Characterization of the interaction between Basigin and the pattern recognition receptor TLR4

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CHARACTERIZATION OF THE INTERACTION BETWEEN BASIGIN AND THE PATTERN RECOGNITION RECEPTOR TLR4

by

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Figure 3.2  **Basigin is present in mouse heart.**
Wells were coated with mouse heart proteins or bovine serum albumin (BSA) at 100 µg/mL. An antibody specific for the Basigin protein (Ochrietor et al., 2003) was applied directly to the proteins and an alkaline phosphatase-conjugated goat anti-mouse (AP-GAM) antibody (Pierce/Thermo Scientific) was subsequently applied. Binding was measured using an alkaline phosphatase detection system and subsequent spectrophotometric analysis at 405 nm. Error bars represent standard deviations of the mean. A Student’s T test was performed to compare detection of Basigin in mouse heart to the negative control (BSA). * indicates a p-value of less than 0.05.
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Figure 3.7 **Binding affinity assays for BasTM segment binding to TLR4.**
Endogenous TLR4 was captured and incubated with recombinant Basigin transmembrane domain segments (BasTM 1-6, BasTM 7-18, BasTM 7-12, BasTM 13-18, BasTM 19-24) at 0.5, 1.0, 1.5, 2.5, and 5.0 μM. Binding between endogenous TLR4 and the recombinant Basigin protein was measured using an alkaline phosphatase detection system and spectrophotometric assay at 405 nm. Percent binding was calculated for each concentration and the binding affinity of the recombinant Basigin protein for TLR4 was determined from the slope equation. All runs were performed in triplicate. Error bars represent standard deviations of the mean.

Table 3.1 **Comparison of Basigin transmembrane segment binding affinities for TLR4.**
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ELISA analysis comparing BasTM 1-6 to recombinant proteins containing consecutive amino acid substitutions to glycine (G) within the BasTM 1-6 segment.

All of these recombinant proteins were compared by the amount of binding to TLR4. An antibody specific for the TLR4 protein was plated followed by an application of mouse heart lysate. The appropriate recombinant Basigin proteins were applied (BasTM 1-6 M1G 6XHis, BasTM 1-6 A2G 6XHis, BasTM 1-6 A3G 6XHis, BasTM 1-6 L4G 6XHis, BasTM 1-6 W5G 6XHis, and BasTM 1-6 P6G 6XHis) and a primary antibody specific for the six histidine tag was applied. An alkaline phosphatase-conjugated goat anti-mouse (AP-GAM) antibody was subsequently applied. Binding was measured using an alkaline phosphatase detection system and subsequent spectrophotometric analysis at 405 nm. Error bars represent standard deviations of the mean. A Student’s T test was performed to compare binding of Basigin transmembrane segments to TLR4 in mouse heart to the control protein. * indicates a p-value of less than 0.05.

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ELISA analysis comparing BasTM 13-18 to recombinant proteins containing consecutive amino acid substitutions to glycine (G) within the BasTM 13-18 segment.

All of these recombinant proteins were compared by the amount of binding to TLR4. An antibody specific for the TLR4 protein was plated followed by an application of mouse heart lysate. The appropriate recombinant Basigin proteins were applied (BasTM 13-18 E13G 6XHis, BasTM 13-18 V14G 6XHis, BasTM 13-18 L15G 6XHis, BasTM 13-18 V16G 6XHis, BasTM 13-18 L17G 6XHis, and BasTM 13-18 V18G 6XHis) and a primary antibody specific for the six histidine tag was applied. An alkaline phosphatase-conjugated goat anti-mouse (AP-GAM) antibody was subsequently applied. Binding was measured using an alkaline phosphatase detection system and subsequent spectrophotometric analysis at 405 nm. Error bars represent standard deviations of the mean. A Student’s T test was performed to compare binding of Basigin transmembrane segments to TLR4 in mouse heart to the control protein. * indicates a p-value of less than 0.05.
Abstract

Toll-like receptors (TLRs) are a major group of pattern recognition receptors expressed on the surface of immune cells that recognize molecular patterns associated with all classes of pathogenic microorganisms. TLR4 recognizes the lipopolysaccharide component of Gram-negative bacterial cell walls and is the only TLR known to induce signaling through both the MyD88 and TRIF pathways. Basigin, a ubiquitous cell adhesion molecule, is a member of the immunoglobulin superfamily that has the ability to influence cell signaling mediated by the MyD88 and TRIF pathways, the same signaling pathways induced by the TLR4 receptor protein. Analysis of the Basigin protein sequence indicates the presence of a hydrophilic glutamate residue within the hydrophobic transmembrane domain, but no consensus binding sites for MyD88 or TRIF. The purpose of this study was to determine if Basigin uses TLR4 for signal transduction. It is hypothesized that Basigin interacts with TLR4 and that the glutamate residue plays a role in the interaction. Enzyme-linked immunosorbent binding assays were performed using endogenous TLR4 and recombinant Basigin proteins. These analyses demonstrated that binding of Basigin to TLR4 was significantly greater than that of the control protein and that the glutamate residue in the Basigin transmembrane domain does play a role in the interaction between Basigin and TLR4 as well as many hydrophobic residues in the Basigin transmembrane domain. The data suggest that Basigin interacts with TLR4 to influence signaling cascades using MyD88 and TRIF.
Chapter 1 - Introduction

Immune-mediated pathogen recognition and destruction occurs via collaboration of the innate and adaptive immune responses. The immune system is collectively comprised of these two processes to facilitate host protection from deleterious factors within the environment. The innate immune response is a rapidly generated early line of defense that occurs as a result of pathogen recognition and works using antimicrobial peptides and the complement system to eliminate pathogens (reviewed by Hendry et al., 2013). The innate response is often sufficient in eliminating the identified invading pathogen, but in circumstances where the innate response is insufficient, the adaptive response ensues and functions until the pathogen is successfully eliminated (reviewed by Hendry et al., 2013). Unlike the immediate response of innate immunity, adaptive immunity is long lasting and responsible for the production of antibodies (reviewed by Hendry et al., 2013). The innate immune system is initiated by the recognition of pathogenic organisms such as bacteria, viruses, fungi, or parasites and involves various proteins including immune cell surface receptors. Immune cell surface receptors directly interact with the pathogen or products of the pathogen for the purpose of self- vs. non-self-discrimination, and thus these recognizable products are specific to pathogenic entities. After pathogen recognition, immune cell receptors are known to induce signaling cascades that result in the production of pro-inflammatory cytokines and subsequent inflammation. Cytokines generate inflammatory responses by acting as signaling proteins that can recruit additional cells to the site of injury or by increasing the number of white blood cells known as macrophages at the site of infection for the purpose of increased phagocytosis (reviewed by Hendry et al., 2013). Inflammation is the process by which heat, redness, pain, swelling, and even
loss of function can occur at the local site of infection. A superabundance of inflammation can result in septic shock (reviewed by Wiersinga et al., 2014).

Many key molecules are involved in the intricate processes of both innate and adaptive immunity. Cell adhesion molecules are thought to play essential roles in the immune response in addition to facilitating cell attachment, specifically by influencing pro-inflammatory intracellular signaling events and subsequent production of cytokines (reviewed by Agrawal and Yong, 2011). Cell adhesion molecules also play large roles in allowing immune cells to travel to and enter tissues from the bloodstream (reviewed by Agrawal and Yong, 2011).

