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A Study of the Interaction Between the Basigin Transmembrane Domain and Monocarboxylate Transporter 1

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By

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Abstract

It is thought that a lactate shuttle complex of Basigin gene products and Monocarboxylate transporter 1 (MCT1) is necessary for photoreceptor cell maturation and function. The purpose of this study was to investigate the assembly of the lactate shuttle by determining which amino acids within Basigin interact with MCT1. It has been hypothesized that these two membrane proteins interact via the transmembrane domain of Basigin. Therefore, a full-length histidine-tagged peptide of the transmembrane domain (24 amino acids), as well as histidine-tagged deletion mutants were generated using the pET102/D vector (Invitrogen) to test for binding to MCT1 via enzyme-linked immunosorbent assay (ELISA). The probe containing the entire transmembrane domain of Basigin (amino acids 1 to 24) did interact with MCT1. An interaction was also observed when a probe containing amino acids 13 to 24 of the Basigin transmembrane domain was used. A comparable interaction was observed when amino acids 19 to 24 were used as a probe, but no signal was observed when amino acids 13 to 18 were used. This indicates that some or all of the six C-terminal amino acids of the Basigin transmembrane domain bind to MCT1. Site-directed mutagenesis of each of the six C-terminal amino acids, and subsequent ELISA analysis, indicates that isoleucine – 20, isoleucine – 21, isoleucine - 23, and tyrosine – 24 interact with MCT1.

Chapter 1 Introduction

The mammalian retina has been extensively studied to understand the processes that occur in the eye. The retina is known to have high rates of oxygen consumption and lactic acid production (1). The retina, like other tissues, has cell surface receptors that participate in cell-cell interactions. Other proteins interact with each other on the surface of the same cell. Here we investigate two such proteins, Basigin and monocarboxylate transporter 1 (MCT1), to determine how they interact on the surface of photoreceptor cells and Müller glial cells of the retina. Basigin is known to be involved in cell-cell interactions and is found throughout the body but the specific role within the retina is not yet known (18). MCT1, a protein involved in lactate transport, is also found in the retina (18). It is thought that these two proteins comprise a lactate shuttle within the retina that functions to move lactate from Müller cells to the photoreceptors, which then use it as an energy source.

Structure of the Eye

The eye is a slightly irregular-shaped hollow sphere that is composed of three tunics, which make up the wall of the eye (2). They are the fibrous, vascular, and sensory tunics. The outermost tunic is the fibrous tunic. The bulk of the fibrous tunic is made up of the sclera, which protects and provides shape

to the eye. The second region of the fibrous tunic is the cornea. The main function of the cornea is to form a window that allows light to enter the eye (2). The middle layer of the eye is known as the vascular tunic, which contains the choroid, ciliary body, and iris. Choroid is highly vascularized and its blood vessels provide nutrients to the eye. The sensory tunic is the innermost layer of the eye. It contains the fragile two-layered retina. The outermost layer of the retina is the pigmented layer and it is composed of a single cell thick lining (2). The pigmented epithelial cells absorb light and prevent it from scattering in the eye. These cells also store vitamin A, which is used by the photoreceptor cells in the form of retinal. The inner layer, or neural layer, of the retina contains the photoreceptor cells, second order neurons, and Müller glial cells. Photoreceptor cells found in the retina can be divided into rods and cones. Rods are more numerous than cones (2). They are used in dim-light and for peripheral vision (2). The cones provide color vision and sharper images (2).

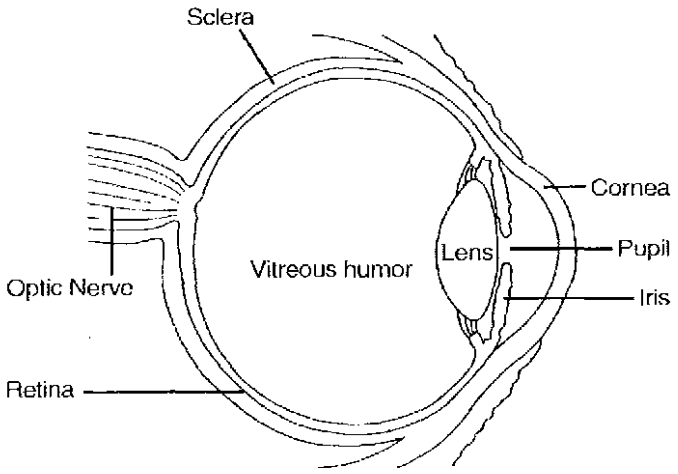
The retinal pigment epithelium (RPE) is an important feature of the retina because it forms the outer blood-retinal barrier (3). The regions of the eye are shown in Figure 1. The RPE is composed of a basolateral surface and an apical surface (4). The basolateral portion of the RPE stays in contact with the blood plasma. The blood plasma is filtered through a collection of porous capillaries within the choroid. The apical surface of the RPE has processes that extend between the photoreceptor cell outer segments and therefore interacts with the neural retina. The RPE and neural retina are separated by the subretinal space (SRS). Regulation of the volume and chemical composition within the SRS is

regulated by the specific proteins that are expressed on the surface of the RPE (3).

The RPE performs many functions that are essential for vision (4). It is used to mediate the transport of metabolites, ions, and fluid between the choroidal blood supply and neural retina (3). The job of the RPE includes supplying the nutrients that are needed to maintain the high metabolic rate of the photoreceptor cells, as well as removing the photoreceptor wastes (4). The RPE is also important for controlling the ionic gradients that are necessary for phototransduction to occur (4). Tight junctions within the RPE impede small water-soluble molecules from moving between cells (3). Evidence shows that the RPE plays a critical role in the maintenance and regulation of an environment that is necessary for retinal function (3).

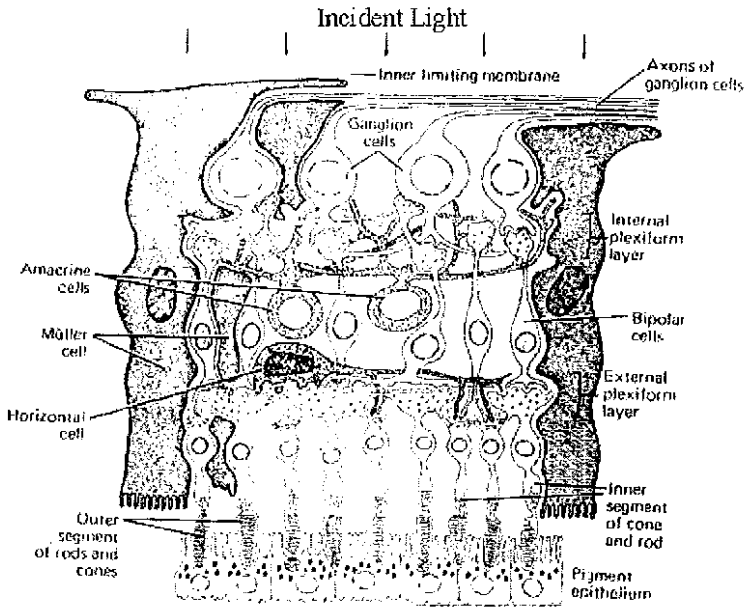
The retina contains photoreceptor cells and other neurons. The photoreceptor cells are located in the outer region of the retina (4). One region of the photoreceptor cells synapses on second-order neurons. When light is detected by the outer segments, an electro-chemical signal is transduced through the cell body to the synaptic terminal (Figure 2) (4). Glial cells play a critical role in the maturation and function of the nervous system, including the neural retina. These cells regulate the microenvironment for proper neuronal activity to occur (5).

Figure 1: .Diagram of the eye. The major components of the organ are indicated. The sclera provides protection and shape to the eye. The cornea is transparent and allows light to enter through the pupil. The size of the pupil is controlled by the iris, or colored-part of the eye. Refracted light from the line hits the retina, which contains photoreceptors. The photoreceptors detect the light and transduce a signal to the brain via the optic nerve. Image obtained from the National Eye Institute (32).



The primary glial cell in the adult retina is the Müller glial cell (5). A mature Müller cell is characterized as having a centralized soma and processes that extend out such that they form the inner- and outer-limiting membranes of the tissue (Figure 2; 5). The many processes of the Müller cells contain branches that are able to completely surround and insulate the neuronal somata and the neuronal processes (5). The structure of the Müller cells enables them to interact with every neuron within the retina (Figure 2). Within the retina the Müller glial cell is radially oriented and extends from the vitreal surface to anywhere from 50% to 70% of the retinal depth (7). In order for the Müller cell to mature, close contact with the neurons is required (5). Müller cells and photoreceptors are linked embryologically and physically, as a single retinal progenitor cell develops into a columnar arrangement of Müller cells, photoreceptors, and a subset of inner retinal neurons (8). When the Müller cell differentiates, altered protein expression is seen (5). The Müller cells continue to interact with photoreceptors after they mature as they surround the photoreceptors from the synaptic terminals to the inner segments (8). The interaction allows Müller cells to regulate the extracellular environment by recycling neurotransmitters, maintaining gas and ion homeostasis, and modulating pH (5). Müller cells are also able to mediate Ca^{2+} levels (5). Increases in neuronal Ca^{2+} influence cell movements, signal transduction, and gene expression (5). It can therefore be argued that Müller glial cells directly modulate neuronal activity (5).

