Epitope presentation to detect human autoantibodies to CTL2/SLC44A2

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Foreword

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Abstract

Choline Transporter-Like Protein 2 (CTL2) is a multi-transmembrane protein that is highly expressed in the supporting cells of the cochlea and vestibular system. It has been implicated as a possible target antigen in autoimmune hearing loss (AHL). Recent studies demonstrated that a significant proportion of suspected AHL patients have antibodies that recognize recombinant human CTL2 (rHuCTL2) in Western blots. To further elucidate the mechanisms behind autoantibody mediated hearing loss and the role of CTL2 in this disorder, an assay is being developed to present the rHuCTL2 molecule in three different topologies to maximize detection of antibodies in patients and control subjects and to begin to map the important immunogenic and functionally important epitopes of CTL2 that are recognized by patient sera. Deglycosylated his-tagged rHuCTL2 expressed in Sf9 insect cells is purified using Ni column chromatography for use as the substrate for this assay. The purified protein is used in a sandwich ELISA, in which the protein is anchored by polyclonal antibodies directed against the N-terminus, C-terminus and Third Outer Loop of CTL2. It was hypothesized that these antibodies would present the protein in multiple confirmations with different epitopes available for binding. In preliminary studies a biotinylated antibody directed against the N-terminus of CTL2 was used to assess the availability of the N-terminal epitope after anchoring with the three antibodies. We observed a high level of background binding of the biotinylated antibody that partially obscured evaluations of epitope presentation. The antibody to the N-terminus bound well to protein anchored by the Third Outer Loop antibody, and surprisingly, to protein anchored by N-terminus antibody. The biotinylated N-terminus antibody bound less well to protein anchored by the C-terminal antibody. N-terminal antibody binding to protein anchored by each of the antibodies was partially inhibited by the N-terminal peptide, supporting a specific
interaction (and a non-specific binding that was not inhibited by the peptide). The observed results indicate that there are unbound sites in the wells that must be blocked to increase sensitivity and specificity. The result is also consistent with CTL2 protein dimers or multimers being anchored by one epitope but leaving the same epitope on a dimerized molecule available for presentation to the biotinylated test antibody. Alternative approaches to rHuCTL2 purification, antibody purification and epitope presentation need to be addressed to increase the sensitivity and specificity of this assay.
Introduction

Autoimmune Disorders

Autoimmune disorders are a set of diverse and poorly understood diseases characterized by loss of immune self-tolerance leading to an organ specific or systemic disease. Classification of a disorder as autoimmune in origin requires that more than one of the following criteria are met: presence of circulating auto-antibodies or autoimmune cells, a defined autoantigen, the ability to passively transfer the disease using autoantibodies or autoimmune cells in experimental animals, the production of disease by immunization with the autoantigen, and the production of autoantibodies or autoreactive cell mediated immunity after immunization with an autoantigen[1]

The pathology of autoimmune disorders can be best understood by investigating the mechanisms involved in the maintenance of self-tolerance in the immune system. There are two main theories detailing the mechanism by which the immune system maintains tolerance. Burnet’s clonal deletion theory states that autoreactive B and T cells are deleted in utero upon exposure to self antigens. According to this theory, only those immunized with a sequestered self antigen or those with an autoimmune disease will have autoantibodies or autoreactive cells. In contrast, Jerne’s theory suggests that both autoreactive cells and autoantibodies are present within the body and regulate one another. For example, anti-antibodies can recognize, bind to and inhibit autoreactive T cells or autoantibodies. Both these theories are complementary as antibodies against autoantigens are found in many normal and autoimmune individuals and clonal deletion is the primary method by which the body maintains tolerance against autoreactive T cells during development[2]. The process of clonal deletion, however, is not comprehensive.
Self reactive B and T cells exist in normal individuals. These B and T cells persist, most frequently, because the cells recognize cryptic epitopes that are present in low concentrations or have low affinity to the MHC complex [3].

In autoimmune disorders, physiological and environmental factors can be responsible for the breakdown of tolerance, and result in the activation of self reactive B or T cells and the production of autoantibodies. Environmental factors such as a bacterial or viral infection can lead to an autoimmune response when these foreign antigens mimic self antigens. This is the case with rheumatic fever in which infection with *Streptococcus pyogenes* can result in the production of humoral and cell-mediated responses against cardiac proteins, causing damage to heart valves that can result in heart failure[4]

There is also evidence to indicate that defects in physiological processes like protein folding, processing or clearance of cellular debris in apoptosis can lead to autoimmunity [5]. Defects in the tyrosine kinase protein MER in mice, lead to an impairment in the clearance of apoptotic cells by macrophages, this can result in production of autoantibodies, particularly anti-DNA antibodies, [6]. Apoptosis also plays an important role in Hashimoto’s thyroiditis. Fas mediated apoptosis of thyrocytes compromises the sequestration of normally immune privileged antigens in the thyroid and promotes the development of autoantibodies and the resulting hypothyroid disorder [7].

