubMed: 20410607]
21. O'Regan S, Meunier FM. *Neurochem Res* 2003;28:551–555. [PubMed: 12675144]
22. O'Regan S, Traiffort E, Ruat M, Cha N, Compaore D, Meunier FM. *Proc Natl Acad Sci USA* 2000;97:1835–1840. [PubMed: 10677542]
23. Traiffort E, Ruat M, O'Regan S, Meunier FM. *J Neurochem* 2005;92:1116–1125. [PubMed: 15715662]
24. Uchida Y, Inazu M, Takeda H, Yamada T, Tajima H, Matsumiya T. *J Pharmacol Sci* 2009;109:102–109. [PubMed: 19122366]
25. Wang HT, Chang JW, Guo Z, Li BG. *Int J Mol Med* 2007;20:501–510. [PubMed: 17786280]
26. Yabuki M, Inazu M, Yamada T, Tajima H, Matsumiya T. *Arch Biochem Biophys* 2009;485:88–96. [PubMed: 19236841]

27. Yuan Z, Tie A, Tarnopolsky M, Bakovic M. *Physiol Genomics* 2006;26(1):76–90. [PubMed: 16609143]
28. Zajic G, Nair TS, Ptak M, Van Waes C, Altschuler RA, Schacht J, Carey TE. *Hear Res* 1991;52:59–71. [PubMed: 2061214]
29. Zeitoun H, Beckman JG, Arts HA, Lansford CD, Lee DS, El-Kashlan HK, Telian SA, Denny DE, Ramakrishnan A, Nair TS, Disher MJ, Sataloff RT, Fisher SG, Carey TE. *Arch Otolaryngol Head Neck Surg* 2005;131:665–672. [PubMed: 16103296]

Abbreviations

CTL2	Choline transporter-like, protein 2
SLC44A2	Solute carrier protein 44 A2
CTL2 P1	Promoter 1 isoform of CTL2
CTL2 P2	Promoter 2 isoform of CTL2
HEK293	Human embryonic kidney cells 293
KHRI-3	A monoclonal antibody named for Kresge Hearing Research Institute
AHL	Autoimmune hearing loss
TRALI	Transfusion related acute lung injury
5'RACE	Five-prime rapid amplification of complementary DNA ends
C-ter AS	Carboxy-terminal alternate splice form
pIRES hr-GFP1a vector	This vector was purchased from Invitrogen; it has an internal ribosomal entry site (IRES) after the cloning site followed by green fluorescent protein sequence which will be present in the mRNA of the cloned CTL2 sequences. IRES elements are recognized by ribosomes allowing simultaneous translation of CTL2 and GFP as separate proteins

Fig. 1.

The gene structures of three expressed human CTL2 isoforms are shown as *line diagrams*. The genomic organization of the human CTL2-P1 and CTL2-P2 isoforms spans 42 kb and contains 24 possible exons (*white rectangular boxes*) of which 22 are expressed in each of these full length isoforms. The predicted promoter sequences P1 and P2 are shown as *square black boxes*. Spliced out exons are shown as *gray rectangles*. In the P1 isoform exon 1a, which encodes 10 amino acids, is spliced to exon 2 (**a**) and in the P2 isoform exon 1b, which encodes 12 amino acids, is spliced to exon 2 (**b**); both lead to a full length transcribed sequence. A third human C-terminal alternate splice isoform (CTL2 C-ter AS) was found in the NCBI database (**c**). This isoform contains an alternate splice site in intron 21 reading into exon 22 [23]. This C-terminal alternate splice form was confirmed in cDNA from a human squamous carcinoma cell line

Fig. 2.

Semi quantitative RT-PCR of CTL2-P1 and CTL2-P2 isoforms in murine tissues and human inner ear. Primers specific for the murine and human P1 and P2 isoforms were used to amplify cDNA. Representative results from one experiment of four is shown. Beta Actin is used as a loading control and the P1 and P2 primer sets serve as controls for one another in each tissue. The PCR amplification signal for CTL2-P2 is greater than CTL2-P1 in tongue, heart, colon, lung, kidney and cochlea in the mouse. The weak signal from the cochlea in panel **a** reflects the tiny amount of RNA available for this tissue. In panel **b** an example of CTL2-P1 and P2 amplification from inner ear cDNA is shown from an experiment with more PCR cycles (40 vs. 30). In contrast to other tissues, P1 amplifies better in liver and spleen suggesting that P1 is more abundant or even exclusively expressed in these two tissues. In panel **c**, RT-PCR shows that CTL2-P1 is abundantly expressed in human inner ear vestibular mRNA from 2 patients (patient 2 and patient 11). CTL2-P2 is poorly expressed relative to CTL2-P1 in human vestibular tissue and no P2 amplification signal could be detected even at 40 cycles

Fig. 3.

