The retina-specific basigin isoform does not induce IL-6 expression in mouse monocytes

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Purpose: Basigin gene products are positioned on adjacent cell types in the neural retina and are thought to compose a lactate metabolon important for photoreceptor cell function. The Ig0 domain of basigin isoform 1 (basigin-1) is highly conserved throughout evolution, which suggests a conserved function. It has been suggested that the Ig0 domain has proinflammatory properties, and it is hypothesized to interact with basigin isoform 2 (basigin-2) for cell adhesion and lactate metabolon formation. Therefore, the purpose of the present study was to determine whether the Ig0 domain of basigin-1 binds to basigin-2 and whether the region of the domain used for binding is also used to stimulate interleukin-6 (IL-6) expression.

Methods: Binding was assessed using recombinant proteins corresponding to the Ig0 domain of basigin-1 and endogenously expressed basigin-2 from mouse neural retina and brain protein lysates. The proinflammatory properties of the Ig0 domain were analyzed with exposure of the recombinant proteins to the mouse monocyte RAW 264.7 cell line and subsequent measurement of the IL-6 concentration in the culture medium via enzyme-linked immunosorbent assay (ELISA).

Results: The data indicate that the Ig0 domain interacts with basigin-2 through a region within the amino half of the domain and that the Ig0 domain does not stimulate the expression of IL-6 in mouse cells in vitro.

Conclusions: The Ig0 domain of basigin-1 binds to basigin-2 in vitro. In addition, contrary to previous reports, there was no evidence that the Ig0 domain potentiates IL-6 expression in a mouse monocyte cell line in vitro. However, it is possible that the Ig0 domain stimulates the expression of proinflammatory cytokines other than IL-6, or that the potential involvement of the Ig0 domain of basigin-1 in the acute inflammatory response is dependent on species.

The inflammatory response protects the body from foreign entities, such as microbes and viruses. During an acute response, innate immune cells, such as macrophages and monocytes, are recruited to the site of invasion to initiate pathogen destruction [1]. Using small proinflammatory cytokines, such as tumor necrosis factor-alpha (TNFα), interleukin (IL) IL-1, and IL-6, the response is rather quick, and the white blood cells return to an inactivated state soon after the foreign molecules are removed [2]. However, in some situations, chronic inflammation occurs, which leads to detrimental effects on surrounding tissue due to the prolonged immune cell activation and cytokine production [2]. Chronic inflammation is a major hallmark of many diseases, including COVID-19, cancer, heart disease, neurodegenerative diseases, and diabetes [2,3]. In some cases, specifically for diabetes, a chronic inflammatory state facilitates damage to the neural retina, which can lead to vision loss [4].

The neural retina is considered an immune-privileged area, in that the eye attempts to suppress the inflammatory response to preserve vision [reviewed in 5]. The blood–retina barrier is composed of inner and outer barriers to regulate fluid and molecular movement between the ocular vascular beds and the retinal tissues. The inner blood–retina barrier consists of tight junctions between blood vessel endothelial cells within the retina, which protects it from the inner retinal vasculature [6]. The tight junctions of the RPE form the outer blood–retina barrier to protect the retina from direct contact with systemic blood circulation of the choroid [7]. If there is damage to the blood–retina barrier, such as in diabetes or macular degeneration, it is possible for monocytes to travel out of blood vessels and into the retina.

The protein basigin, also known as EMMPRIN and CD147, is a transmembrane glycoprotein that belongs to the immunoglobulin superfamily and functions as a cell adhesion molecule [reviewed by 8]. The basigin gene (Gene ID 682, OMIM 111380) is located on chromosome 19 in humans (chromosome 10 in mice) and produces four isoforms via differential splicing. Two isoforms of basigin are expressed in the neural retina [9,10]. Both possess the same transmembrane domain and short cytoplasmic tail; however, they differ in the extracellular region [Figure 1; reviewed by 8]. Basigin isoform 1 (basigin-1) possesses three extracellular Ig-like domains (Ig0, Ig1, and Ig2), whereas basigin isoform 2 (basigin-2) possesses only the Ig1 and Ig2 domains [10]. Basigin-1 is specifically expressed by photoreceptor cells in the neural retina, and basigin-2 is expressed in most, if not
all, tissues of the body [10]. In the neural retina, basigin-2 is expressed by Müller glial cells, blood vessel endothelial cells, and the RPE. Both proteins have been implicated in metabolic and immunologic functions [11-13].