Deficiencies in the clearance of apoptotic cells and subsequent autoimmunity can also be attributed to defects in the complement system. The complement system is a part of the innate immune system that consists of serum components like C1q, C3, C4 and groups of proteins called pentraxins and collectins. These proteins are responsible for opsonization of invading
pathogens for engulfment or processing by macrophages and dendritic cells. But, the complement system is also vital for the clearance of apoptotic cells[8]. Complement proteins, like C1q, can recognize the phosphatidyl serine on the cell membrane, an early signal of the loss of membrane lipid asymmetry and apoptosis[9]. Binding of C1q to an apoptotic cell initiates the complement system to signal for opsonization of the apoptotic cell. Defects in this system can lead to necrosis, inadequate clearance of cellular debris and result in autoimmunity[8]. Additionally, signals released from dying or injured cells, like uric acid, have been shown to signal an inflammatory response that can trigger autoimmunity[10]. Systemic autoimmune disorders, like systemic lupus erythematosus, have been associated with defects in the complement system using knock-out mice lacking components of the complement system such as C1q, C4 and serum amyloid protein[8].

In addition to defects of the complement system, other defects of the immune system that contribute to autoimmunity can be categorized as being antibody mediated or T cell mediated. Well characterized antibody mediated disorders include pemphigus vulgaris and myasthenia gravis. These disorders are organ specific because the antibodies recognize organ specific antigens. In contrast, systemic antibody mediated disorders often involve antibodies that recognize free molecules and cause damage via the formation of immune complexes that can activate the complement system [11]. Pemphigus vulgaris is a dermatological disorder characterized by mucosal and skin lesions that result from the loss of cell adhesion in the epidermis. Serum from patients with pemphigus vulgaris contains IgG antibodies that recognize and bind to desmosomes, specifically desmoglein 3, and interfere with interactions between suprabasal epidermal cells and the basal layer. Many studies have revealed that the titers of desmoglein antibodies vary with the severity of the disease and treatment requires suppression of
the immune system[12] Myasthenia Gravis is a disease that causes muscle fatigue and weakness. Like pemphigus vulgaris, it is characterized by the production of disease-causing antibodies. These antibodies are directed against the acetylcholine receptors of the neuromuscular junction, although pathogenic antibodies can also be produced against the muscle tyrosine kinase protein, MuSK. The antibodies produced in myasthenia gravis are both diagnostic and pathogenic. Treatment for myasthenia gravis involves immune suppression or thymectomy in younger patients[13]. In addition to pathologic antibodies, the disease is also characterized by an inflammatory environment in the thymus. This is frequently a result of impaired T-regulatory CD4+CD25+ cells that are unable to suppress peripheral B and T cells. This can result in chronically activated lymphocytes that are postulated to be significant in maintaining an inflammatory environment, thymic hyperplasia and predisposition to myasthenia gravis [14], [15].

T cell mediated autoimmunity is an important aspect of disorders like rheumatoid arthritis, multiple sclerosis and type I diabetes. Many T cell subtypes can be responsible for T cell mediated autoimmunity. \( T_{h1} \) CD4+ helper T cells, which control cell mediated immunity are pro-inflammatory and are suspected of playing a role in autoimmune disease while \( T_{h2} \) cells are not suspected to be pathogenic in autoimmunity[16, 17]. In diseases like diabetes, researchers have shown that transfer of cultured helper T-cells expressing a diabetogenic T-cell receptor into neonatal non-obese diabetic mice resulted in diabetes when \( T_{h1} \) cells were transferred but not when \( T_{h2} \) cells were transferred[18].

CD4+CD25+ regulatory T cells play an important role in maintaining self tolerance by regulating T cells that recognize self antigens. Defects or deficiencies in these regulatory T cells
have been recognized as an aspect of T cell mediated autoimmunity[16, 19]. Deficiencies in regulatory T cells, in their quantity or in their regulatory capacity have been noted in individuals with autoimmune diseases like multiple sclerosis and type 1 diabetes. Mutations in Foxp3, a transcription factor expressed in regulatory T cells are associated with the fatal autoimmune disorder IPEX (Immunedysregulation, polyendocrinopathy, enteropathy, X-linked syndrome). Foxp3 is expressed in the presence of the cytokine TGF-β and is responsible for maintaining regulatory T-cells in the induced state and a disorder like IPEX demonstrates the significance of activated regulatory T cells in maintaining self tolerance [19, 20].

Another T cell subtype that has been implicated to play a role in autoimmune disease is the T_{H}17 cell type. T_{H}17 cells are a type of helper T cells that produce the cytokine IL-17. They are in an antagonistic relationship with regulatory T cells and this relationship is mediated by the cytokine IL-6. T_{H}17 cells are induced in the presence of cytokines IL-6 and TGF-β while regulatory T cells are inhibited by IL-6 but activated by TGF- β alone. Mouse models of autoimmune disease have demonstrated that T_{H}17 cells are more potent mediators of the inflammatory response than T_{H}1 cells and are more capable of transferring experimental autoimmune encephalomyelitis from affected mice to naïve wild type mice[20].

Autoimmune Hearing Loss

Autoimmune diseases are the product of various genetic and environmental factors and are mediated by many diverse mechanisms. Both T cell and antibody mediated mechanisms as well as other mechanisms may be involved in autoimmune hearing loss.

Autoimmune hearing loss, a progressive and often bilateral form of sensorineural hearing loss...
hearing loss, is not as well characterized as other autoimmune disorders. The disorder which is most likely heterogeneous in etiology was first described by McCabe in 1979 when he observed 18 patients with unique presentations of hearing loss that all responded to autoimmune disease therapy involving treatment with cortisone and cyclophosphamide [21]. Autoimmune hearing loss was later replicated in experimental animal models. Guinea pigs immunized with bovine inner ear extract developed hearing loss, lesions in the inner ear and were found to have circulating titers of antibody to inner ear antigen [22]. In another experiment, mice and guinea pigs immunized with chick and guinea pig inner ear tissues developed temporary hearing loss and antibodies to stereocilia [23].