Cell adhesion molecules (CAMs) are transmembrane proteins involved in extracellular recognition and cell attachment and can be categorized according to whether or not they utilize calcium (Sonderegger, 1998). Molecules that are members of the integrins, selectins, or cadherins are all calcium dependent, whereas members of the immunoglobulin superfamily are calcium independent molecules (Sonderegger, 1998; Figure 1.1). Molecules of the immunoglobulin superfamily are known for their roles in facilitating cell adhesion in addition to activating signaling pathways within the cell (Pollerberg et al., 2013). The members of the immunoglobulin superfamily can be defined by extracellular regions that contain immunoglobulin-like domains formed by anti-parallel β strands connected by loops of various lengths (Sonderegger, 1998). Disulfide bridges link β-strands to form the characteristic immunoglobulin-like domains and are therefore the distinguishing factors for this class of calcium-independent cell adhesion molecules (Sonderegger, 1998).
Figure 1.1 Comparison of calcium dependent and calcium independent cell adhesion molecule domains. Members of the immunoglobulin superfamily are calcium independent and contain disulfide bridges known to form the characteristic immunoglobulin loop-like domains (top row). Other cell adhesion molecule families are calcium dependent and do not contain disulfide bridges (bottom row; Sonderegger, 1998).
Basigin gene products are highly glycosylated members of the immunoglobulin superfamily and function as cell adhesion molecules. Basigin gene products contain immunoglobulin-like domains within the extracellular region, a hydrophobic single-pass transmembrane domain, and a short cytoplasmic tail. The Basigin gene contains eight exons from which two isoforms are produced via alternative splicing (Ochrietor et al., 2003; Figure 1.2). Exons 1 through 7 encode the protein known as Basigin, which contains two immunoglobulin-like domains (Ig 1 and 2) that comprise the extracellular region. Exons 1 through 7, in addition to exon 1A (located between exon 1 and 2), encode the Basigin-2 isoform, which contains three immunoglobulin-like domains that comprise the extracellular region of the protein. Basigin-2 is a retina-specific isoform, while Basigin is ubiquitously expressed (Figure 1.3). Although Basigin is best known for its role in cell adhesion, various studies have indicated that Basigin is also involved in cancer metastasis, regulation of associated signaling cascades, and pro-inflammatory intracellular signaling cascades via interactions with the cyclophilin protein (Wei et al., 2010; Sidhu et al., 2010).

Basigin is thought to play an essential role in proper retinal function (Ochrietor et al., 2001; Philp et al., 2003). In the retina, the Basigin gene codes for two polypeptides via splice variation known as Basigin and Basigin-2 (Ochrietor et al., 2003). Basigin contains two immunoglobulin-like loop domains and is ubiquitously expressed, whereas Basigin-2 contains three immunoglobulin-like loop domains and is a retina-specific isoform of the Basigin gene (Ochrietor et al., 2003). It has been demonstrated that Basigin interacts with Basigin-2 in the retina for the purpose of cell attachment (Ochrietor et al., 2008). It is proposed that this interaction exists to facilitate proper nutrition of photoreceptor cells so that phototransduction, and thus, proper vision can occur (Philp et al., 2003). Both
Figure 1.2 Coding regions of the Basigin gene. The Basigin gene contains eight exons. Exons 1 through 7 encode the Basigin protein consisting of two extracellular immunoglobulin domains. Exons 1 through 7 in addition to exon 1A encode the Basigin-2 protein consisting of the two identical immunoglobulin domains found in Basigin in addition to a third immunoglobulin domain that is specific to the Basigin-2 protein (adapted from Ochrietor et al., 2003).
Figure 1.3  Western blot showing the expression of Basigin gene products in mouse tissue. An antibody that recognized both isoforms of Basigin indicates that the Basigin protein is ubiquitously expressed (top panel). Basigin is approximately 45 kDa in molecular weight while Basigin-2 is approximately 55 kDa in molecular weight. An antibody specific to the Basigin-2 isoform shows that Basigin-2 is a retina-specific isoform (bottom panel). R = retina, B = brain, Lu = lung, Li = liver, K = kidney, H = heart, S = spleen, U = uterus, T = testis, SM = skeletal muscle (Ochrietor et al., 2003).
Basigin and Basigin-2 interact with monocarboxylate transporter 1 (MCT-1) via hydrophobic interactions of the membrane-spanning domains (Finch et al., 2009). Members of the MCT family transport lactate and other monocarboxylates across the plasma membrane (Halestrap and Price, 1999). The expression pattern of Basigin and Basigin-2 in the retina differ in that Basigin is expressed by Müller glial cells and Basigin-2 is expressed specifically by photoreceptor cells (Ochrietor et al., 2003). It is thought that Basigin interacts with Basigin-2 to tether these two cell types together so that lactate is able to move from Müller glial cells and into the photoreceptor cells (Philp et al., 2003). Lactate can be utilized by photoreceptor cells to fuel oxidative phosphorylation and generate energy for the process of phototransduction (Gladden, 2004).

Behavioral studies based on visual cues showed sensory malfunctions associated with Basigin-null animals, which are thought to exist due to the absence of Basigin-mediated cell adhesion in the brain (Naruhashi et al., 1997). Basigin-null animals are also less sensitive to changes in light when compared to wild type animals in that the null animals did not show any electroretinogram (ERG) responses (Hori et al., 2000; Ochrietor et al., 2002). Studies have shown that animals in which the gene for Basigin is deleted lack proper maturation of the retina and exhibit retinal degeneration (Ochrietor et al., 2001).

In addition to cell attachment, members of the immunoglobulin superfamily are known to influence cell signaling. In humans, Basigin has been implicated as an essential regulatory component in signaling pathways associated with cancer development and metastasis (Sidhu et al., 2010). Lung epithelial tumor cells in which Basigin was increased showed increased amounts of signaling through the Wnt/beta-catenin signaling pathway, while decreases in Basigin resulted in decreased intracellular signaling (Sidhu et al., 2010).
Basigin mRNA and protein were shown to be the most frequently up-regulated products in metastatic cancer cells isolated from bone marrow, a correlation indicative of tumor progression (reviewed by Nabeshima et al., 2006). These findings demonstrate that Basigin may behave as a regulatory component in the intracellular signaling and cell attachment involved in tumor metastasis.

There is strong evidence for the involvement of Basigin in immune response signaling and subsequent inflammation. In humans, Basigin was shown to induce pro-inflammatory intracellular signaling cascades in response to cyclophilin, a protein that is characterized as a cytosolic peptidylprolyl isomerase that functions to catalyze the isomerization of peptide bonds from trans to cis at proline amino acid residues (Wei et al., 2010). Elevated cyclophilin levels are associated with atherosclerotic plaques, in which monocytes and other cells secrete cyclophilin in response to reactive oxygen species. Cyclophilin acts as a chemoattractant to recruit other immune cells and causes induction of signaling inside the cell (Wei et al., 2010). Reactive oxygen species in immune cells are commonly associated with pathogen internalization and precedes the inflammatory response (Wei et al., 2010). Cyclophilin was shown to induce the MAP kinase pathway through the Basigin protein, resulting in the activation of the transcription factor NF-kappaB and subsequent release of pro-inflammatory cytokines. (Wei et al., 2010). The study by Wei et al. demonstrated that the effects of cyclophilin were terminated when Basigin was down-regulated, thus indicating that Basigin is required for mediating the effects of cyclophilin.

Pattern recognition receptors (PRRs) are proteins expressed on the plasma membrane of macrophages and other immune cell types that are extensively involved in
pro-inflammatory signaling cascades associated with the innate immune response (reviewed by Kawasaki et al., 2014). Some PRRs can be internalized within the cell for the purpose of recognizing intracellular pathogens (reviewed by Kawasaki et al., 2014). PRRs function to recognize common patterns that are characteristic of groups of pathogens known as pathogen associated molecular patterns (Owen et al., 2013). These receptors elicit pro-inflammatory signaling cascades that ultimately work to generate and secrete chemical signals known as cytokines. Cytokines label pathogens appropriately for immune-mediated destruction, recruit immune cells to the site of infection, increase vascular permeability, and allow immune cells to communicate effectively, all of which elicits inflammation as part of the innate immune response (Owen et al., 2013). Pattern recognition receptors often function as the first line of defense against foreign invaders and include four classes of receptors: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), Retinoic acid-inducible gene I-like receptors (RLRs), and Nod-like receptors (NLRs) (Owens et al., 2013).