Immunoprecipitation, deglycosylation and western blots of CTL2 protein expression in guinea pig and murine tissues. **a** Guinea pig tissues, **b–d** mouse tissues. The relative abundance and varying glycosylation patterns of CTL2 in various tissues can be appreciated. Tissue extracts were incubated with rabbit anti-CTL2 antibody, and precipitated with protein-A Sepharose beads. After washing the beads were washed and divided into two parts one of which was incubated with PNGase F, an enzyme that removes N-linked carbohydrates, the other with mock digestion in buffer, after which the samples were subjected to SDS-PAGE and western blotted with anti-CTL2. The *upper bands* in lanes marked as negative for PNGase F treatment are the glycosylated forms of the CTL2 proteins. Note the variation in extent of glycosylation in various tissues. **c** Tongue and liver are shown with higher protein loading to show the glycosylated forms. In all cases, PNGase F treatment reduced the size of the CTL2 band to 62 kDa. **d** Using inner ear as a positive control, this figure demonstrates that CTL2 is highly glycosylated (~80 kDa) and is abundantly expressed in the spleen

Fig. 4.

Immunofluorescence staining of guinea pig and mouse tissues with rabbit anti CTL2-NT antibody. **a-f** Guinea pig tissues. **g-l** Murine tissues. **a** CTL2 is prominently expressed on differentiating oral keratinocytes (*arrows*). **b** Anti-CTL2 specific membrane binding is completely inhibited by preincubation with the immunizing peptide. **c** Strong CTL2 expression staining in the nerve sheath and staining of Schwann cell membrane and cytoplasm of individual myelinated nerve cells of a large motor nerve in the mouse tongue (*arrows*). Anti CTL2 stains peripheral nerves (*arrow*) within tongue muscle; *inset* shows high power view of the nerve, *long arrow*, perineurium, *short arrow*-nerve cytoplasm staining. **d** Anti-CTL2 binding to nerve is inhibited by the immunizing peptide. **e** Tongue epithelium incubated with only second antibody. **f** Tongue musculature incubated with only secondary antibody. Panels **g** shows CTL2 staining of a mouse glomerulus with expression in the lining of Bowman's capsule (*arrowhead*) and the mesangial cells surrounding the vessels (*arrow*) within the glomerulus. Panel **i** Inhibition of specific CTL2 binding to kidney by the immunizing peptide. **h** CTL2 staining of murine lung; membrane and granular cytoplasmic CTL2 staining of the cells of the lung parenchyma. **j** Inhibition of specific CTL2 binding to lung tissue by the immunizing peptide. **k-l** CTL2 staining of murine spleen showing mostly membrane and some cytoplasmic staining; **k** 100 $\times$, **l** 40 $\times$. **m** Murine Spleen with second antibody only

Fig. 5.

Expression of CTL2-P1 and CTL2-P2 in HEK 293 cells as assessed by immunoprecipitation and western blotting. **a** HEK293 cells transfected with rHuCTL2-P1 or P2, or HEK293 cells transfected with the empty vector control were lysed and the extracts were immunoprecipitated with rabbit anti-CTL2. The immunoprecipitates were western blotted with anti-CTL2 (*lanes 1, 2, 3*) or anti flag antibody (*lane 4*). CTL2-P1 migrates as a broad band of 80 kDa. CTL2-P2 migrates more slowly because it contains the 3× flag tag and it also migrates as two prominent bands of 77 kDa and a second of 88 kDa. Endogenous CTL2 cannot be detected by immunoprecipitation and western blotting in the cells transfected with the empty vector (*lane 3*). **b** Treatment of CTL2-P1 with PNGase F removes the N-glycosylation resulting in a core protein of 62 kDa. **c** Treatment of CTL2-P2 3X-Flag with PNGase F reduces both the 75 and 90 kDa bands to a core protein of 70 kDa (CTL2 core of 62 kDa plus the 3× flag tag 8 kDa). The more slowly migrating glycosylated band for each isoform has a relative mass that is 15–18 kDa greater than the core protein

Fig. 6. Immunofluorescence staining of exogenous CTL2 expression in transfected HEK293 cells. Flag tagged CTL2-P2 expressed from a vector containing IRES-GFP was detected in transfected HEK293 cells using anti-flag antibody. Panel **a** CTL2 expression (*red staining*) is distributed over the cell membrane especially at sites of cell–cell contact. Punctate cytoplasmic staining is also apparent (*Green staining* shows GFP expressing cells). Panels **b, c** show small cell clusters in which only the red channel, anti-flag (panel **b**) or anti-CTL2 (panel **c**) is shown. Note the presence of both membrane CTL2 staining and multiple punctate particles containing CTL2 within the cytoplasm. Both antibodies show similar distribution of expressed protein. Panels **d, e** are HEK293 cells transfected with empty GFP vector (*green* is GFP in **d** and **e**). Panel **d** Cells were incubated with anti-flag (*red*). Panel **e** HEK293 cells incubated with anti-CTL2 (*red*). (Color figure online)

Fig. 7. Choline transport activity of CTL2 compared to CTL1. **a** Human CTL1a and CTL1b (reported to be choline transporters) and **b** Human CTL2-P1 and CTL2-P2 were expressed in *X. laevis* oocytes by microinjection of respective cRNAs. Water-injected oocytes served as controls. Uptake of [³H]-choline [5] in oocytes was measured for 60 min. Data represent means $\pm$ SE ($n = 8-10$)