Antibody as a mechanism of hearing loss is supported by the frequent identification of antibodies reactive with a 68 kDa inner ear protein in patients with progressive sensorineural hearing loss. Harris et al. demonstrated that 35% of patients with progressive sensorineural hearing loss had antibodies against a 68 kDa antigen from bovine inner ear extracts compared to only 7% of normal individuals [24]. Moscicki et al. demonstrated that 89% of patients with progressive, bilateral sensorineural hearing loss had antibodies that detected a 68 kDa antigen in bovine inner ear extract. They also determined that these antibody-positive patients were more responsive to corticosteroid treatment than antibody-negative patients [25].

Subsequently, the 68 kDa antigen recognized by patient sera was prematurely identified to be the ubiquitous Heat Shock Protein 70 (HSP70) [26]. However, HSP70 antibodies do not appear to be unique to patients with progressive bilateral sensorineural hearing loss. Yeom et al. tested 20 patients with rapidly progressive sensorineural hearing loss and 20 normal donors and found that 16 patients and 17 controls had antibodies that recognized HSP70,
demonstrating that the presence of HSP70 antibodies in human sera is not diagnostically significant[27]. Animal models have shown that anti-HSP70 antibodies are not pathogenic. Trune et al. inoculated mice with bovine HSP70 and observed elevated levels of HSP70 antibodies but no corresponding hearing loss suggesting that elevated levels of HSP70 antibodies in sensorineural hearing loss patients are unlikely to play a significant role in the development of antibody mediated hearing loss [28].

Alternatively, other proposed antigenic targets of autoimmune hearing loss include cochlin and Choline Transporter-Like Protein 2 (CTL2).

*Cochlin*

Cochlin, a protein that is highly expressed in the extracellular matrix of fibrocytes of the spiral limbus and spiral ligament of the inner ear, is a product of the COCH gene. Mutations in the COCH gene have been linked to DFNA9, an autosomal dominant, non-syndromic form of sensorineural hearing loss. The mechanism for cochlin dependent autoimmune hearing loss is suspected to be T cell mediated. Mice immunized with cochlin peptides exhibit significant hearing loss and selectively activated CD4+ T cells[29]. Patients with sensorineural hearing loss also had higher frequencies of T cells producing IFN-γ in response to recombinant human cochlin[30]. Mutations of the COCH gene in DFNA9 suggest an important function for cochlin in hearing and its predominant localization within the inner ear in adult humans strengthens the evidence for cochlin’s role as a target antigen in autoimmune hearing loss. Cochlin may also interact with CTL2. Cochlin was found to co-immunoprecipitate with CTL2 in inner ear extracts and cochlin and CTL2 are closely distributed in certain regions of the inner ear suggesting that
these proteins may form functional complexes[31]. However, there have been very few studies to support autoimmune targeting of cochlin in autoimmune hearing loss and this is still an important research question. In our laboratory 27 to 40 percent of sera from suspected autoimmune hearing loss patients have circulating antibodies that bind to two proteins in guinea pig inner ear extracts that migrate with the same mass (44 and 40 kDa) as two of the major cochlin polypeptides. However, those same individuals rarely had antibody that binds to a protein with the 60 kDa mass of the full length cochlin polypeptide. Further studies of these observations will be necessary before any clinical correlates can be drawn.

**CTL2**

Choline Transporter-Like Protein 2 (CTL2), also referred to as Solute Carrier Protein 44A2, is a multi-transmembrane protein that is highly expressed in the supporting cells of the cochlea, vestibular system, in the cells of the scala media and in proximity with cochlin expression[31, 32].

The CTL2 protein was identified as a possible autoantigen in antibody mediated hearing loss by a murine monoclonal antibody. This monoclonal antibody, KHRI-3, was developed in our laboratory from mice immunized with cells isolated from the inner ear. KHRI-3 binds to supporting cells in guinea pig cochlea and to a 68, 72 kDa doublet in immunoblots of guinea pig cochlea. Mice carrying the KHRI-3 hybridoma, that developed high circulating antibody titers developed high frequency hearing loss as compared to mice with control hybridomas of which only one in seven developed hearing loss [33]. Further, intra-cochlear infusions of KHRI-3 antibody into guinea pigs led to 25-55 dB hearing loss while animals infused with control antibody showed no change from baseline. The infused KHRI-3 bound to
the organ of Corti in six of the nine animals tested with four of these animals developing hearing
loss. There was a high frequency of hair cell loss in these infused ears compared to few or no
hair cells losses in mice infused with control saline or immunoglobulin [33]. KHRI-3 localized to
regions of scar formation in the supporting cells suggesting that its epitope plays a role in
facilitating scar formation following damage to the inner ear [34].

Studies of sera from patients suspected of autoimmune hearing loss further implicated
a role for the antigen recognized by KHRI-3 in mediating antibody induced autoimmune hearing
loss. Approximately ½ of patients suspected of autoimmune hearing loss had antibodies that
bound to a 68, 72 kDa protein in immunoblots of guinea pig extract. KHRI-3 antibody binds to
inner ear supporting cells in a wine-goblet staining pattern, that corresponds to the phalangeal
processes of the outer pillar cells. The patient sera stained supporting cells of the guinea pig
organ of Corti in the same wine-goblet staining pattern as KHRI-3 and recognized the guinea pig
epitope when it was immunoprecipitated by KHRI-3 [35].