Toll-like receptors are perhaps the most well understood and extensively studied PRRs. Collectively, the toll-like receptor family recognizes all of the major classes of pathogens including those from bacteria, viruses, fungi, and parasites (reviewed by Kawasaki et al., 2014; Figure 1.4). TLRs have single membrane-spanning domains and extensive extracellular domains consisting of many leucine-rich repeats that make up the ligand-binding region of the receptor. The cytoplasmic domains interact with adaptor proteins via toll-interferon-1 receptor (TIR) domains to induce signaling (Lu et al., 2008). When members of the TLR family bind to their specific ligands, the receptors dimerize either with themselves or, in some cases, with other members of the TLR family (Lu et al.,
Figure 1.4 Toll-like receptor family members and their ligands. All toll-like receptors contain leucine rich repeats that comprise the extracellular domain and a TIR signaling domain comprising the cytoplasmic tail (Armant and Fenton, 2002).
TLR4 specifically recognizes lipopolysaccharide, a component of Gram-negative bacterial cell walls (Lu et al., 2008). TLR4 is the only family member that can be internalized from the plasma membrane or remain at the plasma membrane after binding to lipopolysaccharide, and thus has the ability to induce two different intracellular signaling cascades, one from each of the two locations (Owen et al., 2013). If TLR4 binds to LPS at the plasma membrane, the MyD88 (Myeloid differentiation factor 88) signaling pathway is used (Owen et al., 2013). If TLR4 is internalized in an endosome, the TRIF (TIR-domain-containing adaptor-inducing IFN-beta factor) signaling pathway is used (Owen et al., 2013). The transcription factor NF-kappaB is activated via both the MyD88 and TRIF signaling pathways and initiates the transcription of pro-inflammatory cytokines. These cytokine proteins function as part of the innate immune system.

TLR proteins form protein complexes via their extracellular domains that function in the recognition of and binding to their specific ligands. TLR4 forms a complex with CD14 and MD2 via its extracellular domain (Parham, 2015). CD14 transfers lipopolysaccharide to the TLR4 extracellular domain (Parham, 2015). This transfer is then recognized by MD-2, which allows for the protein to dimerize and subsequently induce signaling (Parham, 2015). TLRs have unique associated signaling pathways determined by the TLR region (TIR domain) and the associated adaptor protein involved in the TLR cytoplasmic domain complex (Owen et al., 2013). When members of the TLR family dimerize after binding to their ligand, TIR domains change conformation and bind to the specific adaptor protein that will be associated with that particular TLR cytoplasmic domain, which activates the appropriate signaling cascades specific to the associated adaptor protein (Figure 1.5). Two of these key adaptors are MyD88 and TRIF (reviewed by
Figure 1.5 TLR4 signaling. Intracellular signaling through the MyD88 pathway in response to activation of TLR4 by LPS (Parham, 2015).
Most TLRs (especially those involved in bacterial recognition) bind to the adaptor MyD88 to initiate MyD88-dependent signaling pathways (reviewed by Kawasaki et al., 2014). TLR4 has the unique ability to bind to both MyD88 and TRIF adaptor proteins and initiate both MyD88 and TRIF-dependent signaling pathways (reviewed by Kawasaki et al., 2014). TLR4 binds MyD88 when it is embedded in the plasma membrane or binds TRIF when it is located within endosomes following internalization (Zanoni et al., 2011). Two other adaptors are necessary for proper functioning of the TLRs and include TIRAP (TIR-domain containing adaptor protein) and TRAM (TRIF-related adaptor molecule; Parham, 2015). Both are adaptor proteins that recruit either MyD88 (TIRAP) or TRIF (TRAM), depending on where the TLR protein is located. Thus, TIRAP and TRAM dictate which signaling pathway is utilized in addition to the receptor location.

The TLR4 protein is expressed on the surface of immune cells of both myeloid and lymphoid subsets, including monocytes, macrophages, and dendritic cells (both mature and immature) in mice (Vaure and Liu, 2014). TLR4 is expressed exclusively by microglia in mouse brain and is also expressed in mouse heart, kidney, liver, lung, pancreas, smooth muscle, and spleen (Vaure and Liu, 2014). TLR4 had greater expression in mouse heart and lung compared to other mouse organs (Vaure and Liu, 2014). In rats, TLR4 is detected in individuals with normal blood pressure but is upregulated in individuals with hypertension, suggesting that it may play a role in cardiovascular disorders (Eissler et al., 2011; Figure 1.6). This upregulation was reversed when the hypertension was reduced with a dose of Ramipril (Eissler et al., 2011).
Figure 1.6 TLR4 is expressed in rodent cardiomyocytes and is upregulated in response to hypertension. Immunohistochemistry showing expression of TLR4 in rat cardiomyocytes. A. TLR4 was detected in control rats that were normotensive (WKY). B. Rats that experienced hypertension (SHR) showed an upregulation of TLR4 in cardiomyocytes compared to normotensive rats. C. Ramipril was given as a treatment to reduce hypertension in SHR rats simultaneously reversing TLR4 upregulation (Eissler et al., 2011).
In the retina, TLRs were characterized as fully functional receptors with the capability to induce an innate immune response following stimulation with pathogenic ligands in a 661W photoreceptor cell line (Singh and Kumar, 2015). In that study, TLRs were localized to both Müller glial cells and photoreceptor cells (Singh and Kumar, 2015).

In 2014, Jeong et al. reported that Basigin influenced the signaling pathways mediated by accessory proteins MyD88 and TRIF. Basigin was linked to NF-kappaB activation via both MyD88 and TRIF pathways, in that signaling occurred in the presence of Basigin, but when Basigin was knocked down, signaling was decreased by about 85 percent (Jeong et al., 2014). The same study indicated that when an antibody specific for Basigin was applied, signaling was also decreased by about 40 percent (Jeong et al., 2014).

Although the MyD88 signaling pathway is commonly induced by most of the TLRs in response to ligand binding, the only member of the TLR family that has the ability to induce signaling through both MyD88 and TRIF signaling pathways is TLR4.

Despite the influential nature of the Basigin protein in the pro-inflammatory signaling cascades associated with MyD88 and TRIF pathways, Basigin has not been shown to directly interact with the TLR4 protein. Both Basigin and TLR4 are expressed by many of the same tissues in mice (Ochrietor et al., 2003, Eissler et al., 2011). The TLR4 protein forms extensive complexes with other proteins via both the extracellular and cytoplasmic domains to induce signaling in response to the recognition of LPS.

The purpose of this study is to determine if an interaction exists between Basigin and TLR4 and to determine which amino acid residues in the Basigin transmembrane domain may be important for stabilizing the interaction. It is hypothesized that Basigin will interact with TLR4 and that the charged glutamate residue within the Basigin
transmembrane domain will be important for stabilizing the interaction. Investigating these aspects of immune-mediated pro-inflammatory signaling events will collectively increase the understanding of the interactions that occur at the cellular level during the process of inflammation. Understanding the role that Basigin plays in TLR4 signaling pathways and how it is connected to the development of inflammation through TLR4 could be crucial for a better understanding of how these processes occur.
Chapter 2 - Methods

Expression plasmids

Expression plasmids containing the cDNA for the entire Basigin transmembrane domain (pET102-BasTM-all), as well as the truncated portions of the domain (pET102-BasTM-1-6, pET102-BasTM-7-12, pET102-BasTM-13-18, and pET102-BasTM-19-24) were generated previously (Finch et al., 2009). In addition, the plasmid containing the cDNA for the entire Basigin transmembrane domain with the coding sequence for glutamate mutated to glycine (pET102-BasTM-all-E13G) and the plasmids in which the coding sequences for the individual amino acids are each mutated to glycine (pET102-BasTM-1-6-M1G, pET102-BasTM-1-6-A2G, pET102-BasTM1-6-A3G, pET102-BasTM1-6-L4G; pET102-BasTM1-6-W5G, and pET102-BasTM-1-6-P5G) were previously generated (Finch et al., 2009). For these plasmids, the cDNA was cloned into the bacterial expression plasmid pET102 (Invitrogen Corporation, Carlsbad, CA). This plasmid allows for recombinant protein expression using BL21 cells (Invitrogen Corporation) in which the protein product contains six histidines at the carboxy-terminus. The plasmid codes for 157 amino acids, which allows the six to 24 amino acids of the hydrophobic transmembrane domain to be expressed as soluble proteins (Finch et al., 2009).