Figure 2: Diagram showing the photoreceptor structure of both rods and cones. Light comes in from the top of the diagram and then travels through the layers of the retina until the light reaches the rods and cones which transduce the light energy to an electrical signal. (6)



The mature Müller glial cell is an important feature of the light-sensing retina because it is involved in many of the biochemical reactions within the eye (5). These new proteins are necessary for the Müller glial cell's role in vision (5). These proteins include glutamine synthetase, carbonic anhydrase, the anion exchanger, S-100 protein, and CRALBP (5). Several researchers have hypothesized that Müller cells offer metabolic and trophic support to promote photoreceptor survival (8). When the RPE is removed physically both Müller cells and photoreceptors undergo degenerative or reactive changes (8). Müller cells also express many types of neurotransmitter receptors that include GABA receptors, glutamate receptors and receptors for amino acids, catecholamines, neuroactive peptides, hormones, and growth factors (9). Müller glial cells have been found to take up and break down glucose extensively when stimulated by neuronal activity (7).

Importance of Basigin gene products

Basigin was first identified by Linser *et al.* as a protein that appeared to be Müller cell-specific within the chick retina (10). They actually found this protein on the Müller cells and mature RPE in the chick retina (11). It was also identified in many other animals including the mouse (10, 16). Basigin is a member of a subset of the immunoglobulin super family proteins that are involved in cell-cell interactions in the developing neural retina (12-14). This family has two other members, embigin and neuroplastin (12). The members of this family are characterized as possessing two extracellular immunoglobulin (Ig) C2 domains, a

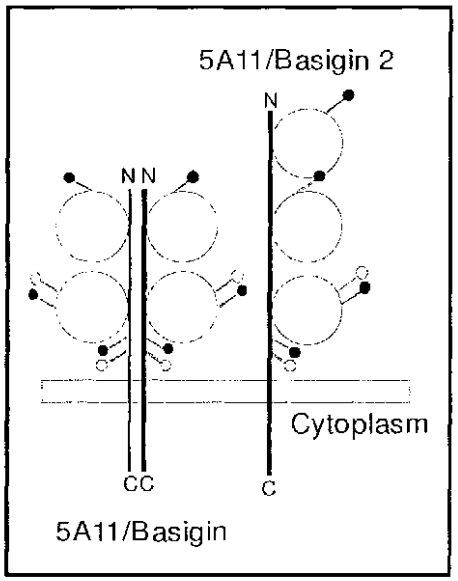
hydrophobic transmembrane domain, and a short cytoplasmic tail (Figure 4) (15). Basigin is known by many names, including M6/EMMPRIN in the human (16), CD147, OX-47, CE9, PE2 in the rat (16), RPE7 in bovine retina (16), and HT7 and 5A11 in chicken (10,11). The known homologues share extensive amino acid sequence identity in the transmembrane and cytoplasmic regions (15). This cross-species conservation within the transmembrane domain is thought to have an important function in this protein (12). Also within this region is a centrally located glutamic acid residue (12). This is common in proteins with multiple transmembrane domains but unusual for a protein with a single transmembrane domain (12). A common feature of the Basigin homologues is that they contain a putative leucine zipper motif within the transmembrane domain (15). The Basigin protein is 27 kDa, with a range of 16-39 kDa of carbohydrates when glycosylated (17). The range of glycosylation occurs because of differential glycosylation patterns in different tissues throughout the body (15).

Basigin is located on chromosome 10, centimorgan position 42.2 (Figure 5a) and has eight exons (Figure 5b) (15). The Basigin gene codes for two splice variants, named Basigin and Basigin-2 (15). The two splice variants differ by the addition of 116 amino acids in the extracellular domain of Basigin-2 (Figure 4 and 5b) (15). Basigin is found throughout the body with the highest expression in the testes and retina (15). Basigin-2, on the other hand, is only found in the retina (15). In the mouse neural retina, Basigin is expressed on the plasma membrane of Müller glial cells and blood vessel endothelial cells (14, 20). Expression is also seen on the apical and basal membranes of the RPE (14, 15). Basigin-2 is seen

only on the surface of the photoreceptor cell bodies and their inner segments (15). The amino-terminus of the extracellular Ig C2 domain of Basigin is critical for the homodimerization to occur (18). Basigin is thought to facilitate correct insertion of other membrane proteins in the plasma membrane (1).

In order to determine the function of Basigin, a strain of mice was generated in which the Basigin gene was deleted and is known as $Bsg^{-/-}$ (21). These mice perform poorly in learning and memory tasks (21). A decreased sensitivity to irritating odors and increased sensitivity to electric footshock is also seen (21). The retinas of $Bsg^{-/-}$ mice appear anatomically normal upon eye opening (2 weeks of age), but retinal degeneration begins at 8 weeks of age (20, 21). The first sign of abnormality in Basigin null mice is at three weeks of age when a difference in the outer segments is noticed because they were partially disordered when compared to the control mice (14). The retinas degenerate progressively and there are also characteristic changes observed in the photoreceptor cell layer (19). When the mice reach 35 weeks of age, the length of the outer segments is reduced to less than half the normal length (21). The thickness of the outer nuclear layer is reduced to four layers (21). By the time the mice are 43 weeks of age, most of the photoreceptor cells are absent from the retina (21). These observations suggest that Basigin plays an important role in maintaining the photoexcitation process (21).

Figure 3: Structure of the Basigin gene products, Basigin and Basigin-2. This diagram shows each protein possesses a single transmembrane domain. Immunoglobulin (Ig) loops are the represented by the large circles, the small open circles represent O-linked glycosylations, and the small shaded circles represent N-linked glycosylations. Basigin-2 is identical in sequence to Basigin except for the third Ig loop. Basigin is known to form a homodimer; however the stoichiometry of Basigin 2 is not known. (18)

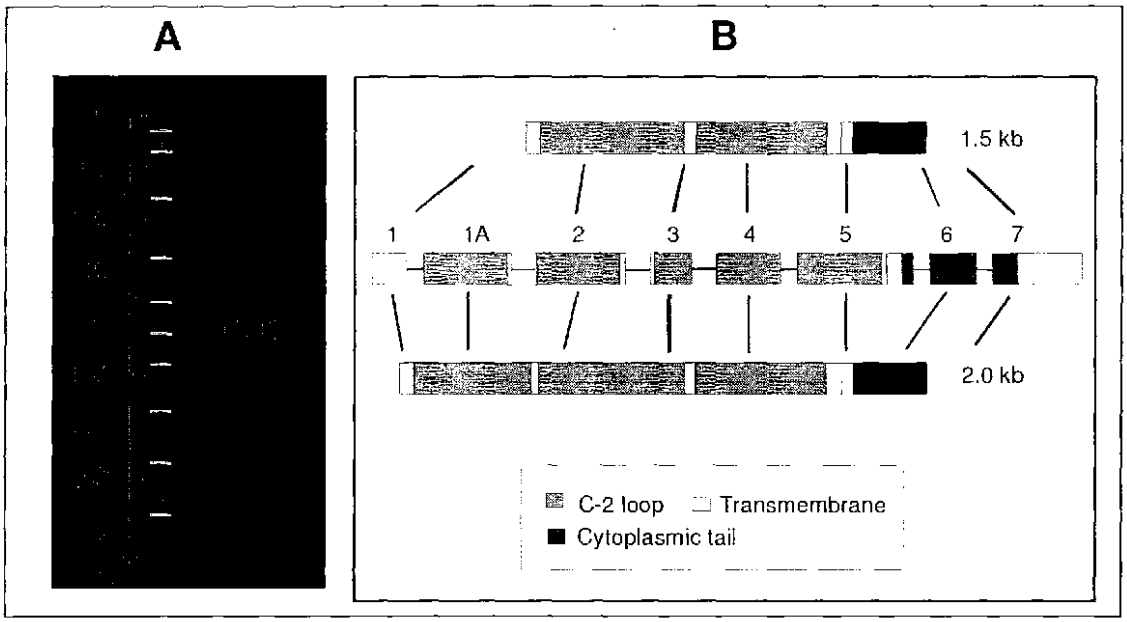


Electroretinograms (ERGs) were used to compare the differences between $Bsg^{-/-}$ mice and littermate controls (20). The ERGs of heterozygous mice, $Bsg^{+/-}$, mice were similar to those that are homozygous for the Basigin gene $Bsg^{+/+}$ (21). The ERGs of the $Bsg^{-/-}$ mice showed a distinct difference when compared to the ERGs of control mice (21). They had a higher stimulus intensity threshold and the amplitudes were lower (21). They never exhibited normal retinal activity and the mice are considered blind from the time of eye opening (14).

Experiments performed on Basigin null mice have shown that Basigin is necessary for normal retinal maturation in the mouse (21). In order to develop, maintain, and have a mature functioning visual apparatus, there must be contact-mediated interactions between the various cells of the retina such as the Müller cells, photoreceptor cells, and the RPE. It has been hypothesized by Ochrietor *et al.* that the two parts of the Basigin null mice phenotype are linked, in that the lack of retinal activity leads to the degeneration (14). It is thought that cell contacts mediated by Basigin gene products are crucial for proper retina function.

Basigin and the Basigin homologues are involved in many different activities. They are implicated in the construction of the blood-brain barrier and in neuronal-glial interactions within the retina during development (21). The homologues are induced during activation of leukocytes, and they are seen to induce matrix metalloproteases (MMP) in fibroblasts and tumor cells (13, 21). Basigin is also found in a wide variety of metastic tumors, where it helps enhance

Figure 4: Chromosome location and structure of Basigin gene products. (a) Chromosome 10 of the mouse genome. The position of Basigin is indicated in red at position 42.2 on the chromosome. (19) (b) Diagram representing Basigin gene structure. In the center of the diagram the Basigin gene encodes eight exons which are indicated in orange. When all eight exons are spliced together they form the 1.8-kb gene product known as Basigin-2, which is shown below the gene. Basigin consists of only seven exons in the 1.5-kb gene product. Exon 1A is not included in Basigin, which is shown above the gene product. The transmembrane domain is indicated in yellow and the cytoplasmic tail is indicated in green. (18)



tumor growth and invasiveness (13). This occurs because it stimulates the secretion of MMPs which promotes tumor cell invasion (13). Basigin has also been implicated in HIV attachment and T cell activation (22).