Additional support for the hypothesis that CTL2 is a target antigen in antibody
mediated hearing loss came from a study of sixty-three patients with idiopathic hearing loss
suggestive of autoimmune sensorineural hearing loss. The study determined that patients with
sera that recognized a 68, 72 kDa protein in immunoblots of guinea pig cochlea or had a “wine-
glass” immunofluorescent staining pattern on a guinea pig cochlea were more likely to improve
their hearing with corticosteroid therapy. This was especially true of patients with IF-positive
sera who were three times more likely to experience improved hearing after corticosteroid
therapy than patients with sera that was IF-negative [36].

The inner ear supporting cell antigen recognized by KHRI-3 was determined to be the
guinea pig homologue of human CTL2. There is a high sequence homology between guinea pig and human CTL2 cDNA and approximately 90% homology between the amino acid sequences. The conserved sequence and expression pattern of CLT2 across species implies an important function for the protein in the inner ear. CTL2 is a member of the solute carrier family, and although its function is unknown, the presence of a KOG1362 homology domain suggests a functional role for CTL2 as a lipid transporter [32]. It is also of interest that the locus of the gene for CTL2 maps to 19p13.2, a region that has been implicated in DFNB68, an autosomal recessive non-syndromic type of hearing loss [37].

Although autoimmune hearing loss is almost certainly a heterogeneous disorder, CTL2 is suspected to be one of the likely targets of autoimmune hearing loss. Preliminary evidence suggests that a significant portion of patients suspected of having autoimmune hearing loss have antibodies that recognize the recombinant form of human CTL2 expressed in Sf9 insect cells. A recent study of sera from 12 patients previously tested for serum reactivity with a 68, 72 kDa protein in immunoblots of guinea pig cochlea or demonstrating a positive IF staining pattern was carried out on rHuCTL2. Sixty-percent of the patients with antibody to the inner ear antigens detected by the forgoing tests, also had antibody that bound strongly to rHuCTL2 expressed in Sf9 insect cells. Sera from normal individuals without any suspected hearing loss predominantly did not recognize rHuCTL2 with only 20% of the normal sera exhibiting barely detectable binding with rHuCTL2[38].

For the present study, the open reading frame of CTL2 isoform P1, the more highly expressed isoform in the inner ear, was cloned into baculovirus and expressed in Sf9 insect cells. Recombinant human CTL2, expressed in Sf9 insect cells, containing an N-terminal hexa-
histidine tag, was purified with Ni column chromatography to test the hypothesis that there are specific target epitopes on rHuCTL2 that are recognized by autoantibodies in patient sera. A sandwich ELISA was to function as the assay to recognize autoantibodies in patient sera. A strategy was devised to anchor the recombinant protein using polyclonal antibodies directed to each of the three most antigenic regions on the protein. These antibodies were directed against the N-terminal (NT), C-terminal (CT) and Third Outer Loop (TOL) regions of the protein and enabled the presentation of rHuCTL2 in multiple conformations with different epitopes available for recognition by sera (Figures 1 and 3).

Such an assay, which exposes multiple epitopes potentially recognized by autoantibodies in patient sera, could provide clues to the etiology of the autoantibody response if the dominant epitope is shared by an infectious agent. This information could also provide a basis for the development of an accurate and rapid test to identify patients with hearing loss of unknown etiology, who may have an autoimmune basis for their disease. Such an assay would also be relevant in monitoring treatment of these individuals. They could be immediately treated with immunosuppressive therapy and the disappearance of antibody from the serum could be used to determine the time for tapering off therapy. A similar strategy is being successfully implemented in the treatment of the antibody mediated autoimmune disorder pemphigus vulgaris [12]. Identification of the significant epitopes in CTL2 could also lead to new treatment strategies to block autoantibodies from reaching their targets in vivo.

Methods

Sf9 Cell Culture
Low passage ATCC Sf9 insect cells were cultured in suspension at 26°C at a density of 2x10^6 cells/mL in Invitrogen Sf9-900 II media supplemented with 1% heat inactivated fetal bovine serum and 10 µg/mL gentamicin (Invitrogen, 15710072). Sf9 cells in the logarithmic growth phase were infected with a baculoviral construct containing the P1 isoform of human CTL2 at a multiplicity of infection (MOI) of 0.1. The cells were cultured with or without tunicamycin (Sigma, T1152) and harvested at various time points after infection.

Harvested cells were treated with lysis buffer (Phosphate Buffered Saline and 1% NP-40 supplemented with protease inhibitors) for fifteen minutes at 4°C before the lysed cells were pelleted by centrifugation at 2000 rpm for ten minutes. Cell supernatant was assayed for protein concentration using the Bradford method and frozen at -80°C.

*Ni Column Chromatography*

Thirty milligrams of Sf9 whole cell lysate was applied to a 4 mL volume column of ProBond Nickel-chelating resin (Invitrogen, R80101). The lysate was incubated with the Ni column beads overnight at 4°C and allowed to flow through the column after incubation (*Figure 2: Step 1*). Flow through was collected and stored at 4°C for further analysis (*Figure 2: Step 2*). The column was washed with 15 to 20 volumes of wash buffer (0.5 M NaCl, 50 mM NaH₂PO₄, 20 mM imidazole, pH 8.0) to remove nonspecific proteins and the first four fractions were collected and stored with the flow through for further analysis (*Figure 2: Step 3*). The recombinant CTL2 protein still bound to the Ni column was eluted using elution buffer (0.46 M NaCl, 46mM Na H₂PO₄, 500 mM imidazole, pH 8.0) and saved for further analysis (*Figure 2: Step 4*). The protein concentrations of all fractions including the flow through, washes and elution were determined by Bradford assay and the samples were analyzed by Western blotting.
to determine the adequacy of the isolation procedure.