Basigin gene products have been ascribed metabolic functions. The proteins are thought to associate with each other in the neural retina to form a lactate metabolon between photoreceptor neurons and Müller glial cells. It is thought that the two cell adhesion molecules interact to allow for the efficient transfer of nutrients from the Müller cells to the photoreceptor cells via monocarboxylate transporters [11]. The absence of basigin gene products in the neural retina, as in the basigin null mouse, results in reduced expression of MCT1 at the plasma membrane, limited electrical signaling by the photoreceptors, and blindness [10,11,14]. The Ig0 domain of basigin-1 is highly conserved across species [10], which suggests a conserved function for the domain.

As immune molecules, basigin gene products are thought to induce proinflammatory responses. Basigin-2 aids in the recruitment of neutrophils to a wound site by acting as a signaling receptor for the chemottractants cyclophilins A and B [15]. In addition, basigin-2 expression is heightened in activated lymphocytes, where it acts as a suppressor of subsequent T-lymphocyte activation [15,16]. Basigin-2 also regulates T lymphocytes through its characteristic expression on regulatory T lymphocytes that possess suppressive activities [17]. Most recently, basigin-2 has been implicated in COVID-19 infections, as it serves as a receptor for viral Spike P protein/cyclophilin A on T lymphocytes, which exhibit abnormal metabolic regulation after severe infection [18]. Additionally, basigin-1, specifically the extracellular Ig0 domain, induces the expression of the proinflammatory cytokine IL-6 [13]. Although this is an intriguing finding, basigin-1 is expressed only in the neural retina, which is an immune-privileged tissue that carefully restricts immune cell migration into the retina. Therefore, the biologic relevance of this observation is unknown.

Figure 1. Gene and protein structure of Basigin gene products expressed in the neural retina. A: An illustration of the gene structure is shown. Exons are represented by colored rectangles, and introns are represented by the gray line. Exons are numbered and color-coded to show sequences translated into the extracellular immunoglobulin (Ig) domains. B: An illustration of the protein structures of basigin-1 and basigin-2. Although they are depicted on the same membrane, they are expressed by different cell types in the neural retina. Exon 1 (black) contains the leader sequence found in both proteins. Exon 2, shown in purple, codes for the Ig0 domain specific to basigin-1. Exon 3, shown in green, codes for the Ig1 domain found in both proteins. Exon 4, shown in green and orange, codes for Ig1 and Ig2, found in both proteins. Exon 5, shown in orange, codes for Ig2. Exon 6 contains the coding sequence for the transmembrane domain. Exons 6, 7, and 8 (all black) code for amino acids contained in both proteins and correspond to regions proximal to the membrane, the transmembrane domain, and the cytoplasmic domain, respectively.
It is likely that the basigin-1 Ig0 domain is important for the lactate-based metabolism and immunological aspects of the protein. In a healthy neural retina, basigin gene products interact to hold photoreceptors and Müller glial cells together. Each interacts with adjacent MCT-1 on their respective cell to aid in the efficient transfer of metabolites from the Müller cells to the photoreceptors [11]. However, upon damage to the blood–retina barrier, as seen in macular degeneration and diabetic retinopathy, basigin-1 may serve as a ligand for basigin-2 on monocytes or other immune cells that infiltrate the neural retina to stimulate the production of IL-6.

Basigin-1 and basigin-2 are thought to interact in the neural retina [10]; however, the specific region of the basigin-1 protein used for this interaction is unknown. Therefore, the purpose of the present study was to test whether the Ig0 domain of basigin-1 binds to basigin-2, and whether this domain is used to stimulate IL-6 production in immune cells. Specifically, one aim was to determine whether the basigin-1 Ig0 domain binds to basigin-2, and if so, the region of the domain used for the interaction. Another aim of the study was to assess whether the same region that binds basigin-1 is used to stimulate the expression of IL-6, as demonstrated previously [13]. The results of this study contribute not only to a better understanding of interactions between Müller cells and photoreceptor cells but also to an exploration of the proinflammatory proteins within the immune-privileged neural retina.