Basigin homologues are multifunctional and able to interact with many other proteins (18). Basigin is able to oligomerize with itself and can also interact with intergrin α3β1 in a cell-cell contact manner, but not at focal adhesions (13). secretion of MMPs and that helps promote tumor cell invasion (13). Basigin has also been implicated in HIV attachment and T cell activation (22).
Basigin homologues are multifunctional and able to interact with many other proteins (18). Basigin is able to oligomerize with itself and can also interact with intergrin α3β1 in a cell-cell contact manner, but not at focal adhesions (13). Integrins play a role in cell attachment, cell migration, and cell-cell interactions (13). Basigin has been reported to bind to oligomannosidic carbohydrates which are also involved in cell-cell recognition (23). A binding partner for Basigin in the retina has not been found, but it has been shown that antibodies specific for Basigin are able to inhibit avian retina reaggregation *in vitro* (11). Basigin interacts with other proteins in both the *cis* and *trans* conformation (18). When in the *cis* conformation, it interacts in the plasma membrane of the same cell, and in the *trans* conformation the interaction occurs between cells (18). While many functions for Basigin have been observed, there is little known about the function of Basigin-2. Basigin-2 has been found to interact with itself or homophillically (22). The biological roles of Basigin and Basigin-2 in the neural retina have not

been determined, but several *in vitro* binding studies indicate that Basigin associates with monocarboxylate transporter-1 (MCT 1) (12, 24).

Cyclophilins are chaperone proteins

Cyclophilins have been established in the regulation of protein trafficking in cells (25). Cyclophilin A (CyPA) is a universally distributed intracellular protein that is a member of the immunophilin family (62). It is recognized as a host cell receptor for the potent immunosuppressive drug cyclosporin A (26). The protein CyPA possesses peptidylpropyl *cis-trans* isomerase activity and it is thought to play an important role in protein folding (27). Cyclophilins form a specific stable complex with substrates like heparins, but only a transitory complex with Basigin (25). An interaction between CyPA, found on the cell surface and Basigin, has been observed in HIV-1 virions. This interaction enhances HIV-1 infection at the entry or uncoating step (26). The protein known as CyPA has two distinct binding interactions, one with heparins and the other with Basigin (26). If there is a high expression of heparins on the cell surface this interferes with the interaction between CyPA and Basigin (13). Basigin interacts with extracellular cyclophilins in a cyclosporin-sensitive fashion (25).

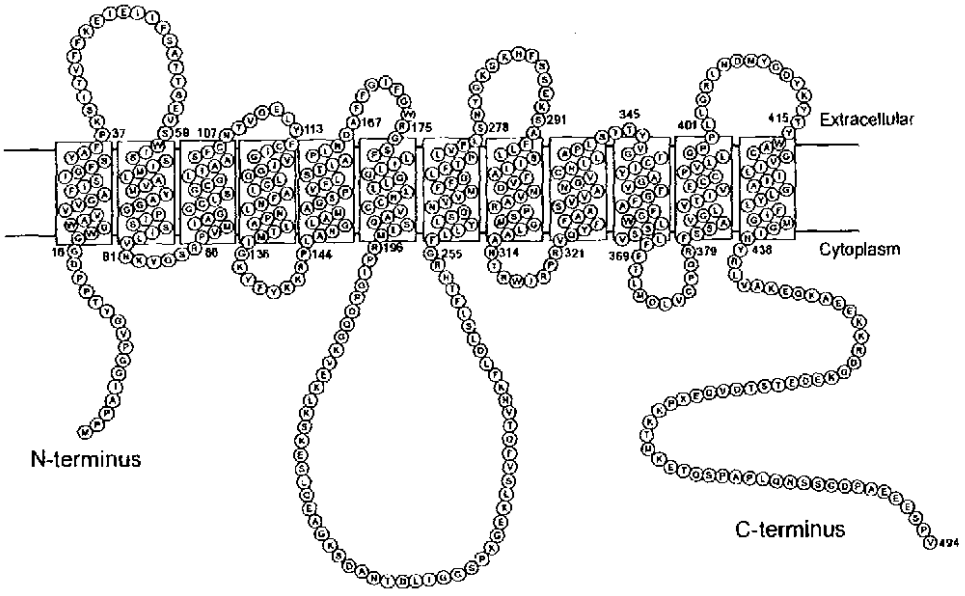
Importance of MCT1 and the other isoforms

Monocarboxylate transporter 1 (MCT1) is a 55 kDa glycoprotein that contains 12 membrane-spanning regions (12; Figure 6). This protein belongs to the proton-linked monocarboxylate transporter family that contains either 10 or

12 transmembrane α-helical domains (3). The N- and C- termini are located within the cytoplasm and a 12 transmembrane helix is common among plasma membrane transporters (3). This family contains at least eight mammalian members but only four of the isoforms (MCT1-MCT4) have been functionally characterized (21). Two of the isoforms, MCT1 and MCT4, are known to transport lactate, pyruvate, acetoacetate, and β-hydroxybutyrate (12). This wide range of metabolites suggests that MCTs are critical for metabolic communication between cells (3).

The isoform, MCT1, has also been cloned from many animals including human, rat, and mouse (3). These homologues of MCT1 share about a 95% sequence identity with each other (3). The isoform MCT2 was cloned from hamster, rat, mouse, and human whereas the isoform, MCT3 was found in chicken RPE (3). The last isoform to be characterized was MCT4 which was cloned from human, rat, and frog (3). Sequence conservation is greatest between MCT1 and MCT4 (3). Sequence conservation between MCT1 and MCT2 is 60% but MCT2 is not as broadly distributed (3). When a comparison was made with MCT3 it was found that it shares 43% and 45% sequence identity with MCT1 and MCT2 respectively (3). The greatest amount of sequence conservation within this family is found within the putative transmembrane regions and the shorter loops between them (3). Little conservation is observed in the hydrophilic regions, which are unlikely to be directly involved in transport (3).

Figure 5: Proposed topology of rat MCT1. This integral membrane protein has the characteristic 12 transmembrane domains and short cytoplasmic tails at both the N- and C-termini. The amino acid sequence of MCT1 is also indicated. (31)



Distribution of MCT1-MCT4 in the retina has been determined by immunofluorescence microscopy (3). The tissue distribution for MCT1 is very broad (3). It is expressed on the apical surface of the RPE, on Müller cells microvilli, the photoreceptor inner segments, and all retinal layers between the inner and external limiting membranes (3). The protein MCT2 is expressed on the inner plasma membrane of Müller cells, on glial cell processes surrounding microvessels, on the synaptic layers of the neural retina, and on the nuclear layers of the neural retina (3). The tissue distribution for MCT3 was found only on the basolateral surface of the RPE (3). Expression of MCT4 was found in the neural retina on the Müller cells microvilli (3). Within the loop between helix 4 and helix 5 an arginine residue is conserved in almost all members of the family (28). This area is the only place where there is a conserved charged residue within the transmembrane regions of the MCT family (28). Analysis of the charged residue indicates that it is crucial for lactate binding and coupling of lactate to proton translocation (28).

Co-immunonprecipitation and chemical cross-linking studies indicate that there is a specific and strong interaction between Basigin and two of the monocarboxylate transporters, MCT1 and MCT4 (12). It is thought that the transmembrane domain of Basigin is important for this interaction to occur (Figure 7; 12). In the neural retina the expression pattern of MCT1 expression overlaps both Basigin and Basigin-2. In cells that express both a Basigin glycoprotein and MCT1, the proteins target to the plasma membrane and have the correct function (29, 26). *In vivo* studies of the Basigin null mice, which have

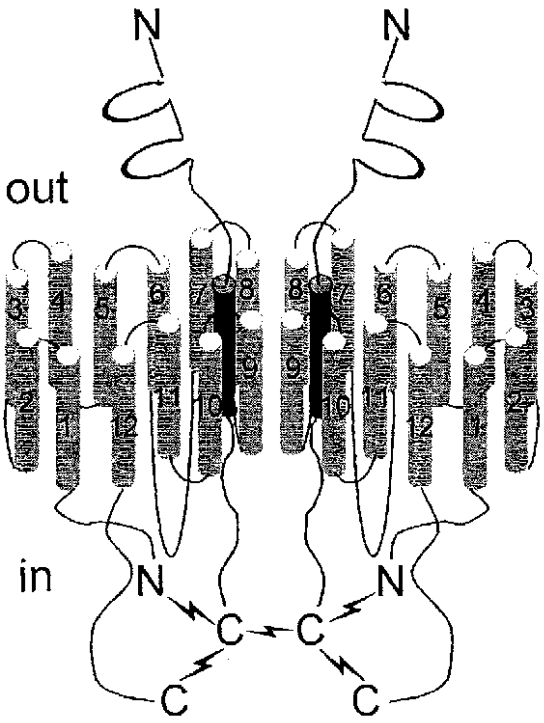
the two forms of Basigin deleted, demonstrated that MCT1 did not locate to the plasma membrane (21). Instead it remained in the intracellular vesicles of both the Müller cells and the photoreceptor cells (29). Therefore, it has been proposed by this laboratory and others that Basigin is necessary for the translocation of MCT1 to the plasma membrane (29, 26). Kirk *et al.* proposed that the transmembrane domain of Basigin is required for the translocation of MCT1 (12).