Eluted protein was dialyzed into phosphate buffered saline containing 1mM β-mercaptoethanol and stored at -80°C in 500 μl aliquots. The Ni column purified protein was assessed for concentration and purity using Coomassie blue staining, immunoprecipitation and Western blotting.

*Immunoprecipitation and Western Blotting*

Samples were immunoprecipitated with anti-CTL2 antibodies targeted against the C-terminus (CT), N-terminus (NT) or Third Outer Loop (TOL) regions. Samples were also loaded directly onto a 3-10% gradient SDS-PAGE gel after mixing with loading buffer (Tris-Cl, pH 6.8, 2 % SDS, 10 % glycerol, 0.005 % bromophenol blue). Samples were electrophoresed for 2 hours at 125 mV and transferred to a nitrocellulose membrane overnight at 25 mV in transfer buffer (20 % methanol, 150 mM glycine, 20 mM Tris buffer). Nitrocellulose blots were blocked with 5 % milk in PBS-Tween and incubated for 1.5 hours with rabbit polyclonal primary antibodies against the CT, NT or TOL regions of CTL2 (1:500, 1:1000 and 1:500 of 1 mg/mL antibody solution, respectively) followed by incubation with secondary antibody (1:2500 horseradish peroxidase conjugated AffiniPure mouse anti-rabbit IgG, Jackson ImmunoResearch). Primary and secondary antibodies were diluted in 5% PBS-T milk. Detection was performed with enhanced chemiluminescence Western blotting reagent (GE Healthcare, RPN 2109)). Blots were then exposed to Hyblot photofilm and developed using a Konica-Minolta Medical Film Processor.

*Sandwich ELISA: Antigen Presentation Strategy*
Recombinant human CTL2, partially purified on a Ni column, was anchored to NUNC 96-well ELISA plates using specific polyclonal antibodies targeting three antigenic regions of the CTL2 protein (Figure 3). The antibodies directed to the N-terminal, C-terminal and Third-Outer Loop regions were designed against highly antigenic regions of CTL2 to facilitate the presentation of rHuCTL2 in different conformations to the probe antibody or patient sera.

*Enzyme Linked Immunoabsorbant Assay*

Flat bottom NUNC 96-well plates were coated with 1 µg/well of an AntiCTL2 antibody by diluting the antibody in 50 mM sodium bicarbonate buffer at a pH of 9.6 and incubated overnight at 4°C. After 12-18 hours of incubation, the plates were decanted and washed three times with 300-400 µL of TBS-T (pH 7.5). Coated wells were blocked with 350 µL of 5% TBS-T milk for one hour and subsequently incubated with 2-4 µg/well of rHuCTL2 protein diluted in 5% TBS-T milk. After one hour, the rHuCTL2 solution was decanted and the plates were washed with TBS-T three times. Wells containing rHuCTL2 were probed with patient sera or the polyclonal control antibody, biotinylated anti-CTL2 NT. Following 1.5 hours of incubation with the primary antibody the plates were washed three times with TBS-T and probed with either alkaline phosphatase conjugated rabbit anti-human antibody (Jackson ImmunoResearch) or streptavidin tagged with alkaline phosphatase (Jackson ImmunoResearch). After 1.5 hours incubation with the secondary antibody or streptavidin, the plates were washed four times with TBS-T and 50 µL of a 2 mg/mL solution of PNPP was added for visualization. ELISA plates were developed in the dark at 37°C for up to 30 minutes and the reaction was stopped by the addition of 50 µL of 0.5 N NaOH. The plates were read by measuring absorbance at 405 nm on a Bio-Tek instruments plate reader.
Results

Recombinant human CTL2 was expressed in Sf9 insect cells, characterized and used in epitope mapping experiments.

Expression of rHuCTL2 in Sf9 insect cells

CTL2 is expressed as a protein doublet of 68 and 72 kDa in guinea pig and human inner ear. The core protein, when deglycosylated, migrates at 62 kDa in immunoblots[32]. In Sf9 insect cells, however, rHuCTL2 is expressed as a triplet of 62, 66 and 70 kDa[38]. Glycosylation in Sf9 insect cells is different from mammalian cells. N-linked glycosylation occurs in insect cells at the Asn-X-Ser/Thr tripeptide, just as in mammalian cells, but recombinant proteins are often unglycosylated or underglycosylated with short oligosaccharide chains of a few mannose or fucose residues[39]. Because glycosylation of rHuCTL2 in the Sf9 insect cell system is different from mammalian cells and because patient sera have been shown to recognize the core deglycosylated protein in Western blots, the rHuCTL2 was deglycosylated for use in ELISAs.