METHODS

Generation of bacterial expression vectors: Recombinant protein expression constructs of the N-terminal Ig domain of basigin isoform-1 (Bsg-1; NM_001728.4) were generated with PCR cloning. The cDNA for the full-length domain was obtained from a mouse retina cDNA library generously provided by Robert Nickells (presently at the University of Wisconsin) and Donald Zack (Johns Hopkins Medical School) via PCR amplification using EX Taq polymerase (Panvera/ Takara, Madison, WI) in conditions suggested by the manufacturer and 50 pmoles each of B1Ig0-Fwd (5'-CAC CGG TTT CCT CAA GGC ACC AC-3') and B1Ig0-RV (5'-GTT CAA GGA CCA CGC TCG CC-3'). The cycling parameters for the amplification were 96 °C for 5 min, followed by 30 cycles of 96 °C for 1 min, 55 °C for 1 min, and 72 °C for 3 min. The PCR product was cloned into pET102/D-TOPO (Invitrogen Corporation, Carlsbad, CA) and transformed into Top 10 cells (Invitrogen Corporation) following the manufacturer's protocol. The resulting colonies were screened via PCR, using the protocol described. The cDNA sequences of the positive clones were verified using GenomeLab Dye Terminator Cycle Sequencing on a Beckman Coulter CEQ 8000 Genetic Analysis system (Fullerton, CA). The resulting plasmid, containing the full-length Bsg-1 Ig0 domain, was named pET102-Ig0.

The pET102-Ig0 plasmid served as the template for the generation of the “deletion constructs” via PCR. Primers were designed to generate cDNA fragments of the Bsg-1 Ig0 domain coding sequence using the cycling parameters described. The primers Ig0-Fwd and B6911RV (5'-CCC TTC AAA CCA CAG G-3'; 50 pmoles each) were used to amplify the amino half of the Ig domain (Ig0N), whereas the primers B6911F (5'-CAC CCC AGT GGT TTG AAG GG-3') and Ig0-RV (50 pmoles each) were used to amplify the carboxy half of the Ig0 domain (Ig0C). The primers L1Ig0-F (5'-CAC CGG GGG CGG CGT GGT CCT G-3') and L1Ig0-RV (5'-TTC AAA CCA CTA GCT CTC GGG-3'; 50 pmoles each) were used to amplify the region of the Ig0 domain, with significant homology to L1cam (Ig0-L1). The cycling parameters for amplification were 96 °C for 2 min, followed by 30 cycles of 96 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The resulting PCR products were separately cloned into pET102/D-TOPO (Invitrogen Corporation) and transformed into Top 10 cells (Invitrogen Corporation) following the manufacturer’s protocol. The resulting colonies were screened via PCR, using the protocol described. The cDNA sequences of the positive clones were verified as described.

The L1cam construct was generated with reverse transcription (RT)–PCR cloning. Mouse brain RNA was isolated using the TRI Reagent protocol (MRC, Inc., Cincinnati, OH), following the manufacturer’s instructions. Total RNA (2 μg) was reverse transcribed using the SuperScript IV first-strand synthesis system (Invitrogen Corporation). The primers L1cam-F (5'-CTC GAG GGC AGT ACT GCT TACT TG-3') and L1cam-RV (5'-CTC GAG TTC ATC CAG CCA CTG GAC-3'; 50 pmoles each) were used to amplify the region of the L1 cell adhesion molecule (L1cam; NM_008478) with significant homology to the Ig0 domain of Bsg-1. The cycling parameters for amplification were 96 °C for 2 min, followed by 30 cycles of 96 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The resulting PCR product was cloned into pET102/D-TOPO (Invitrogen Corporation) and transformed into Top 10 cells (Invitrogen Corporation) following the manufacturer’s protocol. The resulting colonies were screened via PCR, using the protocol described. The cDNA sequences of the positive clones were verified as described.