Site Directed Mutagenesis

For mutation of the coding sequences of individual amino acids within the pET102-BasTM 7-12 and pET102 BasTM 13-18 plasmids, 1 μl dNTP mix, 1 μl pET102 BasTM 7-12 plasmid DNA or pET102 BasTM 13-18 plasmid DNA (Finch et al., 2009), 5 μl 10X Buffer, 1 μl forward primer (Table 2.1), 1 μl reverse primer (Table 2.1), and 3 μL Quick Solution
Table 2.1 Basigin transmembrane domain mutagenesis primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BasTM 7-12 F7G F</td>
<td>5’-GGGAATTGATCCCTTCACCGGCCTAGGCACTCGTGCTGTAAG</td>
</tr>
<tr>
<td>BasTM 7-12 F7G RV</td>
<td>5’-CTTAGCCACGATGCTAGGGCCTAGGCACTCGTGCTGTAAGGGATCAATCCC</td>
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<tr>
<td>BasTM 7-12 L8G F</td>
<td>5’-GAAATTGATCCCTTCACCGGCCTAGGCACTCGTGCTGTAAGGGGCGAG</td>
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<tr>
<td>BasTM 7-12 L8G RV</td>
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<td>BasTM 7-12 I10G F</td>
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<tr>
<td>BasTM 7-12 I10G RV</td>
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<tr>
<td>BasTM 7-12 V11G F</td>
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<tr>
<td>BasTM 7-12 V11G RV</td>
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</tr>
<tr>
<td>BasTM 13-18 E13G RV</td>
<td>5’-CTTAACCAACACCAGGACCCCGGCTAGGCACTCGTGCTGTAAGGGGATCAATTC</td>
</tr>
<tr>
<td>BasTM 13-18 V14G F</td>
<td>5’-GAAATTGATCCCTTCACCGGCCTAGGCACTCGTGCTGTAAGGGGCGAG</td>
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<td>5’-CTCGCCCTTAGACGATGCTAGGGCCTAGGCACTCGTGCTGTAAGGGGCGAGCTCAAGC</td>
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<tr>
<td>BasTM 13-18 V16G RV</td>
<td>5’-CTCGCCCTTAGACGATGCTAGGGCCTAGGCACTCGTGCTGTAAGGGGCGAGCTCAAGC</td>
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<td>BasTM 13-18 L17G F</td>
<td>5’-CCCTTCACCGAGGTCCGCTGCGATGCTAGGGCCTAGGCACTCGTGCTGTAAGGGGCGAGCTC</td>
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<td>BasTM 13-18 L17G RV</td>
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<td>BasTM 13-18 V18G RV</td>
<td>5’-GAGCTCGCCCTTAGACGATGCTAGGGCCTAGGCACTCGTGCTGTAAGGGGCGAGCTCAAGC</td>
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</table>
(QuickChange® II XL Site Directed Mutagenesis Kit, Stratagene, La Jolla, CA) were combined in a final volume of 50 μl. One microliter of PfuUltra™ high-fidelity DNA Polymerase (Stratagene) was added and the solution was subjected to thermal cycling in which the following cycling parameters were utilized: 95°C for 1 minute followed by 18 cycles of 95°C for 50 seconds, 60°C for 50 seconds, 68°C for 5 minutes and finally 1 cycle of 68°C for 7 minutes. A restriction digest was performed in which 1 μl of the restriction enzyme Dpn I was added to each reaction sample followed by incubation at 37°C for 1 hour.

The resulting plasmids were then transformed into bacterial cells. Two microliters of the appropriate plasmid DNA were added into 50 μl XL10-Gold® Ultracompetent cells (Strategene). The solution was incubated on ice for 20 minutes followed by incubation at 42°C for 30 seconds. Two hundred fifty microliters SOC broth was added and the cells were incubated at 37°C for 1 hour with shaking. The contents of the solution were transferred to an LB agar plate containing carbenicillin (50 μg/mL) and incubated at 37°C overnight.

Selected colonies of XL10-Gold® Ultracompetent cells (Strategene) were isolated from the LB agar plate and added to 3 mL LB broth. The solution was incubated overnight at 37°C with shaking.

Plasmids were purified using the QIAprep miniprep protocol (QIAGEN Corporation, Valencia, CA). One thousand five hundred microliters of culture (from each culture incubated overnight) was centrifuged at 13,000 rpm for 1 minute. Two hundred fifty microliters buffer P1 (QIAprep® Miniprep, Qiagen) were added to resuspend the pelleted
bacterial cells, followed by the addition of 250μL buffer P2. The tubes were inverted 6 times, followed by the addition of 350μL buffer N3. The tubes were inverted 6 times, followed by centrifugation at 13,000 rpm for 10 minutes. Supernatants were applied to spin columns, followed by centrifugation at 13,000 rpm for 1 minute. The flow-through was discarded and 500μL buffer PB was applied to the spin columns. The spin columns were centrifuged at 13,000 rpm for 1 minute and the flow-through was discarded. Seven hundred fifty microliters buffer PE were applied to the spin columns, followed by centrifugation at 13,000 rpm for 1 minute. The flow-through was discarded and the spin columns were centrifuged once again in the absence of buffer at 13,000 rpm for 1 minute. Fifty microliters buffer EB were applied to the spin columns, followed by incubation at room temperature for 1 minute and subsequent centrifugation at 13,000 rpm for 1 minute. The flow-through containing eluted plasmid DNA was saved.

Sequences were verified by the Operon-Eurofin Corporation (Louisville, KY) and subsequently analyzed using the ExPASy Bioinformatics Research Portal maintained by the Swiss Institute of Bioinformatics (web.expasy.org/translate).

**Recombinant Protein Expression and Purification**

Two microliters of appropriate plasmid were added into 50μL of BL21 *E.coli* cells (Invitrogen Corporation, Carlsbad, CA). The solution was incubated on ice for 20 minutes followed by incubation at 42°C for 30 seconds. Two hundred fifty microliters of SOC broth was added and the cells were incubated at 37°C for 1 hour with shaking. The cells were transferred to 10 mL LB broth containing carbenicillin (50 μg/mL) and incubated at 37°C overnight with shaking. The entire overnight culture containing plasmid was added to 250
mL LB broth containing carbenicillin (50 μg/mL) and incubated for 3 hours at 37°C with
shaking. Two hundred fifty microliters of 1M IPTG was added and the culture was
incubated overnight at 37°C with shaking.

For purification, two hundred fifty milliliters of overnight culture were centrifuged
at 3,000 rpm for 15 minutes. Pelleted bacterial cells were re-suspended in 10 mL Talon
Xtractor buffer (Clontech, Mountain View, CA) in addition to lysozyme (100 microliters of
10 mg/mL) and DNase I (20 microliters) and the solution was incubated for 10 minutes at
room temperature. The solution was centrifuged for 20 minutes at 10,000 rpm to form a
protein lysate. The protein lysate was then applied to Talon metal affinity resin (Clontech)
and purified via affinity chromatography. Protein was collected in 0.5 mL fractions and the
content was analyzed at 280 nm.

Protein concentration was determined using the Bradford Coomassie protein assay
(Pierce Biotechnology, Rockford, IL, USA). A standard curve was generated by using serial
dilutions of bovine serum albumin (BSA) from 0.2 mg/mL to 2.0 mg/mL. Five microliters
of each BSA dilution was transferred to the wells of a 96 well plate in addition to 5
microliters of recombinant protein fractions. Absorbance at 595 nm was measured using
a Bio-Tek plate reader (Biotek Instruments, Winooski, VT) and the average absorbance
was obtained for each duplicate BSA dilution. The averages were used to generate a graph
in which the y-axis was labeled absorbance at 595 nm and the x-axis was labeled protein
concentration in mg/mL. An equation was obtained from the logarithmic regression of the
average BSA dilution absorbance values and the unknown recombinant protein
concentrations were determined from the equation.
**Mouse Tissue Protein Lysates**

Mice were sacrificed according to accepted protocols and tissue (retina, brain, or heart) was removed and immediately placed into 2 mL PBS. The tissue was homogenized and the protein concentration was determined via the Bradford Coomassie protein assay previously described.