Lactate Metabolism

Lactate is considered to be the most important metabolite that MCTs transport because of its use in metabolic processes (12). Lactate has a pKa of 3.86, which causes it to dissociate entirely to the lactate anion (3). This charged species cannot cross the plasma membrane by free diffusion but instead requires a specific transport mechanism (3). This is provided by proton-linked MCTs (3). The retina is a known producer of large amounts of lactate (3). The transport of lactate across the plasma membrane is important for the retina to maintain proper functions (3). This is done in the presence and absence of oxygen (30).

A unique feature of Müller cells is that they have a high rate of glucose utilization and lactate formation in the presence of oxygen (7). This is supported by research done on the Guinea pig retina that found that Müller cells metabolize glucose aerobically to primarily form lactate (7). The lactate is then transported out of the cells and used by the photoreceptor cells for fuel in oxidative phosphorylation (30). Active retinal neurons, especially photoreceptors, release

Figure 6: Proposed interaction between Basigin and MCT1. Basigin is represented by the single pass transmebrane protein that has the black barrel. MCT1 is shown as the twelve transmembrane protein and has the lighter shaded barrels. They are drawn forming dimers where two Basigin and two MCT1 interact. The proteins are thought to interact via their transmembrane domains (24)



CO_2 that leads to high concentrations of extracellular CO_2 (9). This increase causes a rapid intracellular acidification in the Müller cells (9). This acidification is neutralized by lactate being released with a proton (30). The lactate that is not used by the photoreceptor cells is transported out through the SRS and into the choroid by the RPE (30). It is believed that a chemical gradient exists for lactate within the retina (3).

The purpose of this project is to determine whether the Basigin transmembrane domain interacts with MCT1, and if so, to determine which amino acids within the transmembrane domain interact with MCT1. The significance of the study is to determine whether a lactate shuttle exists within the retina. Lactate is an important metabolite and the photoreceptors use lactate as a form of energy. Therefore, it is thought that there is a complex of MCT1, cyclophilin A, and Basigin on the Müller cells that interacts with another complex on the photoreceptor cells that contains Basigin-2, cyclophilin A, and MCT1. These two complexes interact and form a complex that transports lactate from Müller cells to the photoreceptor cells. This study determined that the Basigin transmembrane domain, specifically the residues within the six C-terminal amino acids of the domain, interact with MCT1. Site-directed mutagenesis of each of the six C-terminal amino acids and subsequent binding assays indicate that when isoleucine at position 20, isoleucine at position 21, isoleucine at position 23, and the tyrosine at position 24 were all individually mutated to glycine, they significantly lowered the ability of the probes to bind to MCT1. Therefore, it is

likely that all four of these amino acids are important for the interaction with MCT1.

Chapter 2 Materials and Methods

Amplification of cDNA

Amplification of the transmembrane region of Basigin (NM 009768) was performed with Platinum *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA). PCR setup included: 5µl of 10X *Pfx* amplification buffer, 1.5µl 10mM dNTP mixture, 1µl 50mM $MgSO_4$, 1.5µl primers at a final concentration of 10µM, 200ng of template DNA, and 0.5µl *Pfx* DNA polymerase; in a total volume of 50µl. [This was accomplished by primers that were designed to amplify the region of interest.] The primers used are listed in Table 1. The full-length probe was generated first from mouse retina cDNA. The deletion mutants used the full-length transmembrane domain of Basigin as the template. A three step cycling protocol was used: 95°C for 30 seconds, 55°C for 30 seconds, 68°C for two minutes; for a total of 30 cycles.

The constructs containing the first and last six amino acids of the Basigin transmembrane domain could not be generated via PCR. Rather a primer annealing procedure was used. This was accomplished by annealing the forward primer (100 pmol/µl) and the reverse primer (100 pmol/µl) using a 1 µl of a 20X annealing buffer (0.2M Tris-HCl pH 7.9, 40mM $MgCl_2$, 1M NaCl, 20mM EDTA) in a total volume of 20 µl. The reaction was heated to 100°C for 5 minutes and then allowed to anneal by slowly cooling to room temperature.

Table 1: Primers used for amplification of the Basigin transmembrane domain.

Name	Sequence
TM1047-NF	5' CACCACCATCATCTTTATCTAT 3'
TM1065R-NF	5' ATAGATAAAGATGATGGT 3'
pET102-TM-RV	5' ATAGATAAAGATGATGGTAACCAACA 3'
pET102-TM-FWD	5'CACCATGGCAGCCCTCTGGCCC 3'
pET102-TM1011-RV	5' GGGCCAGAGGGCTGCCAT 3'
pET102-1047RV-long	5' AACCAACACCAGGACCTCAGCCACGA 3'
pET102-1029F-NF	5' CACCGAGGTCCTGGTGTTT 3'
pET102-1047RV-NF	5' AACCAACACCAGGACCTC 3'
pET102-1029RV-NF	5' AGCCACGATGCCTAGGAA 3'
pET102-1011F-NF	5' CACCTTCCTAGGCATCGTGGCT 3'
pET102-1011F-long	5' CACCTTCCTAGGCATCGTGGCTGAGG 3'

DNA gel extraction

All PCR products were analyzed by gel electrophoresis using a 2% agarose gel with 1X Tris-boric acid-EDTA (TBE) buffer. After documenting the gels, the PCR products were excised with a razor blade and placed into a 1.5 ml microcentrifuge tube. DNA was purified from gel fragments via the QIAquick gel extraction kit (Qiagen, Valencia, CA). Buffer QG (180µl) was added to the gel slice and the tube was incubated at 57°C for 10 minutes to dissolve the gel. Isopropanol (60µl) was added to the 1.5 ml tube and the solution was mixed by vortexing. The solution was then placed into a QIAquick gel column in a 1.5 ml collection tube and centrifuged at 13,000 rpm for one minute. The flow-through was discarded and the column was placed back into the tube. Buffer QG (500µl) was added to the column and centrifuged for one minute at 13,000 rpm. The flow-through was once again discarded and the column was placed back into the tube. The column was then washed by adding PE buffer (750µl) and centrifuged at 13,000 rpm for one minute. Flow-through was then discarded and the column was centrifuged again at 13,000 rpm. The column was then placed into a clean 1.5 ml microcentrifuge tube and 50µl of EB buffer was added to the column. The column was incubated at room temperature for one minute and then centrifuged again at 13,000 rpm for one minute to elute the DNA.

Ligation and Transformation

After the DNA was extracted from the agarose gel, it was subcloned into the pET102/D-TOPO vector (Invitrogen). The annealed cDNA was subcloned

directly into the vector without agarose gel analysis. For each of the amplified regions of the transmembrane domain, 4 µl of purified DNA, 1µl of the salt solution and 1µl of the pET102 TOPO vector were combined. This mixture was mixed gently and incubated for five minutes at room temperature. The recombinant DNA probe was transformed into Top 10 cells (Invitrogen). The ligation mixture (2µl) was added to 50µl of cells and incubated on ice for 30 minutes. The cells were heat-shocked by incubating at 42°C for 30 seconds. The tube was immediately placed on ice for one minute and then 250µl of Super Optimal Catobolite repression broth (S.O.C.) medium was added. The transformed cells were incubated at 37°C with shaking (225 rpm) for one hour. The entire mixture was then spread onto Luria-Bertani Agar (BD/Fisher) plates containing 50 µg/ml of carbenicillin. These plates were inverted and incubated at 35°C overnight. The next day, colonies were picked with a toothpick and placed in 25µl of water in a 500µl microcentrifuge tube (a water culture).

Colony screen

The colonies were screened using M13R and PD13 primers (Table 2). The reaction included 25µl EX-*Taq* (Takara, Otsu, Shiga, Japan), 5µl of the water culture, and 0.5 µM of each primer in a total volume of 50µl. The reaction was subjected to PCR amplification using a protocol as follows: 95°C for 2 minutes, and 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for two minutes. The amplified PCR products were then analyzed on a 1% agarose gel in 1X TBE buffer. Colonies producing PCR products of the approximate number

of base pairs were considered positive for the recombinant DNA insert. Positive colonies (5µl of water culture) were grown overnight at 37°C with shaking (225 rpm) in 3ml of Luria Bertani broth that contained carbenicillin (50µg/ml).

Plasmid purification

The plasmids contained in positive colonies were purified using the QIAprep spin miniprep protocol (Qiagen). An aliquot of the overnight culture (1.5 ml) was placed into a 1.5 ml microcentrifuge tube and centrifuged at 13,000 rpm for one minute. The supernatant was removed and the pellet was resuspended in P1 buffer (250µl). Buffer P2 (250µl) was added to the cells and mixed by gently inverting the tube five times. Buffer N3 (350µl) was added to the solution and also mixed gently by inverting the tube five times. The mixture was centrifuged at 13,000 rpm for 10 minutes. The supernatant was removed from the tube and placed into a QIAprep spin column in a 1.5 ml collection tube using a fine transfer pipette. The column was centrifuged at 13,000 rpm for one minute. The flow-through was discarded. The plasmid DNA was washed with 500µl of PB buffer and the column was centrifuged for one minute at 13,000 rpm. The flow-through was discarded. The spin column was washed with 750µl of buffer PE and centrifuged for one minute at 13,000 rpm. The flow-through was discarded. The column was centrifuged for one minute at 13,000 rpm to remove residual ethanol.