The control (C) construct was generated as described [19]. The pET102/D-TOPO plasmid (Invitrogen Corporation) was ligated in the absence of the PCR product. The circularized plasmid was transformed into Top 10 cells (Invitrogen Corporation) and verified by PCR using the protocol described. The cDNA sequences of the positive clones were verified as described.
Immunocytochemistry: Immunocytochemistry was performed to verify the expression of basigin-2 in the RAW 264.7 cells. The cells were plated on Lab-Tek chamber slides (Nunc; Thermo Fisher Scientific, Rochester, NY) and incubated overnight at 37 °C with 5% CO₂. The cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and washed with several changes of PBS. The cells were incubated in 500 μl blocking solution (TBS containing 0.1% Tween-20; Sigma Chemical Company, St. Louis, MO; and 2% normal goat serum; Pierce/Thermo Scientific) overnight at 4 °C. The cells were incubated with an antibody specific for mouse basigin gene products ([10]; diluted to 1 μg/ml in blocking solution) for 1 h at 37 °C and
Statistical analyses were performed as described [19,20]. The wells were coated with an antibody specific for basigin gene products ([10]; 100 μl of 500 ng/ml in PBS) and incubated overnight at 4 °C. The capture antibody solution was removed, and the wells were blocked with incubation with bovine serum albumin (BSA; Pierce/Thermo Scientific, Rockville, IL; 100 μl of 200 μg/ml in PBS) for 30 min at 37 °C. The BSA was removed, and the wells were washed three times with PBS-T. For the affinity binding assays, recombinant proteins (100 μl of 5 μM for proteins made in bacteria and 100 μl for proteins made in vitro) were added to appropriate wells (100 μl of 200 μg/ml in PBS) and incubated for 30 min at 37 °C. The unbound antibodies were removed, and the wells were washed three times with PBS-T. Recombinant proteins were serially diluted (1:2 in PBS) from 5 μM to 0.3125 μM. The diluted recombinant proteins and a 0 μM sample consisting of PBS were added to appropriate wells (100 μl) and incubated for 30 min at 37 °C. The recombinant proteins were removed, and the wells were washed three times with PBS-T. Recombinant proteins (100 μl diluted 1:500 in PBS) was added to the wells and incubated for 30 min at 37 °C. The unbound antibodies were removed, and the wells were washed three times with PBS-T. Alkaline phosphatase (AP)-conjugated secondary antibody (goat-anti-mouse; Pierce/ThermoScientific; 100 μl diluted 1:1,000 in PBS) was added to the wells and incubated for 30 min at 37 °C. The unbound antibodies were removed, and the wells were washed three times with PBS-T. A reaction was initiated by adding 100 μl of AP substrate (PNPP, Pierce/ThermoScientific; 100 μl diluted 1:1,000 in PBS) was added to the wells and incubated for 30 min at 37 °C. The solution was incubated until a color change from clear to yellow was seen, typically within 20 min, and the reactions were stopped by the addition of 50 μl of 2N NaOH (Sigma Chemical Company). The wells serving as the “blank” received PBS at every step until the AP substrate and 2 N NaOH were added. The absorbance at 405 nm in each well was measured using a Bio-Tek plate reader (Winooski, VT) and reported as binding units. Three technical replicates were performed for each recombinant protein produced in bacteria, and two technical replicates were performed for each recombinant protein produced in vitro.

**IL-6 assay:** Human and mouse IL-6 ELISAs (Thermo Fisher Scientific) were used to quantify IL-6 expression in the appropriate cell culture medium, following the manufacturer’s instructions. The standards and culture media samples were plated in duplicate. The concentration of each sample was calculated using an IL-6 standard curve and plotted as a bar graph.

**Statistical analysis:** Statistical analyses were performed using GraphPad Prism 9.0 software (San Diego, CA). Unless indicated, the data were normally distributed, and graphs show the means and standard deviations of the mean for each group. The results for the binding assays were compared using one- or two-way ANOVA with subsequent Tukey’s multiple comparison tests. Results for the affinity binding assays and the IL-6 assays were compared using an unpaired t test or a Kruskal–Wallis test.