Infection at 0.1 MOI with baculovirus containing rHuCTL2 typically yields optimal rHuCTL2 expression in Sf9 insect cells 48 hours post infection in approximately one third of all cells[38]. In Sf9 insect cells treated with 10 µg/mL of tunicamycin to block glycosylation of N-linked modifications, optimal expression of rHuCTL2 was delayed from 48 hours to 72 hours (Figure 4). Three doses of tunicamycin were tested to determine the minimum dosage required for maximal inhibition of glycosylation of the protein. The three tested treatments of 2.5, 5 and 10 µg/mL resulted in fairly complete blockade of glycosylation of rHuCTL2 at 72 hours post
infection (Figure 4).

**Purification of rHuCTL2 in Sf9 insect cells**

Nickel column purification of whole cell lysates from tunicamycin treated Sf9 insect cells expressing rHuCTL2 yielded partially purified protein (Figure 5). The protein yields ranged from 40 µg/mL to 186 µg/mL after three separate purifications of 30 milligrams of Sf9 lysate on a four milliliter Ni column. The wide variation in protein yields was not a result of variability in expression of rHuCTL2 in Sf9 insect cells but variability in Ni column binding capacity after multiple uses.

Sf9 lysate purified by Ni column chromatography was analyzed by immunoblotting and Coomassie blue staining. Immunoblotting revealed that rHuCTL2 was enriched from Sf9 cell lysate after one passage through the Ni column (Figure 6). Whereas, loading 100 to 200 micrograms of Sf9 lysate onto the SDS-PAGE gel was required to detect rHuCTL2 in the crude material, only one to two micrograms of the eluent protein was required for detection of column purified rHuCTL2. Purity of the Ni column eluent containing rHuCTL2 was assessed with gel electrophoresis and Coomassie blue staining (Figure 5). Coomassie blue staining revealed that although rHuCTL2 was represented in the final eluate from the Ni column, there were many contaminating proteins that were not detected by immunoblotting. However, this partially purified protein was used in ELISA assays despite the presence of impurities because anchor antibodies are expected to further purify the sample.

**Immunoprecipitation of rHuCTL2 expressed in Sf9 insect cells**

Recombinant human CTL2 was immunoprecipitated from 100-200 µg of Sf9 cell
lysate using polyclonal rabbit antibodies targeted against the NT, CT and TOL regions of human CTL2. From previous analysis of guinea pig cochlear lysate, it was known that the NT antibody recognizes guinea pig CTL2 with the greatest efficiency in Western blots. In contrast, TOL and CT antibodies bind to guinea pig CTL2 with lower efficiency and are unable to bind to the glycosylated form of the protein while NT successfully recognizes both the glycosylated and unglycosylated forms. The binding affinity of these rabbit polyclonal antibodies for rHuCTL2 expressed in Sf9 insect cells was unknown. Immunoprecipitation followed by immunoblotting with these three antibodies allowed for a qualitative comparison of the binding efficiency of NT, TOL and CT antibodies for rHuCTL2 in the glycosylated and unglycosylated forms (Figure 7).

Qualitative comparisons of the binding efficiency of NT, TOL and CT antibodies for rHuCTL2 reveal that the binding of these antibodies to the recombinant protein is similar to binding to guinea pig cochlear protein. Of the three antibodies, NT recognizes rHuCTL2 with greater efficiency and at a lower concentration than TOL and CT antibodies. The antibody directed at the N-terminus also recognizes the glycosylated forms of rHuCTL2. The antibody directed against the Third Outer Loop recognizes rHuCTL2 with the least efficacy. It binds weakly to only the core protein, the unglycosylated form of rHuCTL2, and immunoprecipitates relatively less rHuCTL2 when compared to the amount of protein immunoprecipitated by the NT and CT antibodies. The antibody targeted to the C-terminus recognizes the unglycosylated core protein of rHuCTL2 most efficiently but can also recognize the glycosylated forms when the rHuCTL2 is first immunoprecipitated by the NT antibody.

Enzyme Linked Immunoabsorbant Assay (ELISA) using partially purified rHuCTL2

The characterized antibodies, targeting the N-terminal, C-terminal and Third Outer
Loop regions of CTL2, were used in an ELISA to anchor and present rHuCTL2 in multiple confirmations in a strategy designed to assist in mapping the epitopes that are predominantly recognized by antibodies from patient sera. The anchor antibodies were coated at concentrations of 1 µg per well onto a 96 well ELISA plate. After addition of 2 to 4 µg per well of partially purified rHuCTL2 the samples were probed using a biotinylated antibody against the N-terminus of CTL2. The biotinylated antibody was used to test the hypothesis that anchor antibodies would present CTL2 in variable confirmations and that the anchor site would be unavailable for binding by a probe antibody.

Up to 1 µg/well of biotinylated anti-CTL2 NT antibody was added to probe for rHuCTL2. The antibody was detected with the addition of 1 µg/well of streptavidin conjugated with alkaline phosphatase. Peptide blocking was used as a specificity control by incubating the biotinylated antibody for one hour with NT peptide, at concentrations fifty times in excess of the antibody. In an additional control, background binding of the polyclonal biotinylated anti-CTL2 NT antibody to a well coated with anchor antibody alone (i.e. no rHuCTL2) was assessed.

Biotinylated anti-CTL2 antibody targeted against the N-terminus of CTL2 was able to bind to the partially purified protein anchored by antibodies in the ELISA plate (Figure 8). The binding of the biotinylated anti-CTL2 had a high level of background with alkaline phosphatase activity even in the wells in which partially purified rHuCTL2 was not added. However, the addition of peptide specific to the N-terminal anti-CTL2 antibody partially eliminated binding of the antibody to anchored rHuCTL2 protein, suggesting that some of the binding is epitope specific.