Table 2: Vector primers used for sequencing

Name	Sequence
PD13	5' CTGGCCGTCGTTTTAC 3'
M13 Rev	5' CAGGAAACAGCTATGA 3'
TrxFus	5' TTCCTCGACGCTAACCTG 3'
T7 Rev	5' CCACCGCTGAGCAATAACTA 3'

The spin column was then placed into a clean 1.5 ml microcentrifuge tube. Elution buffer (50µl) was added to the spin column and incubated at room temperature for one minute. The column was centrifuged for one minute at 13,000 rpm. The collected purified plasmid DNA was then analyzed to determine the concentration.

Plasmid DNA quantification

A 1:20 dilution of plasmid DNA in nuclease-free water was used to determine the plasmid concentration on a BioTek plate reader. The concentration was calculated using the following equation:

$$\text{Concentration } (\mu g/ml) = A_{260} \cdot 50\mu g/ml \cdot \text{dilution factor}$$

Sequencing

The sequence of each construct was verified via automated DNA sequencing. Sequencing was performed using the GenomeLab Dye Terminator Cycle Sequencing with Quick start kit (Beckman Coulter, Fullerton, CA). Cycle sequencing was performed using 4µl Quickstart mix, 2 µl of TrxFus primer (1 pmol/µl), 2 µl T7 Reverse primer (1 pmol/µl) (Table 2) and 0.165 µg plasmid in 10 µl total volume. The following cycling parameters were used: 96°C for 20 seconds, 50°C for 20 seconds, 60°C for 4 minutes for 30 cycles. The PCR reaction was placed into a clean 0.5 ml micro centrifuge tube containing 5µl of stop solution (2 µl of 3M sodium acetate, pH 5.2; 2µl of 100mM Na_2-EDTA, pH 8.0; and 1µl of 20 mg/ml of glycogen). Cold 95% ethanol was added and the

solution was thoroughly mixed. The tube was then immediately centrifuged at 14,000 rpm for 15 minutes. The supernatant was removed with a fine transfer pipette, making sure that the pellet was not removed. The pellet was rinsed with 200µl of 70% ethanol and centrifuged at 14,000 rpm for two minutes. A fine transfer pipette was used to remove the supernatant while making sure that the pellet was not removed. This step was repeated. The tube was then dried in a speed vacuum for 10 minutes. Sample loading solution (25 µl) was used to resuspend the dried pellet. The DNA was analyzed on a Beckman Coulter CEQ 8000 Genetic Analysis system (Beckman Coulter).

Frozen Stocks

Once the sequence was verified, a frozen stock was made that contained 850µl of the culture and 150µl of glycerol. The stock was then mixed to ensure that the glycerol was mixed throughout the entire solution and stored at -80°C in a cryotube.

Site-Directed Mutagenesis

Primers were designed to individually mutate the six amino acids at the carboxy terminus of the Basigin transmembrane domain (Table 3). Each of the primers was designed to change the wild type amino acid to glycine. The mutagenesis procedures used the QuikChange II XL site directed mutagenesis kit (Stratagene, La Jolla, CA). The reaction mix was assembled in a 0.5 ml tube containing: 5 µl 10X reaction buffer, pET102-TM19-24(50ng), 125 ng of the

forward primer, 125 ng of the reverse primer, 1 µl DNTP, in 50 µl total volume. *Pfu* Ultra HF DNA polymerase (Stratagene; 1 µl) was added to the reaction mixture just prior to PCR amplification. The cycling parameters were 95°C for 30 seconds, followed by 16 cycles of 95°C for 30 seconds, 55°C for 1 minute, 68°C for 5 minutes. After the amplification was completed the reaction mix was placed on ice for two minutes and 1µl of Dpn I (10 U/µl) was added and mixed gently by pipetting up and down. The reaction was centrifuged to ensure that all of the reaction mix was at the bottom of the tube and then incubated at 37°C for one hour. XL1-Blue supercompetent cells (Stratagene; 50 µl) were placed on ice into a pre-chilled polypropylene tube (BD falcon). The Dpn I treated reaction (1 µl) was added to the cells and the mixture was incubated on ice for 30 minutes. The cells were then heat shocked by incubation at 42°C for 45 seconds and then placed back on ice for two minutes. Then S.O.C. medium (250 µl) was added to the cells and incubated at 37°C for one hour with shaking (225 rpm). The cells were then spread onto a pre-warmed Luria Bertani agar plates containing carbenicillin (50µg/ml) and incubated at 37°C overnight. Colonies were picked with a toothpick, transferred into Luria Bertani broth containing carbenicillin (50µg/ml), and incubated overnight at 37°C. Plasmids were purified using a QIAprep spin Miniprep kit (Qiagen) as described previously. Plasmids were cycle sequenced, to verify that the mutation was introduced using the Quickstart reaction, as described previously.

Table 3: Primers used to mutate the carboxy terminus of the Basigin transmembrane domain in the Site-directed mutagenesis reaction.

Name	Sequence
pET102-BsTM-19-F	5' ATTGATCCCTTCACCGGCATCATCTTTATCTATAAGGGC 3'
pET102-BsTM-19-Rv	5' GCCCTTATAGATAAAGATGATGCCGTGGAAGGGATCAAT 3'
pET102-BsTM-20-F	5' ATTGATCCCTTCACCACCGGCATCTTTATCTATAAGGGC 3'
pET102-BsTM-20-Rv	5' GCCCTTATAGATAAAGATGCCGGTGGTGAAGGGATCAAT 3'
pET102-BsTM-21-F	5' ATTGATCCCTTCACCACCATCGGCTTTATCTATAAGGGC 3'
pET102-BsTM-21-Rv	5' GCCCTTATAGATAAAGCCGATGGTGGTGAAGGGATCAAT 3'
pET102-BsTM-22-F	5' TTCACCACCATCATCGGGATCTATAAGGGCGAG 3'
pET102-BsTM-22-Rv	5' CTCGCCCTTATAGATCCCGATGATGGTGGTGAA 3'
pET102-BsTM-23-F	5' ACCACCATCATCTTTGGCTATAAGGGCGAGCTC 3'
pET102-BsTM-23-Rv	5' GAGCTCGCCCTTATAGCCAAAGATGATGGTGGT 3'
pET102-BsTM-24-F	5' ACCATCATCTTTATCGGGAAGGGCGAGCTCAAG 3'
pET102-BsTM-24-Rv	5' CTTGAGCTCGCCCTTCCCGATAAAGATGATGGT 3'

Expression of Histidine-tagged recombinant proteins

Each of the expression vectors containing a Basigin transmembrane domain were transformed into BL21 star (DE3) one shot cells (Invitrogen). One µl of plasmid was added to 50 µl of cells in a 3 ml tube and the mixture was incubated on ice for 30 minutes. The cells were heat shocked by incubation 42°C for 30 seconds. The tube containing the transformation reaction was immediately transferred to ice for two minutes. Room temperature S.O.C. medium (250 µl) was added to the cells and they were incubated at 37°C for 30 minutes with shaking at 225 rpm. The entire reaction mixture was transferred to 10 ml of LB containing 50 µg/ml of carbenicillin and incubated overnight at 37°C with shaking at 225 rpm.

The entire 10 ml of the overnight inoculation was transferred to an Erlenmeyer flask containing 1 liter of LB with 50 µg/ml of Carbenicillin. The culture was incubated at 37°C with shaking at 225 rpm until the cells reached midlog phase of growth (OD_{600} = 0.5-0.8). Once the culture reached the midlog range, the culture was induced with 500 µl of 1M of Isopropyl β-D-thiogalactopyranoside (IPTG, Fisher Scientific) and allowed to grow overnight at 25°C with shaking (225 rpm).

Purification of Histidine-tagged recombinant proteins

The induced overnight culture was divided into four 500 ml centrifuge tubes (approximately 250 ml of the culture in each). The cells were pelleted by centrifugation at 3000xg for 15 minutes at 4°C in a Sorvall centrifuge. Xtractor

buffer (20 ml; Clontech) was added to the pellet for resuspension of the cells. The 20 ml was divided among the four centrifuge tubes and the Xtractor buffer was pipetted up and down to break up the pellets completely. The entire resuspension was then placed into a 50 ml centrifuge tube and 40 µl DNase I and 50X lysozyme solution (200 µl) were added. This solution was mixed by gently pipetting and then allowed to incubate at room temperature for 10 minutes. The solution was then centrifuged in a Sorvall centrifuge at 10,000xg for 20 minutes at 4 °C. The supernatant was collected and placed on ice.

The TALON metal affinity resin (Clontech) was prepared. The TALON metal affinity resin (Clontech) was resuspended by vortexing and 2 ml of the resin was transferred to a 50 ml centrifuge tube. The resin was centrifuged at 700xg for two minutes at 4 °C. The supernatant was discarded. The resin was washed with 10 ml of 1X equilibration/wash buffer (Clontech), mixed briefly and centrifuged at 700xg for two minutes at 4 °C. This equilibration step was repeated.

The bacterial supernatant was added to the resin and allowed to incubate at room temperature with shaking (225 rpm). The mixture was centrifuged at 700xg for five minutes at 4 °C. The supernatant was discarded and the pellet was washed with 10 ml of 1X equilibration/wash buffer. The solution was then centrifuged at 700xg for five minutes and the supernatant was discarded again. This wash step was repeated once. One ml of the 1X equilibration/wash buffer was added to the pellet and the mixture was vortexed briefly. This solution was then transferred to a 2 ml disposable column that is included with the TALON

purification kit (Clontech). The column was held vertically until the resin settled. The buffer was then drained from the column into a waste beaker. The column was washed with 5 ml of 1X equilibration/wash buffer. The purified protein was eluted with 5 ml of 1X elution buffer. Eluent was collected in 0.5 ml fractions in 1.5 ml microcentrifuge tubes. The fractions were analyzed at 280 nm absorbance and the fractions with the highest values were pooled.