**RESULTS**

The first aim of this study was to determine whether the Ig0 domain of basigin-1 interacts with basigin-2. A recombinant form of the basigin-1 protein (Ig0–6XHis) and a control protein (C-6XHis) made from the self-ligation of the expression vector were used in binding assays with endogenous protein lysates derived from the mouse neural retina, the mouse brain, and commercially available BSA. Binding of the recombinant probes to basigin gene products captured from the neural retina lysates, to basigin-2 captured from the brain lysates, or to BSA was assessed (Figure 2A). Binding of the Ig0–6XHis protein to basigin gene products in the neural retina or to basigin-2 in the brain was significantly greater than binding of C-6XHis to these proteins (p<0.0001 and p<0.001, respectively). Binding of the Ig0–6XHis protein to basigin gene products in the neural retina or basigin-2 in the brain was significantly greater than binding of Ig0–6XHis to BSA (p<0.001 and p<0.001, respectively). Conversely, the binding of Ig0–6XHis to BSA was not significantly different from the binding of C-6XHis to BSA (p=0.5469). The Ig0–6XHis recombinant protein interacted with endogenous basigin with an affinity of 1.78±0.07 μM (Figure 2B, Table 1). In summary, these data suggest that the Ig0 domain of basigin-1 interacts with basigin-2.

To determine which region of the Ig0 domain binds to basigin-2, deletion constructs were generated to cut the domain into two halves. Binding of a protein containing the
amino half of the Ig0 domain (Ig0N-6XHis) to the captured endogenous basigin-2 was similar to that of Ig0–6XHis and greater than that of C-6XHis and a protein containing the carboxy half of the Ig0 domain (Ig0C-6XHis; Figure 3A). The Ig0N-6XHis recombinant protein interacted with endogenous basigin with an affinity similar to that of Ig0–6XHis at 1.59±0.01 μM (Figure 3B, Table 1). Further examination of the sequence within Ig0N-6XHis revealed a region with a significant (50%) amino acid sequence identity to the cell adhesion molecule L1cam, a cell adhesion molecule known to interact with basigin-2 in the brain ([21]; Figure 4A). Binding of a protein containing these amino acids (IgL1–6XHis) to captured endogenous basigin-2 was similar to that of Ig0–6XHis (Figure 4B; p=0.9107), with a binding affinity of 1.82±0.02 μM (Figure 4C, Table 1). Additionally, the binding of a protein containing the region of sequence identity within L1cam (L1cam-6XHis) was not significantly different from that of Ig0–6XHis and Ig0L1–6XHis (Figure 4B). When the binding of Ig0L1–6XHis to captured basigin-2 versus commercially available BSA was evaluated, it was determined that the binding to basigin-2 was significantly greater than to BSA (p<0.01; Figure 4D). These data suggest that the L1cam homology region within the Ig0 domain of basigin-1 interacts with basigin-2.

The second aim of the study was to determine whether an interaction of basigin gene products, specifically using the region of the Ig0 domain that binds to basigin-2, accounts for the immunological aspects of the basigin-1 function previously described [13]. To assess this, cell-based in vitro assays were used. The RAW 264.7 cell line, derived from mouse monocytes, was selected for the analyses. Immunocytochemical analyses indicated that the cell line expresses basigin-2 (Figure 5A). RAW 264.7 cells incubated in the presence of LPS expressed significant concentrations of IL-6 when compared to those incubated in the presence of saline (D-PBS; p=0.0013; Figure 5B). However, RAW 264.7 cells incubated in the presence of Ig0–6XHis, Ig0N-6XHis, or Ig0L1–6XHis did not generate detectable concentrations of IL-6 (Figure 6A). In addition, incubation of the RAW 264.7 cells in the presence of protein lysates derived from the mouse neural retina produced a minimal, yet detectable, concentration of IL-6 (approximately 3 pg/ml). Conversely, the C-6XHis protein induced significant expression of IL-6 in