The experiment thus far is a proof of principle, but there are many variables that must
be worked out for the assay to move forward. These variables will be reviewed in the discussion.

**Discussion**

Autoimmune diseases can be mediated by many diverse mechanisms. In Autoimmune Hearing Loss, CTL2 is implicated as a possible target antigen for autoantibody mediated autoimmune hearing loss while the inner ear specific antigen cochlin may be responsible for T cell mediated hearing loss.

Patients with antibodies to CTL2 appear to recognize the deglycosylated form of rHuCTL2 expressed in Sf9 insect cells[38]. Yet, we do not have an understanding of the specific epitopes that patients recognize. Determination of the specific epitopes of CTL2 involved in mediating autoimmune hearing loss is significant to evaluating the mechanism by which autoantibody-mediated autoimmune hearing loss may arise and can provide insight into understanding which antibodies are pathogenic and how their binding can block the function of CTL2.

It has been demonstrated that protein processing, apoptosis and variable expression of proteins can make a protein antigenic and break down self-tolerance[5, 6]. Such mechanisms may play an important role in mediating the antigenicity of CTL2. Using epitope mapping to understand whether patient sera recognize the core protein or glycosylations, intracellular, extracellular or transmembrane regions of CTL2 will provide clues into the specific mechanisms involved in the development of autoantibodies against CTL2.

Additionally, epitope mapping of CTL2 may enable the development of interventional therapies to treat autoimmune hearing loss associated with the development of autoantibodies
against CTL2. In other antibody mediated autoimmune disorders such as pemphigus vulgaris and myasthenia gravis, procedures like plasmapheresis and immunoadsorption have been used to remove pathogenic antibodies. Plasmapheresis, however, is not specific and also removes important plasma components like clotting factors and serum albumin in addition to pathogenic immunoglobulin. An immunoadsorption technique has been developed for pemphigus vulgaris in which desmoglein 3 is anchored onto a solid substrate and used to specifically isolate immunoglobulin recognizing the antigenic protein[40]. In myasthenia gravis, an immunoadsorption strategy has been developed that uses the specific epitope of anti-AChR antibodies to isolate these disease causing antibodies from the blood. The N-terminal extracellular domain of the α subunit of the AChR was immobilized to Sepharose beads and this strategy was able to successfully remove, on average, 35 % of anti-AChR antibodies from patients with myasthenia gravis[41].

An ELISA enables us to investigate the epitopes on CTL2 that are recognized by patient sera more readily than Western blotting[42]. Some epitopes can become obscured in Western blots and using the sandwich ELISA strategy to present rHuCTL2 in different topologies allows for various antigenic regions of the protein to be available to patient sera.

However, the sandwich ELISA approach developed as my thesis project and used in this study remains to be validated. In the current approach, high levels of background and nonspecific binding obscured our ability to draw conclusions about the binding of the anchor antibody to rHuCTL2. The detection antibody appears to have high levels of nonspecific binding, evidenced by the high absorbance values even in the absence of the rHuCTL2 target (Figure 8). The biotinylated anti-CTL2 antibody was not peptide purified and the background
may be a result of nonspecific immunoglobulin binding. Peptide affinity purification of the
detection antibody is underway in the lab and will be necessary to improve the sensitivity and
specificity of the ELISA and will enable us to continue with testing of human sera. There are
additional considerations to evaluate in the design of the ELISA to eliminate background
staining. For example, the blocking reagent used, 5 % non fat dry milk may reduce the sensitivity
of our assay because it has been reported that preparations of 5 % or 10 % nonfat dried milk
inhibit interaction between streptavidin and biotin [43]. Other considerations to increase the
sensitivity and specificity of the epitope mapping assay include modifying the concentrations of
anchor antibody, rHuCTL2 and detection antibody as well as further purifying the rHuCTL2 to
reduce any non-specific binding of the detection antibody. Furthermore the antigen specific
binding observed with the N-terminus antibody to rHuCTL2 anchored by the same antibody
suggest that the recombinant protein may be forming dimers or other multimers that result in
exposure of the N-terminus from the unanchored but bound dimers. Better blocking of the plates
possibly with non-immune immunoglobulins to saturate Ig binding sites on the plate may also be
necessary to reduce background.

However, if we are unable to improve the specificity of the sandwich ELISA,
alternative strategies for epitope presentation and mapping may need to be employed. The N-
terminal, C-terminal and Third Outer Loop regions of CTL2 were determined to be highly
antigenic in a hydrophobicity assessment. Peptides of these regions, as well as other highly
antigenic regions, can be synthesized and immobilized on an ELISA plate to assess the
frequency with which these epitopes are recognized by serum antibodies from individuals
suspected of having autoimmune hearing loss. The benefits of the peptide approach to epitope
mapping are that the peptides can be synthesized in high quantities and with high purity,
increasing the specificity of the assay[42, 44]. However, such a strategy excludes conformational epitopes that are derived from the secondary or tertiary structure of the protein as well as antibodies that recognize epitopes containing posttranslational modifications like glycosylation or ubiquitination[42, 44]. Because conformational epitopes cannot be mapped using this approach, antibodies that recognize and inhibit functional domains in a protein may not be detected[42, 44].
Figures and Legends.