Mouse retina lysates

Mouse eyes were removed from the animals according to approved methods at the Whitney Laboratory for Marine Bioscience in Saint Augustine, Florida. For every mouse eye 100 µl of water was added and the tissue was sonicated until the tissue was dissolved. The concentration of the mouse retina lysates was then analyzed using the Coomassie (Bradford) Protein Assay kit (Pierce, Rockford, IL) as described below.

Bradford Protein Assay

Protein concentrations of the pooled fractions were determined using the Coomassie (Bradford) Protein Assay kit (Pierce). A standard curve was generated using 1:2 serial dilutions of bovine serum albumin (BSA, Pierce) from 0.2 mg/ml to 1 mg/ml. A total of 20 µl was generated for each. Each standard (5 µl) was transferred into two wells of a 96 well microplate (Corning, Lowell, MA). Water (5 µl) was used as the blank. Each of the recombinant proteins that were purified using the TALON purification kit (Clontech) were analyzed both undiluted

(5 µl) and at 1:2 dilution (2.5 µl protein plus 2.5 µl H_2O). All wells received 250 µl of Coomassie reagent (Pierce). The absorbance at 595 nm was then determined using a BioTek plate reader. The average absorbance of each standard was calculated and plotted on a graph that had the x-axis as the concentration of protein in mg/ml and the y-axis was the average absorbance at 595 nm. A linear regression was performed and the concentrations of the purified histidine proteins were determined from the graph.

ELISA Analysis

Sandwich ELISA was performed to test for Basigin transmembrane binding to endogenous mouse MCT1. Wells of a 96 well plate (Corning) were coated with an antibody specific for mouse MCT1 (Gift from Nancy Philp, Thomas Jefferson Institute) at 0.5 µg/ml in 1XPBS (100 µl total volume) overnight at 4°C. The coating antibody was removed with a fine transfer pipette and the wells were washed three times with PBS-T (1X PBS plus 0.25% Tween-20). Next, mouse retina protein was added to the wells at a concentration of 1µg/ml in 1X PBS (100 µl total volume) and incubated at room temperature for 30 minutes. The protein was removed with a fine transfer pipette and the wells were washed three times with PBS-T. Purified histidine proteins were added to appropriate wells at a concentration of 100µg/ml in 1X PBS (100 µl total volume) and incubated for 30 minutes at room temperature. The purified histidine proteins were removed using a fine transfer pipette and the wells were washed with PBS-T. The primary antibody, anti-His (Invitrogen; diluted 1:10,000 in 1X PBS) was

added to the appropriate wells in a total volume of 100µl and incubated for 30 minutes at room temperature. The anti-His antibody was removed using a fine transfer pipette and the wells were washed three times with PBS-T. The secondary antibody, Alkaline Phosphatase Goat anti Mouse (APGAM, Pierce; diluted 1:10,000 in 1X PBS) was added to appropriate wells and incubated for 30 minutes at room temperature. The secondary antibody was removed with a fine transfer pipette and the wells were washed three times with PBS-T. Alkaline phosphatase substrate, PNPP (Pierce), was added to each well at a total volume of 100µl. The substrate was incubated at room temperature for 30 minutes or until color development was observed. Once sufficient color development occurred, 50 µl of 2N NaOH was added to each well to stop the reaction. The results were generated by determining the absorbance at 595 nm. Wells containing only 1X PBS served as the blanks. All runs were performed in triplicate and the results were averaged. The results were plotted in a bar graph using Microsoft Excel software. The standard deviations are represented by error bars.

Chapter 3 Results

The ability of the transmembrane domain of Basigin to bind MCT1 was assayed by ELISA analysis. The cDNA coding the entire transmembrane domain of Basigin (all 24 amino acids) was cloned into the pET102 expression vector and the probe known as TM-all was produced (Table 4). The protein contains a 6X-Histidine tag for detection. Endogenous mouse MCT1 was captured and probed with TM-all. In Figures 8 and 9, it is shown that the transmembrane domain of Basigin does bind to MCT1.

Once it was determined that the Basigin transmembrane domain does bind MCT1, the domain was systematically deleted from the carboxy terminus in increments of six amino acids (Table 4). Deletion probes were generated using the pET102 vector and used in ELISA binding analyses. Figure 8 shows that binding was observed for all constructs. These data suggest that amino acids within TM1-6 bind to MCT1.

The transmembrane domain was also systematically deleted from the amino terminus (Table 4) and tested for binding to MCT1. Figure 8 shows that binding was observed with all deletion probes used. These data suggest that the amino acids within TM19-24 are also involved in the Basigin-MCT1 interaction.

Table 4: Basigin transmembrane probes used in ELISA analysis.

Name	Amino Acid Sequence
TM All	MAALWPFLGIVAEVLVLVTIIFIY
TM 1-18	MAALWPFLGIVAEVLVLV
Tm 1-12	MAALWPFLGIVA
TM 1-6	MAALWP
TM 7-24	FLGIVAEVLVLVTIIFIY
TM 13-24	EVLVLVTIIFIY
TM 19-24	TIIFIY
TM 7-18	FLGIVAEVLVLV
TM 7-12	FLGIVA
TM 13-18	EVLVLV

Figure 7: Interaction between the amino terminus of the Basigin transmembrane domain and MCT1. Sandwich ELISA analysis was performed to test for binding of the transmembrane constructs to endogenous MCT1. Interactions were visualized by incubation with an antibody specific for 6XHis, followed by alkaline phosphatase conjugated secondary antibody and substrate. All runs were performed in duplicate and the average absorbance was plotted. Standard deviations are shown as error bars.

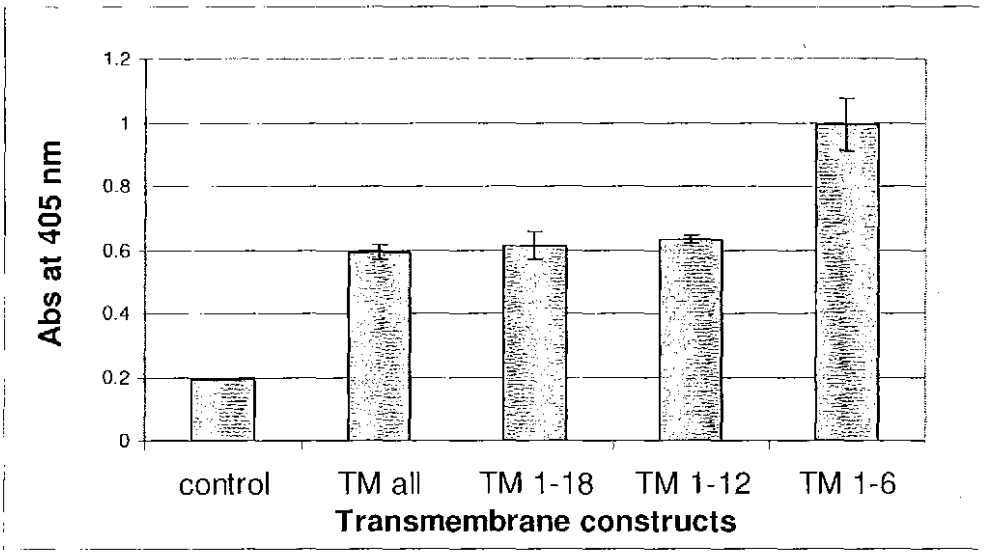
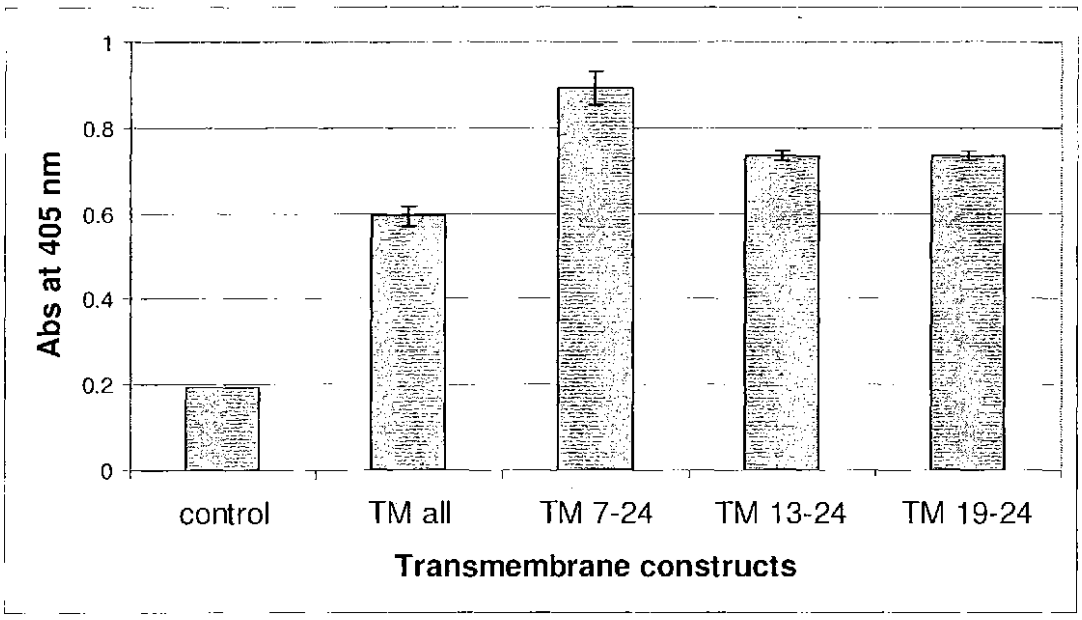


Figure 8: Interaction between the carboxy terminus of the Basigin transmembrane domain and MCT1. Sandwich ELISA analysis was performed to test for binding of the transmembrane constructs to endogenous MCT1. Interactions were visualized by incubation with an antibody specific for 6XHis, followed by alkaline phosphatase conjugated secondary antibody and substrate. All runs were performed in duplicated and the average absorbance was plotted. Standard deviations are shown as error bars.