### Table 1. Affinity of the Basigin-1 Ig0 Domain Recombinant Proteins for Endogenous Basigin-2.

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<th>Protein</th>
<th>Affinity (μM)</th>
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<tr>
<td>Ig0–6XHis</td>
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<tr>
<td>Ig0N–6XHis</td>
<td>1.59±0.01</td>
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<td>Ig0L1–6XHis</td>
<td>1.82±0.02</td>
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**A.** Binding assays were performed using Ig0–6XHis (black circles) and C-6XHis (white circles) recombinant proteins with endogenous basigin gene products captured from the mouse neural retina (NR), basigin-2 captured from the mouse brain (Brain), or bovine serum albumin (BSA). The interaction was measured using an antibody specific to the six-histidine epitope at the C-terminus of the recombinant proteins and a secondary antibody conjugated to alkaline phosphatase (AP). Conversion of the AP substrate to the product was measured at 405 nm, and the absorbance was used as the binding units. The binding units were plotted as the mean of the triplicate runs. The error bars represent the standard deviation of the mean. A two-way ANOVA with Tukey’s multiple comparison test was performed. The p values are indicated (α level = 5%). **B.** An affinity binding curve was generated using serial dilutions of Ig0–6XHis from 5 μM to 0.3125 μM with endogenous basigin gene products captured from the mouse neural retina. Interactions were measured as described for the binding assays. Absorbance at 405 nm was used to determine percent binding in reference to the 5 μM sample. The percent binding was plotted as the mean of duplicate runs. The error bars represent the standard deviation of the mean. The equation from the trendline was used to calculate affinity.
the RAW 264.7 cells (Figure 6A). To determine whether this result is species-specific, the cell-based assay was repeated using the HEK 293T cell line. No significant IL-6 was detected in the HEK 293T cells using any of the recombinant proteins or the mouse neural retina protein lysate (Figure 6B). Similar results were observed using the U937 human monocytic cell line (data not shown), which expresses basigin-2 and responds to LPS by inducing IL-6 expression [22, 23]. These data suggest that the Ig0 domain of basigin-1 does not induce IL-6 expression in the cell lines tested.

DISCUSSION

The purpose of this study was to evaluate the ability of the Ig0 domain of basigin-1 to bind basigin-2 and to induce the expression of IL-6 in monocytes. This was performed using recombinant proteins, mouse protein lysates, and cell-based assays. It was determined that the Ig0 domain of basigin-1 binds to basigin-2; however, the domain does not induce the expression of IL-6 in the cell lines tested. These findings suggest that basigin gene products support the balance of Müller glial cell to photoreceptor cell metabolism in the neural retina by providing a scaffold for the assembly of a lactate metabolon rather than contributing to immunological aspects of retinal eye diseases.

Previous studies on the expression of basigin gene products have indicated that basigin-1 and basigin-2 are closely positioned within the mouse neural retina. Photoreceptor cell bodies and inner segments express basigin-1 on the plasma membrane, while Müller glial cells express basigin-2 on the plasma membrane [10]. It was previously proposed that the two proteins participate in a lactate metabolon with MCT1 to allow for the rapid and efficient transfer of lactate from highly glycolytic Müller cells to energy hungry photoreceptor neurons [11]. Without the metabolon, as in the basigin null mouse, the positioning of the metabolon is disrupted, and the photoreceptor cells do not function [10, 11, 14]. Because of the highly conserved amino acid sequence of the Ig0 domain of basigin-1, it has been proposed that it possesses a conserved function, specifically for cell adhesion [10]. This group hypothesized that the Ig0 domain of basigin-1 binds to basigin-2 as part of the lactate metabolon. The results of the present study support this hypothesis, in that a recombinant version of the basigin-1 Ig0 domain binds to endogenous basigin-2 captured from mouse neural retina and brain protein lysates at moderate affinity [24]. The interaction was specific to the basigin-1 amino acids in the recombinant protein because the binding was significantly greater than that of a control recombinant protein generated from the same bacterial expression vector. In addition, the interaction was specific for basigin-2 because significant binding was observed using mouse brain protein lysates, but not for BSA. These findings are significant because previous studies have
Figure 4. A region of the basigin-1 Ig0 domain with sequence homology to L1cam binds to basigin-2. A: A sequence comparison of the basigin-1 Ig0 domain and the L1cam is shown. Identical amino acids are indicated, and homologous amino acids are highlighted. B: Binding assays were performed using the Ig0–6XHis, Ig0L1–6XHis, and L1cam-6XHis recombinant proteins with endogenous basigin captured from the mouse neural retina. C: An affinity binding curve was generated using serial dilutions of Ig0L1–6XHis from 5 μM to 0.3125 μM with endogenous basigin gene products captured from the mouse neural retina. D: Binding assays were performed using the Ig0L1–6XHis (black circles) and C-6XHis (white circles) recombinant proteins with endogenous basigin from the mouse neural retina (NR) or BSA (BSA). For the binding assays, the interactions were measured using an antibody specific to the six-histidine epitope at the C-terminus of the recombinant proteins and a secondary antibody conjugated to alkaline phosphatase (AP). Conversion of the AP substrate to the product was measured at 405 nm, and the absorbance was used as the binding units. The binding units were plotted as the mean of the triplicate runs. The error bars represent the standard deviation of the mean. A one-way ANOVA (B) or two-way ANOVA (C) with Tukey’s multiple comparison test was performed. The p values are indicated (α level = 5%). For the affinity curve, interactions were measured as described for the binding assays. Absorbance at 405 nm was used to determine percent binding in reference to the 5 μM sample. The percent binding was plotted as the mean of duplicate runs. The error bars represent the standard deviation of the mean. The equation from the trendline was used to calculate affinity.
Figure 5. RAW 264.7 cells express basigin-2 and LPS-induced IL-6. A: Immunocytochemical analyses of RAW 264.7 using an antibody specific for basigin gene products [10] and Alexa 488-conjugated goat-anti-rabbit secondary antibody. Basigin-2 expression is represented by green fluorescence, and nuclear DNA is represented in purple. The magnification bar represents 100 μm. B: Interleukin-6 (IL-6) present within the cell culture medium of RAW 264.7 cells treated with D-PBS or lipopolysaccharide (LPS: 1 μg/ml) for 24 h at 37 °C was measured via quantitative enzyme-linked immunosorbent assay (ELISA) and plotted as the mean concentration (pg/ml) of triplicate runs. The error bars represent the standard deviation of the mean. An unpaired t test was performed. The p values are indicated (α level = 5%).