**Enzyme-Linked Immunosorbent Assay (ELISA)**

One hundred microliters of anti-TLR4 capture antibody (Pierce/Thermo Scientific; 0.05 μl antibody/100 μl PBS) was added to wells of a 96 well plate. Several wells received 100 microliters of PBS and no antibody. The plate was incubated at 4°C overnight. Unbound capture antibody was removed followed by three washes with approximately 200 μl PBS-T. One hundred microliters of bovine serum albumin (BSA; Pierce/ThermoScientific, Rockford, IL; diluted 1:10 in PBS) was added to wells of the plate. The plate was incubated at 37°C for 30 minutes. The BSA was removed from the wells and they were washed three times with approximately 200 μl PBS-T. One hundred microliters of endogenous proteins from mouse heart (diluted to 100 μg/mL in PBS) were added to wells of the plate. The plate was incubated at 37°C for 30 minutes. Unbound mouse heart proteins were removed and the wells were washed three times with approximately 200 μl PBS-T. One hundred microliters of the appropriate recombinant protein were added to wells of the plate. The plate was incubated at 37°C for 30 minutes. Unbound recombinant proteins were removed and the wells were washed three times with approximately 200 μl PBS-T. One hundred microliters of anti-6x-His primary antibody (diluted 1:1000 in PBS) was added to wells of the plate. The plate was incubated at 37°C for 30 minutes. Unbound
primary antibody was removed and the wells were washed three times with approximately 200 μl PBS-T. One hundred microliters of alkaline phosphatase-conjugated goat-anti-mouse secondary antibody (AP-GAM; Pierce/ThermoScientific; diluted 1:1000 in PBS) was added to wells of the plate. The plate was incubated at 37°C for 30 minutes. Unbound secondary antibody was removed and the wells were washed three times with approximately 200 μl PBS-T. One hundred microliters of AP substrate (PNPP; Thermo Fisher Scientific, Rockford, IL) was added to wells of the plate. The reactions were stopped by the addition of 50 μl 2N NaOH to all wells. The absorbance in each well was measured at 405 nm on a Bio-Tek plate reader (Biotek Instruments, Winooski, VT).

To detect the presence of TLR4 and Basigin in mouse heart, one hundred microliters of endogenous proteins from mouse heart (diluted to 100 μg/mL in PBS) were added to wells of the plate. The plate was incubated at 4°C overnight. Unbound mouse heart proteins were removed. One hundred microliters of either anti-TLR4 antibody (Pierce/Thermo Scientific) or anti-Basigin antibody (Ochrietor et al., 2003; 0.05 μl antibody/100 μl PBS) were added. The plate was incubated at 37°C for 30 minutes. Unbound antibody was removed and the wells were washed three times with approximately 200 μl PBS-T. One hundred microliters of the appropriate alkaline phosphatase conjugated secondary antibody (AP-GAM or AP-GAR; Pierce/Thermo Scientific; diluted 1:1000 in PBS) was applied to the wells, followed by an incubation at 37°C for 30 minutes. Unbound secondary antibody was removed and the wells were washed three times with approximately 200 μl PBS-T. One hundred microliters of AP substrate (PNPP; Thermo Fisher Scientific) was added to wells of the plate. The reactions
were stopped by the addition of 50 μl 2N NaOH to all wells. Absorbance in each well was measured at 405 nm using a Bio-Tek plate reader.
Chapter 3 - Results

The aim of the present study was to investigate the relationship between the Basigin transmembrane domain and TLR4. To achieve this aim, binding assays were employed to determine if the transmembrane domain of Basigin physically interacts with TLR4. Expression plasmids containing the cDNA for the entire transmembrane domain of Basigin gene products, or truncated portions of the domain, cloned in frame of a six histidine carboxy-terminal epitope tag were previously generated (Finch et al., 2009) and used in the present study. Recombinant protein expression was achieved by transformation of BL21 cells (Invitrogen Corporation), followed by the induction of protein synthesis and protein purification via affinity chromatography. Although the transmembrane domain sequence for Basigin is highly hydrophobic, a soluble recombinant protein is generated, because the vector codes for 157 amino acids that mask the hydrophobic nature of that sequence (Finch et al., 2009).

To effectively utilize the recombinant Basigin transmembrane domain proteins in ELISA binding assays, it was necessary to find a suitable tissue to serve as a source of endogenous TLR4 protein. Reports indicate that TLR4 is expressed by microglia of the brain, in heart, lung, liver, pancreas, smooth muscle, spleen, and immune cells such as monocytes, macrophages, and dendritic cells, with highest expression in mouse heart and lung (Vaure and Liu, 2014). Additionally, TLR4 has previously been reported to be expressed in retinal cell types such as photoreceptors and Müller glial cells (Singh and Kumar, 2015; Kumar and Shamsuddin, 2012). Therefore, mouse retinas were isolated, homogenized and subjected to an ELISA to detect the presence of endogenous TLR4. The results of that initial ELISA analysis indicated that endogenous TLR4 is not detectable in
mouse retina. No significant difference in TLR4 expression was observed between the retina lysates and bovine serum albumin (BSA), the negative control (p-value = 0.338; Figure 3.1A). Similarly, data from ELISA analyses conducted using mouse brain also demonstrate that TLR4 is not detectable in that tissue (Figure 3.1B). An ELISA analysis was next performed using mouse heart. TLR4 expression was detected in mouse heart at a level significantly greater than that of the BSA negative control (p value = 0.006; Figure 3.1C). The subsequent analyses to investigate the Basigin-TLR4 interaction used mouse heart as a source for TLR4.

In addition, it was necessary to demonstrate that Basigin is also expressed within mouse heart samples. Therefore, an ELISA was performed to assay for the expression of endogenous Basigin in mouse heart. It was determined that Basigin is indeed expressed in mouse heart, as the levels were significantly greater than those of the BSA negative control (p value = 1.205 x 10^{-5}; Figure 3.2).

Once an appropriate source of TLR4 protein was determined, ELISA binding assays were performed to assess the ability of the Basigin transmembrane domain to bind TLR4. The recombinant Basigin protein consisting of the entire transmembrane domain was first used in an ELISA with endogenous mouse heart TLR4. The results indicate that the Basigin transmembrane domain does interact with TLR4 at a level that is significantly greater (p-value = 9.17 x 10^{-5}) than that of the control recombinant protein, which consists of vector-specific amino acids (Figure 3.3). To determine whether the glutamate residue within the Basigin transmembrane domain is used in the interaction with TLR4, a recombinant Basigin transmembrane domain protein in which the transmembrane glutamate (E) residue was mutated to glycine (G) via site-directed mutagenesis was also used in an
Figure 3.1. TLR4 is present in mouse heart and was not detected in retina or brain. Wells were coated with either retina lysates (A), brain lysate (B), or heart lysate (C) and bovine serum albumin (BSA) at 100 µg/mL. An antibody specific for the TLR4 protein (Pierce/Thermo Scientific) was applied directly to the immobilized proteins and an alkaline phosphatase-conjugated goat anti-rabbit (AP-GAR) antibody was subsequently applied (Pierce/Thermo Scientific). Binding was measured using an alkaline phosphatase detection system and spectrophotometric analysis at 405 nm. Error bars represent standard deviations of the mean. A Student’s T test was performed to compare detection of TLR4 in retina to the negative control (BSA). * indicates a p-value of less than 0.05.
Figure 3.2. Basigin is present in mouse heart. Wells were coated with mouse heart proteins or bovine serum albumin (BSA) at 100 µg/mL. An antibody specific for the Basigin protein (Ochrietor et al., 2003) was applied directly to the proteins and an alkaline phosphatase-conjugated goat anti-mouse (AP-GAM) antibody (Pierce/Thermo Scientific) was subsequently applied. Binding was measured using an alkaline phosphatase detection system and subsequent spectrophotometric analysis at 405 nm. Error bars represent standard deviations of the mean. A Student’s T test was performed to compare detection of Basigin in mouse heart to the negative control (BSA). * indicates a p-value of less than 0.05.
ELISA. The assay specifically tested whether the absence of the glutamate would result in decreased binding between Basigin and TLR4. The results indicate that the glutamate residue alone does not appear to be essential for the interaction with TLR4 because binding of the mutated transmembrane domain to TLR4 was not significantly lower than that of the non-mutated transmembrane domain (p value comparing binding of the normal and mutated Basigin proteins = 0.106; Figure 3.3). The p values comparing binding of either normal Basigin or mutated Basigin to the control protein were less than 0.05 in both cases (Figure 3.3).