Kirk *et al.* proposed that the glutamate within the Basigin transmembrane domain is critical for binding to MCT1 (12). This hypothesis was tested by generating probes of the glutamate containing region of the Basigin transmembrane domain for ELISA analysis. Figure 9 shows that no significant binding to MCT1 is observed with the TM 13-18 probe, which contains the glutamate residue in question. The probe containing amino acids 7-12 did not bind to MCT1 as well (Figure 9). Figure 9 shows that TM 1-6 and TM 19-24 are possible candidates for binding to MCT1. The most likely candidate is TM 19-24 because Yurchenko *et al.* showed that the proline of the Basigin transmembrane domain, found in the TM 1-6 probe, interacts with Cyclophilin A (21).

This laboratory recently demonstrated that CyPA interacts with both Basigin and MCT1 in the ELISA analyses (33). The positive signal observed for the TM 1-6 probe may be due to an interaction with CyPA associated with endogenous MCT1 in the wells rather than with MCT1 directly. To test whether TM 1-6 is indeed interacting with CyPA bound to captured MCT1, a competition ELISA was performed using an antibody for Cyclophilin A (Millipore, Billerica, MA) with the TM 1-6. The antibody for Cyclophilin was used at two different concentrations (1:50 and 1:100) to see if it would inhibit the binding of TM 1-6 to captured proteins in the cell. Figure 10, shows that the binding of TM 1-6 was reduced in the presence of the CyPA-specific antibody diluted 1:50 and also at the 1:100 dilution. While this is not statistically significant, these data suggest that the first six amino acids (TM 1-6) are actually binding to CyPA rather than MCT1.

After it was determined that the most likely candidates for interaction with MCT1 were the last six amino acids of the Basigin transmembrane domain, each amino acid was individually mutated to glycine via site-directed mutagenesis. Mutant probes were generated and ELISA analyses were performed. These mutations were compared to the signal for wild-type TM 19-24 to determine which, if any, amino acids were important for the interaction with MCT1. Figure 11 shows that each of the mutagenized amino acids decreases the ability of the transmembrane domain probe to bind MCT1. The probe containing the mutation I20G, significantly reduced the binding of amino acids 19-24 of the Basigin transmembrane domain to MCT1 by 61%. The probes containing the I21G and I23G mutations reduced the binding by 42%, and the probe containing the Y24G mutation reduced binding by 31%. These values are statistically significant but less so than for the I20G mutation.

Figure 9: Analysis of the contribution of the Glutamate at position 13 of the Basigin transmembrane domain in binding to MCT1. Sandwich ELISA analysis was performed. Interactions were visualized by incubation with an antibody specific for 6XHis, followed by alkaline phosphatase conjugated antibody secondary and alkaline Phosphatase substrate. All runs were performed in duplicate and the average absorbance was plotted. Standard deviations are shown as error bars.

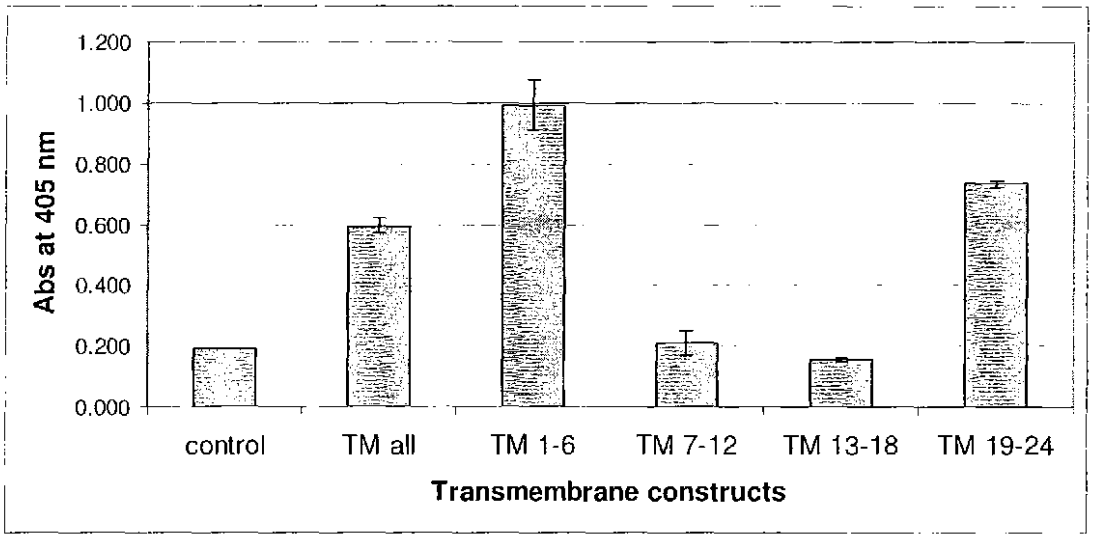


Figure 10: Competition ELISA using an antibody for Cyclophilin A. Sandwich ELISA analysis was performed to test whether an antibody to Cyclophilin A would inhibit binding of the first six amino acids of the transmembrane domain captured mouse retina proteins. Two dilutions of the CyPA-specific antibody were used (1:50 and 1:100). Interactions were visualized by incubation with an antibody specific for 6XHis, followed by alkaline phosphatase conjugated secondary antibody and alkaline phosphatase substrate. All runs were performed in duplicate and the average absorbance was plotted. Standard deviations are shown as error bars.

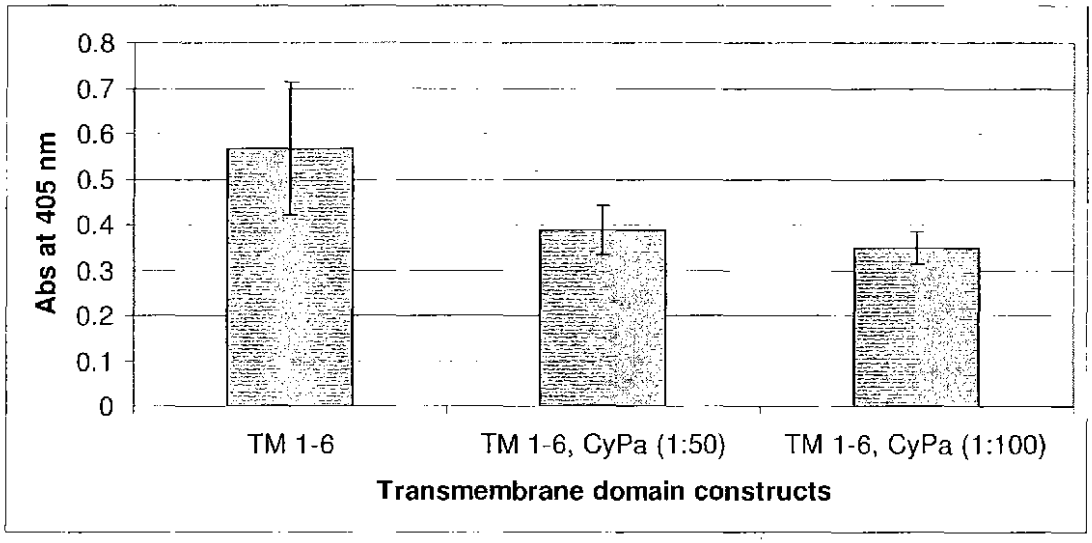
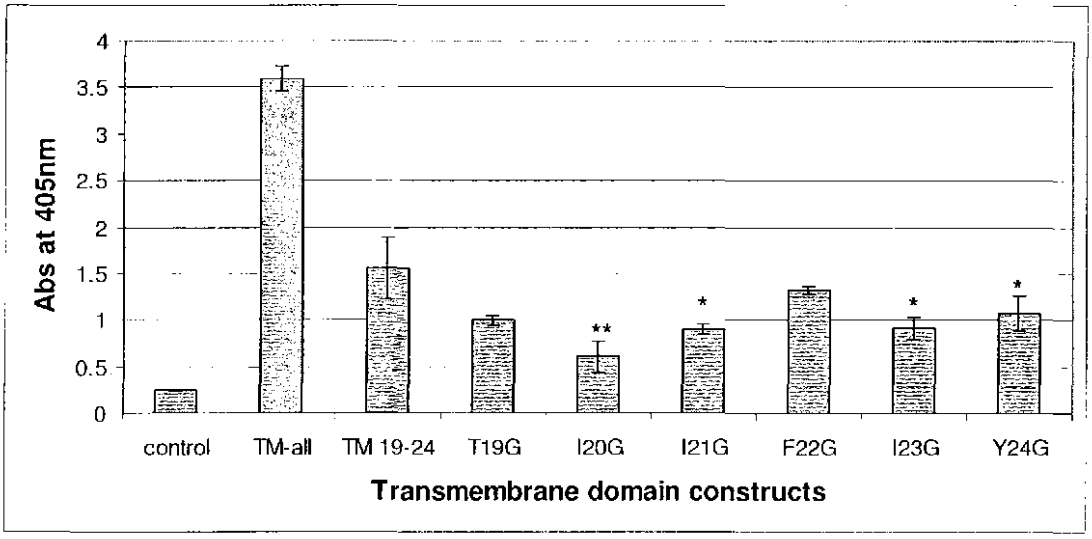


Figure 11: Analysis of the mutagenesis of the last six amino acids of the Basigin transmembrane domain. Amino acids within the Basigin transmembrane domain were individually mutated to the amino acid glycine via site-directed mutagenesis. Probes were generated to test for binding to MCT1 using sandwich ELISA analysis. Interactions were visualized by incubation with an antibody specific for 6XHis, followed by alkaline phosphatase conjugated secondary antibody and alkaline phosphatase substrate. ** indicates p-value< 0.01; * indicates p-value < 0.05 via student T-test. All runs were performed in triplicate and the average absorbance was plotted. Standard deviations are shown as error bars.