Figure 6. The Ig0 domain of basigin-1 does not stimulate the expression of IL-6. Interleukin-6 (IL-6) present in the cell culture medium of (A) RAW 264.7 or (B) HEK293–7 cells treated with D-PBS, C-6XHis, Ig0–6XHis, Ig0N-6XHis, or Ig0L1–6XHis recombinant proteins (5 μM) or mouse neural retina protein lysate (NR, 500 μg/ml) for 24 h at 37 °C was measured via quantitative enzyme-linked immunosorbent assay (ELISA) and plotted as the mean concentration (pg/ml) of triplicate runs. The error bars represent the standard deviation of the mean. A Kruskal–Wallis test was performed. The p values are indicated (α level = 5%).
indicated that basigin-1 self-associates [25]. Self-association of the Ig0 domain would have produced significantly less binding when the mouse brain lysates were used because basigin-1 is not expressed in that tissue.

Binding analyses using recombinant proteins corresponding to the amino and carboxy halves of the basigin-1 Ig0 domain suggest that binding to basigin-2 is specific to the amino half of the domain. Personal observations of recombinant protein production indicate that the generation of the Ig0 domain protein requires specific conditions. The entire domain of the protein appears to be toxic to bacterial cells and requires a short period of protein expression when compared to other recombinant proteins, such as the control protein generated for this study (A. Solstad, personal observation). Truncation of the amino acid sequence to include only the amino half of the domain results in the use of less stringent bacterial culturing conditions, whereas the vector that contains the carboxy half of the domain could not be expressed in vivo (J. Ochrietor, personal observation), suggesting that the toxic portion of the sequence resides within those amino acids. Because of this, the Ig0C protein was produced using only in vitro translation methods. Binding assays indicated that the carboxy-half amino acids of the basigin-1 Ig0 domain do not bind to basigin-2, but the amino acids within the amino half of the domain do.

Alignment analyses of the amino acid sequence of the basigin-1 Ig0 domain identified a region within the amino half of the domain with significant sequence identity to the neural L1 cell adhesion molecule. The L1cam protein interacts with basigin-2 in the brain [21], suggesting biologic relevance for the observation. A recombinant protein corresponding to the amino acids with a significant sequence identity to L1cam (Ig0L1–6XHis) was generated. Assays using the Ig0L1–6XHis protein indicated that binding to basigin-2 was similar to that of the entire Ig0 domain. Conversely, the binding of the recombinant protein corresponding to the homologous region of the L1cam protein (L1–6XHis) to basigin-2 was less than that of the basigin proteins. This analysis suggests that conserved and nonconserved amino acids within the sequences are important for the interaction. However, greater binding occurs with the basigin-1 Ig0-specific amino acid histidine at position 2 (substituted for hydrophobic leucine in L1cam) and glutamate at position 4 (substituted for positively charged lysine in L1cam). Three-dimensional analyses of the structure of the Ig0 domain indicate that the first nine amino acids of this region (to the first proline of the sequence; see Figure 4A) are solvent accessible and on the surface of the protein in beta strand B of the domain [13]. This region is in a different location than the free cysteine within the basigin-1 Ig0 domain, which may be used in the complex formed with rod-derived cone viability factor (RdCVF) and GLUT-1 for cone survival [12,13]. The sequence within the L1cam protein resides in the fourth Ig domain of the protein and may not be positioned on the surface of the protein in its three-dimensional structure (UniProtKB P32004). Previous studies of the interaction between L1cam and basigin examined the ability of basigin-2 to interact with carbohydrates on L1cam, through which it was deemed an oligomannose-binding lectin [21]. However, that previous study did not perform a test of the ability of basigin-2 to bind amino acids within the L1cam protein sequence [21].