The affinity of each recombinant protein for TLR4 was also assessed via ELISA analyses. It was determined that the affinity of the transmembrane domain of Basigin for TLR4 is moderate at 0.742 μM and is similar to affinities for antibody-antigen interactions in the human immune system (Parham, 2015; Figure 3.4). The transmembrane domain of Basigin with the glutamate mutation to glycine had a similar affinity (p value = 0.103) at 0.930 μM (Figure 3.5).

To better understand what segments of the Basigin transmembrane domain were important for the interaction between Basigin and TLR4, recombinant Basigin proteins consisting of various segments of the transmembrane were generated (Finch et al., 2009) and used in ELISA analyses. Figure 3.6 indicates that amino acid residues 7-12 (p value = 0.00013), 13-18 (p value = 0.0201), and 19-24 (p value = 3.55 x 10^-5) are the most essential for establishing the interaction with TLR4 (Figure 3.6). The binding affinity of each of the Basigin recombinant proteins for TLR4 is as follows: BasTM 1-6 6XHis binding affinity = 0.097 μM (Figure 3.7 A); BasTM 7-18 6XHis binding affinity = 0.144 μM (Figure 3.7 B); BasTM 19-24 6XHis binding affinity = 0.236 μM (Figure 3.7 C); BasTM 7-12 6XHis binding
Figure 3.3. The Basigin transmembrane domain binds to TLR4. The TLR4 protein was captured from mouse heart lysates using an antibody specific for TLR4 (Pierce/Thermo Scientific). The appropriate recombinant Basigin proteins were applied (BasTM or BasTM E13G) and a primary antibody specific for the six histidine tag (BD Biosciences) present on the recombinant proteins was applied. An alkaline phosphatase-conjugated goat anti-mouse (AP-GAM) antibody (Pierce/Thermo Scientific) was added and binding was measured using an alkaline phosphatase detection system and spectrophotometric analysis at 405 nm. The control consisted of 157 amino acid residues encoded by the pET102 plasmid. Error bars represent standard deviations of the mean. A Student’s T test was performed to compare binding of each recombinant Basigin protein to TLR4 with that of the control recombinant protein. * indicates a p-value of less than 0.05.
Figure 3.4. A binding affinity assay for Basigin transmembrane domain binding to TLR4. Endogenous TLR4 was captured and incubated with recombinant Basigin transmembrane domain (BasTM) at 0.5, 1.0, 1.5, 2.5, and 5.0 μM. Binding between endogenous TLR4 and the recombinant Basigin protein was measured using an alkaline phosphatase detection system and spectrophotometric assay at 405 nm. Percent binding was calculated for each concentration and the binding affinity of the recombinant Basigin protein for TLR4 was determined from the slope equation. All runs were performed in triplicate. Error bars represent standard deviations of the mean.
Figure 3.5. A binding affinity assay for BasTM E13G binding to TLR4. Endogenous TLR4 was captured and incubated with recombinant Basigin transmembrane domain in which the glutamate residue was mutated (BasTM E13G) at 0.5, 1.0, 1.5, 2.5, and 5.0 μM. Binding between endogenous TLR4 and the recombinant Basigin protein was measured using an alkaline phosphatase detection system and spectrophotometric assay at 405 nm. Percent binding was calculated for each concentration and the binding affinity of the mutated recombinant Basigin protein for TLR4 was determined using the slope equation. All runs were performed in triplicate. A Student’s T-Test was used to compare the mutated transmembrane binding affinity to that of the normal Basigin transmembrane domain. A p-value of less than 0.05 is considered statistically significant. Error bars represent standard deviations of the mean.
Figure 3.6. Basigin transmembrane segments 7-12, 13-18, and 19-24 bind to TLR4. Endogenous TLR4 was captured using an antibody specific to TLR4 (Pierce/Thermo Scientific) and was incubated with the appropriate recombinant Basigin protein (BasTM 1-6 6XHis, BasTM 7-12 6XHis, BasTM 13-18 6XHis, and BasTM 19-24 6XHis) followed by the application of a primary antibody specific for the six histidine tag (BD Biosciences). An alkaline phosphatase-conjugated goat anti-mouse (AP-GAM) antibody (Pierce/Thermo Scientific) was applied. Binding was measured using an alkaline phosphatase detection system and spectrophotometric analysis at 405 nm. The control consisted of 157 amino acid residues encoded by the pET102 plasmid. Error bars represent standard deviations of the mean. A Student’s T test was performed to compare binding of Basigin transmembrane segments to TLR4 in mouse heart to that of the control protein. * indicates a p-value of less than 0.05.
A

% binding vs. µM BasTM 1-6

B

% binding vs. µM BasTM 7-18
C

% binding

μM BasTM 19-24

D

% binding

μM BasTM 7-12
Figure 3.7. Binding affinity assays for BasTM segment binding to TLR4. Endogenous TLR4 was captured and incubated with recombinant Basigin transmembrane domain segments (BasTM 1-6 [A], BasTM 7-18 [B], BasTM 7-12 [C], BasTM 13-18 [D], BasTM 19-24[E]) at 0.5, 1.0, 1.5, 2.5, and 5.0 μM. Binding between endogenous TLR4 and the recombinant Basigin protein was measured using an alkaline phosphatase detection system and spectrophotometric assay at 405 nm. Percent binding was calculated for each concentration and the binding affinity of the recombinant Basigin protein for TLR4 was determined from the slope equation. All runs were performed in triplicate. Error bars represent standard deviations of the mean.
affinity = 0.109 μM (Figure 3.7 D); BasTM 13-18 6XHis binding affinity = 0.160 μM (Figure 3.7 E). Binding affinities of all recombinant Basigin transmembrane segments for TLR4 were compared to the normal Basigin transmembrane domain containing all 24 amino acid residues using standard deviation and the Student's T Test (Table 3.1).