Chapter 4 Discussion

The interaction between Basigin and MCT1 is important, because without the presence of Basigin MCT1 does not reach the plasma membrane (21). The Basigin knockout mice are known to have a novel form of retinal dystrophy (14). With this in mind, researchers have investigated whether there is a correlation with absence of Basigin expression and the effect that has on other proteins, specifically MCT1. Immunohistochemsitry analysis was performed by Philp *et al.* on normal mice and Basigin knockout mice (29). The absence of Basigin may contribute to the loss of the ability of MCT1 to target to the plasma membrane, which may contribute to the loss of electrical activity within the retinas of these mice (29). When Basigin is absent MCT1 is unable to leave the intracellular vesicles of both the Müller cells and the photoreceptor cell bodies (29). It is known that MCT1 is primarily involved in the transport of lactate within the outer retina and in the Basigin null mice the inability of MCT1 to perform its job is thought to lead to a decrease in the electrical activity within the retina (29). Therefore, the investigation of the region within the Basigin transmembrane domain that interacts with MCT1 is an important area of research to fully understand how lactate transport occurs in the retina.

Kirk *et al.* proposed that the transmembrane domain of Basigin is important for the interaction with MCT1 (12). The purpose of this study was to determine if the transmembrane domain of Basigin would interact with MCT1 and, if so, to determine which amino acid(s) are important for the interaction with MCT1. The transmembrane domain of Basigin is 24 amino acids in length and contains a charged glutamate residue in the middle (12). A centrally located charged glutamate residue is commonly seen in proteins with multiple transmembrane domains but unusual in a protein with a single transmembrane domain (12). A few single-pass proteins with a charge residue include FcεRIα, FcγRIII (CD160 and the natural killer cell killer activatory receptors (12). The occurrence of these other single-pass transmembrane proteins having a charge residue lead Kirk *et al.* to hypothesize that the centrally located glutamate residue within the Basigin transmembrane domain was important for binding to MCT1 (12).

This study tested whether the transmembrane domain of Basigin interacts with MCT1 and whether the glutamate residue within the transmembrane contributes to this interaction. It was determined that the transmembrane domain of Basigin does indeed interact with Basigin (Figure 7 and 8). Also it was determined that the charged glutamate residue within the transmembrane domain of Basigin does not participate in this interaction (Figure 9). This is important because it indicates that other amino acid(s) are involved in the interaction with MCT1. This led to the testing of other probes that contain portions of the transmembrane domain of Basigin to determine what is necessary

for the interaction of MCT1 to take place. Initially, there were two likely candidates for the interaction with MCT1: TM 1-6 and TM 19-24 (Figures 7 and 8).

Yurchenko *et al.* showed that the proline within the transmembrane domain of Basigin interacts with Cyclophilin A, CyPA, and this amino acid is in the probe TM 1-6 (25). Therefore, these six amino acids are not likely candidates in the interaction with MCT1. Cyclophilins are known to regulate protein trafficking in cells (25). This group determined that when the single proline residue within the transmembrane domain of Basigin is mutated, it reduces the cell surface expression of Basigin because CyPA is no longer able to bind to Basigin (25). These data suggests that CyPA is important in the regulation of Basigin within the cell, because without the presence of cyclophilin A Basigin is unable to be expressed on the cell surface (25). This suggests that cyclophilins have a role in assisting the transport proteins from the ER to the plasma membrane (25). We have demonstrated that CyPA interacts with both MCT1 and Basigin in ELISA analysis (33). We were also able to inhibit, yet not significantly, the binding of TM 1-6 to captured proteins in the cell using an antibody to Cyclophilin A (Millipore). When this antibody was used in a competition ELISA it reduced the binding of TM 1-6 to the captured proteins suggesting that TM 1-6 binds to Cyclophilin and not MCT1 (Figure 10). Further research is needed to determine the exact role that CyPA has on the interaction with MCT1 and Basigin.

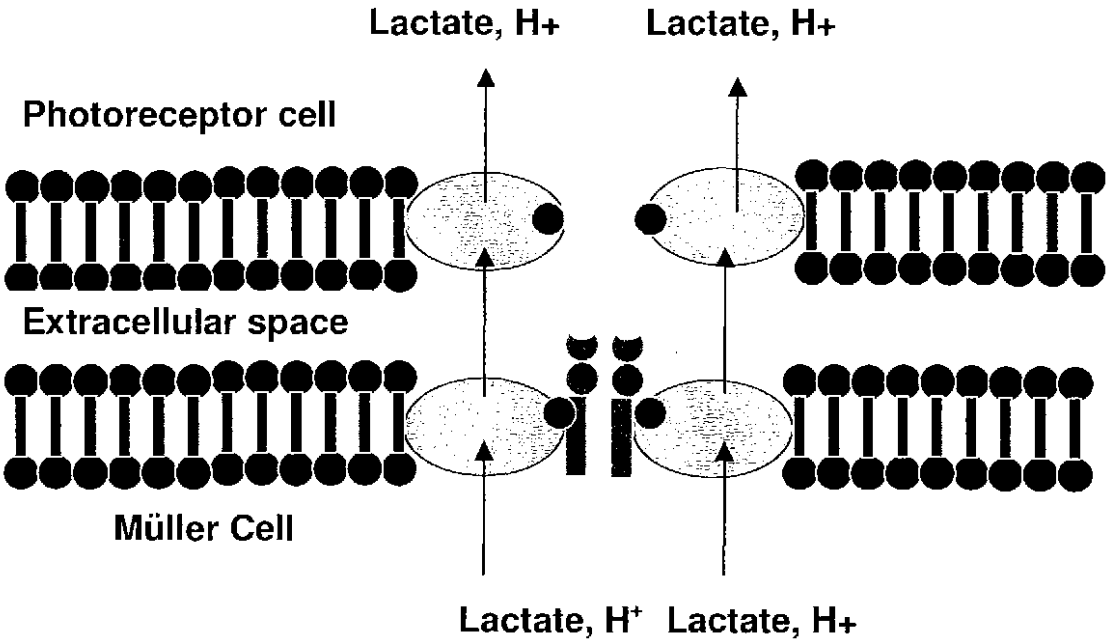
The likely candidates for the interaction with MCT1 are amino acids 19-24 of the transmembrane domain of Basigin (Figure 8). These are good candidates because of their position in the transmembrane of Basigin. It is likely that these amino acids are not embedded in the interior of the lipid bi-layer and stick out into the cytoplasm, making it easy for MCT1 to bind to one or more of those amino acids.

After the likely amino acid candidates were determined to be those last six (TM 19-24), site-directed mutagenesis was performed to determine the contribution of each amino acid to the interaction with MCT1. Each amino acid was mutagenized to glycine and then ELISA analysis was performed to determine if the mutated amino acids would reduce binding to MCT1. We determined that when the amino acid at position 20 (I20G) was mutated it statistically reduced the binding to MCT1 (Figure 11). Mutating amino acids at position 21 (I21G), 23 (I23G), and 24 (Y24G) also significantly reduced binding to MCT1 (Figure 11). Therefore, these data support our hypothesis that the Basigin transmembrane domain does interact with MCT1, and the amino acids that are important for this interaction are isoleucine at position 20, isoleucine at position 21, isoleucine at position 23, and tyrosine at position 24.

The retina produces a large amount of lactate (3). Transport of lactate across the plasma membrane is important for all cells within the retina (24). Wilson *et al.* demonstrated that when Basigin and MCT1 are co-expressed lactate transport increases (24). This is important because the retina transports lactate across the plasma membranes of many cells to maintain proper functions

(3). In the retina, it has been shown that some lactate released by the Müller cells is used for oxidation as respiratory fuel by the photoreceptors. The rest of the lactate is removed via the blood supply (3). Therefore, the presence of both MCT1 and Basigin appears to be necessary for photoreceptor function, because they use lactate as an energy source (30). Lactate that is not used by the photoreceptor cells is transported out of the retina and into the choroid by the RPE (3). This suggests that there is a chemical gradient of lactate within the retina (3). We propose that there is a complex of Basigin, MCT1, and Cyclophilin A on the Müller cells that interacts with a complex of Basigin-2, MCT1, and Cyclophilin A on the photoreceptor cells (Figure 12). This proposed lactate shuttle will enable lactate to be transported to the photoreceptors from the Müller cells. This work determined the amino acids that are important for the interaction between Basigin and MCT1 are isoleucine at position 20, isoleucine at position 21, isoleucine at position 23, and tyrosine at position 24 of the transmembrane domain of Basigin. Further work needs to be performed in order to determine if this also occurs *in vivo.*

Figure 12: Proposed Lactate shuttle within the vertebrate retina. The large blue oval represent MCT 1, the small pink circles represent cyclophilin A, the darker blue structure in the center of the diagram represent Basigin, and the yellow structure in the center of the diagram represents Basigin-2. The Müller cells produce lactate and then lactate is transported through this shuttle to the photoreceptor cells where it is used as an energy source.



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