A previous study of the structure and function of the Ig0 domain of basigin-1 indicated that the domain possesses proinflammatory properties because a recombinant protein corresponding to the sequence of the domain induced the expression of IL-6 in the human monocyte THP-1 cell line as well as the human embryonic kidney HEK 293T cell line [13]. When this laboratory group repeated the analyses using the mouse monocyte RAW 264.7 cell line, no significant IL-6 was detected. To determine whether the results were caused by species differences, the analyses were repeated using the U937 and HEK 293T cell lines, but again, no significant IL-6 was detected. Although the present study followed the methodology of the previous study, there were several differences that may account for the different results. The previous study used the sequence for human basigin-1, and the present study used the sequence for mouse basigin-1. The Ig0 domain of basigin-1 possesses the highest sequence homology of the entire protein, with 35% sequence identity and 56% homology across species [10]. It is possible that species-specific differences in the sequence are required for the proinflamatory properties of the domain. However, one would expect that if human monocytes respond to the human basigin sequence, then mouse monocytes would respond to the mouse basigin sequence unless this is truly a species-specific (human only) response. In addition, the data reported in the previous study were plotted as an “IL-6 fold increase,” whereas the data in the present study were plotted as the concentration. Because different IL-6 ELISAs were used in the studies, it is possible that the assay used in the previous study had a lower threshold of expression (less than 7.8 pg/ml) than the one used in the present study.

The full immunological functions of the Ig0 domain of the basigin-1 domain were not tested in the present study. This study was specific, and perhaps limited in testing for the expression of the proinflammatory cytokine IL-6. This was done because the aim of the present study was to build on the findings of another laboratory [13]. It is possible that
the basigin-1 Ig0 domain can induce the expression of other proinflammatory cytokines or immune molecules. It is also possible that the Ig0 domain of basigin-1 can influence the function or metabolism of blood vessel endothelial cells within the neural retina, as basigin-2 is a known marker for these cells [8]. Additional studies of the immunological functions of basigin-1, specifically the Ig0 domain of the protein, should be undertaken so that a complete story of the molecule can be produced.

It was surprising to see that the control protein (C-6XHis) stimulated the expression of IL-6 in the RAW 264.7 cells. The same result was not observed using HEK 293T cells. The construction of the control protein includes the self-ligation of the pET102/D-TOP vector, which generates a four-amino-acid insert of aspartate–isoleucine–valine–lysine that is not found within the sequence of proteins produced by the vector containing a cDNA insert (J. Ochrietor, personal observation). Monocytes recognize molecular patterns, and this sequence of amino acids likely mimics a pattern recognized by pattern recognition receptors (PRRs) on RAW 264.7 cells [1]. A previous study of basigin-1 proinflammatory properties used a different bacterial expression vector and did not use a control (empty vector) protein in their IL-6 expression study [13]. It is likely that the same result was not observed using the HEK 293T cells because they are not monocytes and therefore would not express PRRs.

Basigin is a multifunctional glycoprotein. This statement typically refers to the basigin-2 isoform, which is expressed throughout the body and plays a role in a variety of processes [8]. For the basigin-1 isoform, which is the retina-specific isoform, although immunological and metabolic properties have been suggested, it appears that the predominant role of the protein is to participate in metabolic processes. Basigin-1 is essential for the proper function of photoreceptor neurons, as it interacts with other proteins within the neural retina to ensure that photoreceptors, especially cones, receive adequate supplies of glucose and lactate to support their function.

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**REFERENCES**