Based on the binding affinity comparison of each Basigin transmembrane segment, Basigin transmembrane residues 1-6 (BasTM 1-6 6XHis), 7-12 (BasTM 7-12 6XHis), and 13-18 (BasTM 13-18 6XHis) have the highest affinity for TLR4 and binding affinities of these segments are significantly greater than the binding affinity of Basigin transmembrane residues 19-24 (BasTM 19-24 6XHis). For each of these Basigin transmembrane segments, individual consecutive amino acids within the segment were mutated to glycine (G) via site-directed mutagenesis and recombinant proteins were generated for each of the amino acids that were mutated within that Basigin transmembrane segment. All of the recombinant proteins subsequently generated containing these mutations were used in ELISA analyses to determine which specific amino acids within those segments are important for the interaction with TLR4. All mutated recombinant proteins were compared to the appropriate normal recombinant Basigin protein segment (Figures 3.8, 3.9, and 3.10). BasTM 1-6 was compared to recombinant proteins containing mutations for each consecutive amino acid within that segment (Finch et al., 2009; Figure 3.8). The methionine at position 1, alanine at position 2, and alanine at position 3 within the Basigin 1-6 transmembrane segment appear to be essential for the interaction with TLR4 (BasTM 1-6 M1G p-value = 0.03; BasTM 1-6 A2G p-value = 0.01; BasTM 1-6 A3G p-value = 0.02). The leucine at position 4, tryptophan at position 5, and
Table 3.1. Comparison of Basigin transmembrane segment binding affinities for TLR4. Statistical analyses were performed to compare the binding affinities of BasTM and BasTM segments for TLR4 using three distinct mouse hearts. Average values were calculated for each protein construct and the percent binding was determined. The concentration at which the percent binding of the recombinant Basigin protein was 50% was calculated. The calculated binding affinities of each recombinant protein were compared using a Student’s T-test to obtain a p-value. The standard deviation was calculated for binding affinities from each mouse heart.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Affinity</th>
<th>p-value</th>
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<tbody>
<tr>
<td>BasTM 6XHis</td>
<td>0.742 μM ± 0.162</td>
<td></td>
</tr>
<tr>
<td>BasTM E13G 6XHis</td>
<td>0.930 μM ± 0.044</td>
<td>0.103</td>
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<tr>
<td>BasTM 1-6 6XHis</td>
<td>0.097 μM ± 0.011</td>
<td>0.012</td>
</tr>
<tr>
<td>BasTM 19-24 6XHis</td>
<td>2.289 μM ± 0.200</td>
<td>0.002</td>
</tr>
<tr>
<td>BasTM 7-12 6XHis</td>
<td>0.109 μM ± 0.006</td>
<td>0.011</td>
</tr>
<tr>
<td>BasTM 13-18 6XHis</td>
<td>0.160 μM ± 0.012</td>
<td>0.013</td>
</tr>
</tbody>
</table>
Figure 3.8. ELISA analysis comparing BasTM 1-6 binding to TLR4 to that of recombinant proteins containing consecutive amino acid substitutions to glycine (G) within the BasTM 1-6 segment. An antibody specific for the TLR4 protein was plated followed by an application of mouse heart lysate. The appropriate recombinant Basigin proteins were applied (BasTM 1-6 M1G 6XHis, BasTM 1-6 A2G 6XHis, BasTM 1-6 A3G 6XHis, BasTM 1-6 L4G 6XHis, BasTM 1-6 W5G 6XHis, and BasTM 1-6 P6G 6XHis) and a primary antibody specific for the six histidine tag was applied. An alkaline phosphatase-conjugated goat anti-mouse (AP-GAM) antibody was subsequently applied. Binding was measured using an alkaline phosphatase detection system and subsequent spectrophotometric analysis at 405 nm. Error bars represent standard deviations of the mean. A Student’s T test was performed to compare binding of Basigin transmembrane segments to TLR4 in mouse heart to the control protein. * indicates a p-value of less than 0.05.
proline at position 6 within the Basigin 1-6 transmembrane segment do not appear to be essential for the interaction with TLR4 because when mutated, they did not show a significant difference in the ability of BasTM 1-6 to bind to TLR4 than the normal Basigin 1-6 transmembrane segment (BasTM 1-6 L4G p-value = 0.42; BasTM1-6 W5G p-value = 0.18; BasTM 1-6 P6G p-value = 0.11).

The BasTM 7-12 recombinant protein was compared to recombinant proteins containing mutations for each consecutive amino acid within that segment (Figure 3.9). The phenylalanine residue at position 7, the leucine residue at position 8, and the valine residue at position 11 appear to be important for the interaction with TLR4 (BasTM 7-12 F7G 6XHis p-value = 0.019; BasTM 7-12 L8G 6XHis p-value = 0.033; BasTM 7-12 V11G 6XHis p-value = 0.025). The isoleucine residue at position 10 and alanine residue at position 12 within the Basigin 7-12 transmembrane segment do not appear to be essential for the interaction between Basigin and TLR4 because when mutated, there was no significant difference in the ability of BasTM 7-12 to bind to TLR4 than the normal Basigin 7-12 transmembrane segment (BasTM 7-12 I10G 6XHis p-value = 0.37; BasTM 7-12 A12G 6XHis p-value = 0.28).

The BasTM 13-18 recombinant protein was compared to recombinant proteins containing mutations for each consecutive amino acid within that transmembrane segment (Figure 3.10). The glutamate residue at position 13, valine residues at position 14 and 16, and leucine residues at position 15 and 17 are important for the interaction with TLR4 (BasTM 13-18 E13G 6XHis p-value = 0.001; BasTM 13-18 V14G 6XHis p-value = 0.017; BasTM 13-18 L15G 6XHis p-value = 0.007; BasTM 13-18 V16G 6XHis p-value = 0.001; BasTM 13-18 L17G p-value = 0.0007). Residue 18 within the Basigin transmembrane...
Figure 3.9. ELISA analysis comparing BasTM 7-12 binding to TLR4 to that of recombinant proteins containing consecutive amino acid substitutions to glycine (G) within the BasTM 7-12 segment. An antibody specific for the TLR4 protein was plated followed by an application of mouse heart lysate. The appropriate recombinant Basigin proteins were applied (BasTM 7-12 F7G 6XHis, BasTM 7-12 L8G 6XHis, BasTM 7-12 I10G 6XHis, BasTM 7-12 V11G 6XHis, BasTM 7-12 A12G 6XHis, and BasTM 7-12 6XHis) and a primary antibody specific for the six histidine tag was applied. An alkaline phosphatase-conjugated goat anti-mouse (AP-GAM) antibody was subsequently applied. Binding was measured using an alkaline phosphatase detection system and subsequent spectrophotometric analysis at 405 nm. Error bars represent standard deviations of the mean. A Student’s T test was performed to compare binding of Basigin transmembrane segments to TLR4 in mouse heart to the control protein. * indicates a p-value of less than 0.05.
Figure 3.10. ELISA analysis comparing BasTM 13-18 binding to TLR4 to that of recombinant proteins containing consecutive amino acid substitutions to glycine (G) within the BasTM 13-18 segment. An antibody specific for the TLR4 protein was plated followed by an application of mouse heart lysate. The appropriate recombinant Basigin proteins were applied (BasTM 13-18 E13G 6XHis, BasTM 13-18 V14G 6XHis, BasTM 13-18 L15G 6XHis, BasTM 13-18 V16G 6XHis, BasTM 13-18 L17G 6XHis, and BasTM 13-18 V18G 6XHis) and a primary antibody specific for the six histidine tag was applied. An alkaline phosphatase-conjugated goat anti-mouse (AP-GAM) antibody was subsequently applied. Binding was measured using an alkaline phosphatase detection system and subsequent spectrophotometric analysis at 405 nm. Error bars represent standard deviations of the mean. A Student’s T test was performed to compare binding of Basigin transmembrane segments to TLR4 in mouse heart to the control protein. * indicates a p-value of less than 0.05.
segment 13-18 does not appear to be essential for the interaction between Basigin and TLR4 because when mutated, there was no significant difference in the ability of BasTM 13-18 to bind to TLR4 than the normal Basigin 13-18 transmembrane segment (BasTM 13-18 V18G 6XHis p-value = 0.46).
Chapter 4 - Discussion

It has been demonstrated that Basigin has the ability to influence signaling through both the MyD88 and TRIF pathways, both of which are utilized by TLR4 to induce pro-inflammatory signaling in response to LPS detection (Jeong et al., 2014). However, the short Basigin cytoplasmic domain does not possess putative binding sites for MyD88 or TRIF (J. Brown, personal observation). In addition, in analyzing the Basigin transmembrane domain sequence, it is questionable as to why a hydrophilic glutamate residue would be located in the hydrophobic environment of the plasma membrane. It has been hypothesized that Basigin interacts with TLR4, using the charged glutamate residue within the transmembrane domain, to initiate MyD88 and TRIF signaling cascades via the TLR4 protein. Therefore, the purpose of the present study was to determine if the Basigin transmembrane domain interacts with TLR4, and if so to analyze which amino acid residues are important for stabilizing the interaction.

ELISA binding analyses indicate that Basigin does interact with TLR4 via the transmembrane domain. These analyses also indicate that when the glutamate residue in the Basigin transmembrane domain is mutated, a slight decrease in binding is observed although that decrease is not considered significantly different from binding of the normal Basigin transmembrane domain.

Additional assays were performed to understand what amino acid residues in the Basigin transmembrane domain are important for the interaction with TLR4. Initially, the Basigin transmembrane domain was separated into segments consisting of six amino acid residues each to determine what segments have a higher affinity for the TLR4 protein. Site-directed mutagenesis was performed on the segments that had a high affinity for TLR4
such that each consecutive amino acid residue within that segment was mutated to glycine. These mutated transmembrane domains were then expressed as recombinant proteins and utilized in ELISA analyses to determine which residues within the segments are important for interacting with TLR4. The binding affinity of the Basigin transmembrane domain for TLR4 is similar to affinities of antibody-antigen interactions in the human immune system (Parham, 2015). BasTM 1-6, BasTM 7-12, and BasTM 13-18 had the highest affinity for TLR4, while BasTM 19-24 had a significantly lower affinity. The transmembrane segments with a high affinity for TLR4 were investigated further to determine what residues were important for stabilizing the interaction between Basigin and TLR4.