Figure 1: Predicted model of the P1 isoform of the human transmembrane protein CTL2
This predictive model of the structure of CTL2 was developed using ExPasy topology prediction software. Portions highlighted in blue represent peptides used to immunize and develop polyclonal antibodies against the N-terminal, C-terminal and Third Outer Loop regions of human CTL2. These regions were selected for their high antigenicity as determined by hydrophobicity predictions. Yellow balls marked by arrows, represent predicted areas of N- linked glycosylation sites on human CTL2.
1. Add Sf9 lysate from cells expressing rHuCTL2 to a 4 mL Ni Column. Incubate lysate with beads overnight at 4°C.

2. Collect the Sf9 cell lysate that flows through the column (FT). Contains other proteins, leaving

FT  Washes  Elution

3. Wash the column with 15 to 20 bed volumes of wash buffer. Collect initial and final fractions for analysis.

4. Elute CTL2 protein from column by adding imidazole (500 mM).

Figure 2: Schematic of Ni column chromatography
Figure 3: Schematic of sandwich ELISA antigen presentation
Partially purified rHuCTL2 was anchored to an ELISA plate using polyclonal rabbit antibodies directed against the N-terminal, C-terminal and Third Outer Loop regions of human CTL2. The primary advantage of the three anchoring strategies lies in that multiple epitopes of the antigen are available for recognition by sera.
Figure 4: Treatment of Sf9 cells with tunicamycin
(A) Expression of CTL2 was assessed in Sf9 cells treated with 10 µg/mL of tunicamycin for 24, 48 or 72 hours. Tunicamycin treatment delayed expression of rHuCTL2 in Sf9 cells from 48 hours to 72 hours and resulted in a single 62 kDa protein band.
(B) The optimal dose of tunicamycin required for inhibition of glycosylation of rHuCTL2 was tested by treating Sf9 cells expressing rHuCTL2 with 0, 2.5, 5 or 10 µg/mL of tunicamycin. A minimum dose of 2.5 µg/mL was sufficient to block glycosylation.
Figure 5: Coomassie blue staining of partially purified rHuCTL2

Samples 1, 2 and 3 represent protein (45 µL) from three separate Ni column purifications of Sf9 cell lysate electrophoresed on a 3-10% gradient gel. For samples 1 and 3, approximately 2 µg of protein was loaded onto the gel and for sample 2, approximately 8 µg was loaded onto the gel. The gel was stained using Coomassie blue. Ni column purifications of the Sf9 lysate resulted in only partial isolation of rHuCTL2 from contaminant proteins. Further purification, such as antibody affinity purification may be necessary prior to use in ELISAs. Relative protein amounts can be compared to 5µg of BSA shown in lane 1.
Figure 6: Ni column purification of rHuCTL2 from Sf9 cells treated with tunicamycin
Recombinant human CTL2 was purified from Sf9 lysate of cells treated with tunicamycin. A Western blot of the lysate, flow through (FT), wash fractions (W1-4) and elution (E) reveals that CTL2 was partially purified using Ni column chromatography. Faint bands corresponding to the molecular weight of CTL2 are present in the flow through as well as wash fractions 1 and 2. The final concentration of the partially purified rHuCTL2 was determined to be approximately 40 μg/mL by Bradford protein assay.
Figure 7: Binding efficiency of αCTL2-CT, TOL and NT antibodies to rHuCTL2
Sf9 cell lysates from tunicamycin treated and non-treated cells were immunoprecipitated with αCTL2-CT*, TOL or NT antibodies and probed on a Western blot with these three antibodies. (*α=anti)

(A) Probe αCTL2-CT recognized only the core 62 kDa band from Sf9 lysates not treated with tunicamycin. αCTL2-NT antibodies recognized the triplet in cells not exposed to tunicamycin and NT antibodies had a greater affinity for rHuCTL2 than either the CT or TOL antibodies. TOL recognizes both the deglycosylated, core 62 kDa band and a faint higher molecular weight band that is not recognized by αCTL2-CT or NT antibodies.

(B) CT and NT antibodies recognize a 62 to 70 kDa triplet of CTL2 from Sf9 cells not treated with tunicamycin. However, CT antibodies are unable to bind to the glycosylated forms of CTL2 on western blots even when though the glycosylated forms of the protein are immunoprecipitated with CT antibodies (A). TOL antibodies mainly recognize the core deglycosylated band in cells treated or not treated with tunicamycin.

(C) TOL antibodies have the weakest binding to CTL2 and mainly recognize the core deglycosylated band. The probe NT antibody recognizes less CTL2 protein in samples immunoprecipitated with TOL antibody with the core being predominantly represented.
Figure 8: ELISA-detection of partially purified rHuCTL2 anchored to anti-CTL2 CT, NT and TOL antibodies using a biotinylated, polyclonal anti-CTL2 NT antibody as a probe

Partially purified rHuCTL2 was anchored to an ELISA plate using antibodies targeted to the CT, NT or TOL regions of the protein. Detection was performed using a biotinylated, polyclonal anti-CTL2 NT antibody to evaluate the binding efficiency of the various anchor antibodies for partially purified rHuCTL2 and to evaluate whether anchoring at the NT epitope prevents binding of the detection antibody at the same epitope. High levels of background obscured these evaluations, however, when N-terminal peptide was added fifty times in excess to the anti-CTL2 N-terminal detection antibody, some of the binding was eliminated suggesting that despite the high background, the binding of the biotinylated anti-CTL2 NT antibody is epitope specific.