Within the BasTM 1-6 segment, the methionine residue at position 1, and the alanine residues at positions 2 and 3 all appear to be essential for interacting with TLR4. Amino acid mutations to residues 4 through 6 did not significantly change the affinity of the BasTM 1-6 segment for TLR4 and did not appear to be as important as residues 1-3 in the Basigin 1-6 transmembrane segment.

Within the BasTM 7-12 segment, the phenylalanine residue at position 7, the leucine residue at position 8, and the valine residue at position 11 appear to be important for the interaction with TLR4 because mutating these residues significantly decreased binding between Basigin and TLR4. The isoleucine residue at position 10 and alanine residue at position 12 within the Basigin transmembrane segment 7-12 do not appear to be essential for the interaction between Basigin and TLR4.

Within the BasTM 13-18 segment, all of the residues including the glutamate at position 13, the valine residues at positions 14 and 16, and the leucine residues at
positions 15 and 17 appear to be important for the interaction with TLR4 because mutating these residues resulted in a significant decrease in binding between Basigin and TLR4. The valine at position 18 does not appear to be important for the interaction between Basigin and TLR4.

Although the ELISA analysis conducted using only the normal Basigin transmembrane domain and the transmembrane domain in which only the glutamate was mutated showed that the glutamate was not important for stabilizing the interaction, that data did show a slight decrease in binding even though it was not considered significantly different. It appears that the glutamate residue is important for stabilizing the interaction with TLR4, along with almost all of the additional hydrophobic residues within the 13-18 transmembrane segment. It would be expected that if several residues are key for stabilizing an interaction, then a single mutation to one of those residues would not result in a large decrease in binding between the two proteins because the additional residues that were not mutated are still contributing to stabilization. When the smaller segment consisting of only amino acids 13-18 was used, a significant difference in binding was observed, likely because the affinity of that region for TLR4 is much greater than that of the entire transmembrane domain, and a single amino acid change was enough to destabilize the interaction.

The data indicate that mutations throughout the Basigin transmembrane domain produced pronounced decreases in binding to TLR4 and suggests that the Basigin-TLR4 interaction is stabilized by both hydrophilic and hydrophobic interactions. The transmembrane domain sequence of TLR4 contains hydrophobic amino acids, as well as
two histidine residues (accession number NM_021297.3), which have the ability to interact with and stabilize the glutamate residue within the Basigin transmembrane domain.

Unlike the Basigin-TLR4 interaction characterized in this study, Finch et al., 2009 showed that the glutamate residue in the Basigin transmembrane domain was not important for establishing an interaction with monocarboxylate transporter 1 (MCT1), and that the Basigin-MCT1 interaction was stabilized solely by hydrophobic interactions. The data from the present study indicate that Basigin does utilize the transmembrane domain glutamate residue to stabilize the interaction with TLR4, in addition to hydrophobic residues. The same study by Finch et al. demonstrated that mutations within the Basigin transmembrane region 19-24 produced dramatic decreases in binding between Basigin and MCT1, while mutations within the Basigin transmembrane segment 1-6 produced less dramatic decreases in binding to MCT1 (Finch et al., 2009). The data presented in this study suggests that mutations within the Basigin transmembrane segment 1-6 produce significant decreases in binding between Basigin and TLR4. In contrast to Finch et al., 2009, the Basigin transmembrane segment 19-24 has a significantly lower affinity for TLR4 compared to transmembrane segments 1-6, 7-12, and 13-18. Mutations within the Basigin transmembrane segments 7-12 and 13-18 produced significant decreases in binding between Basigin and TLR4, however, Finch et al., 2009 indicated that those same transmembrane segments were not utilized in the interaction between Basigin and MCT1.

It is possible that the extracellular and cytoplasmic domains of Basigin, in addition to the transmembrane domain, play a role in influencing signaling through the TLR4-induced signaling pathways MyD88 and TRIF; however, the main purpose of the present
study was to specifically investigate the role of the Basigin transmembrane domain in pro-inflammatory signaling pathways associated with TLR4.

The ability of Basigin to influence the MyD88 and TRIF signaling pathways could be in response to ligand binding via the extracellular domain of the Basigin protein. Jeong et al. demonstrated that when an antibody specific for the Basigin protein was applied to cell cultures, signaling through both the MyD88 and TRIF pathways was significantly decreased. It was specifically shown that the cultures in which the antibody was applied had lower levels of the transcription factor NF-kappaB when compared to cultures in which no antibody was applied (Jeong et al., 2014). It is well established that the TLR4 protein, in addition to the proteins MD2 and CD14, are responsible for the detection of LPS via the formation of a complex with the extracellular domain of TLR4 (Owen et al., 2013). It is not known if Basigin has the ability to interact with LPS or the proteins involved in forming a complex with the extracellular or cytoplasmic domains of TLR4 during the initiation of pro-inflammatory signaling pathways involving MyD88 and TRIF. Because Basigin possesses a short cytoplasmic domain with no known putative binding sites for MyD88 and TRIF, yet has been documented to elicit signal transduction events through these pathways (Jeong et al., 2014), it is possible that the role of Basigin in pro-inflammatory signaling is to establish an interaction with TLR4 in response to binding of yet to be identified ligand to carry out the signaling events. This is reminiscent of the structure and activation process for the T cell receptor, which uses different proteins within the receptor complex to bind ligand and to elicit signal transduction (Birnbaum et al., 2014).
It is known that Basigin is involved in the Wnt/beta-catenin pathway, a pathway extensively involved in embryonic development that regulates cell proliferation and migration (Wei et al., 2010; Logan and Nusse, 2004). The Wnt/beta-catenin pathway is also a prevalent pathway associated with cancer (Reya and Clevers, 2005). It is well understood that Basigin is highly expressed on the surface of tumors and serves as a cell surface marker for the identification of some cancer cells (Davidson et al., 2003; Yan et al., 2005). Unscheduled reactivation of the Wnt/beta-catenin pathway has the ability to cause inflammatory diseases and it is known that both developmental signaling pathways and inflammatory signaling pathways are used by metastatic cancer cells for the purpose of proliferation and the ability to alter cell interactions (Morrisey, 2003; Srikrishna and Freeze, 2009; Hagemann et al., 2005). The full role of Basigin in signaling through pro-inflammatory pathways is unknown, but it is intriguing to speculate that Basigin is necessary for the overlapping of Wnt/beta-catenin and pro-inflammatory pathways necessary for cancer cell proliferation. In non-cancer cells, Basigin may play a role in initiating inflammation through TLR4 in response to LPS or another, yet to be identified ligand.

In conclusion, the results of this study indicate that the Basigin and TLR4 proteins interact and that this interaction via the Basigin transmembrane domain is stabilized by both hydrophilic and hydrophobic interactions. The Basigin transmembrane glutamate residue does play a role in the interaction between Basigin and TLR4 but is not entirely responsible for stabilizing the interaction between Basigin and TLR4, as several other amino acid residues appear to be key for the interaction in addition to the glutamate residue. The TLR4 transmembrane domain contains two positively charged histidine
residues that could be important for establishing electrostatic interactions with the Basigin transmembrane glutamate residue. Basigin transmembrane residues that appear to stabilize the interaction between the Basigin and TLR4 are located within positions 1-18 in the Basigin transmembrane domain. Residues located in the 19-24 Basigin transmembrane region do not appear to be as important as the other residues because the observed binding affinity of residues 19-24 for TLR4 was significantly less than the binding affinities of the other transmembrane regions of Basigin. This phenomenon allows Basigin to interact with TLR4 and the transporter protein MCT1 simultaneously and hence perform multiple tasks within the same cell.
References


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