Retina-Specific Expression of 5A11/Basigin-2, a Member of the Immunoglobulin Gene Superfamily

Judith D. Ochrietor, Tatiana P. Moroz, Leslie van Ekeris, Michael F. Clamp, Stephanie C. Jefferson, Ana C. deCarvalho, James M. Fadool, Graeme Wistow, Takashi Muramatsu, and Paul J. Linser

PURPOSE. 5A11/Basigin has recently been identified as a critical glycoprotein for full maturity and function of the mouse retina. However, the biological function of 5A11/Basigin has yet to be determined. Therefore, in an effort to determine the function of 5A11/Basigin, the molecular diversity of its expression was evaluated.

METHODS. Northern blot and immunoblot techniques were used to evaluate the number of forms of 5A11/Basigin in the mouse retina. cDNA cloning, using a mouse retina library or RT-PCR from rat, chicken, zebrafish, and human retina, was performed to determine the sequence of 5A11/Basigin transcripts. A peptide was generated, based on the deduced amino acid sequence, for subsequent antibody production. Localization of 5A11/Basigin expression was evaluated by immunoblot, immunohistochemistry, and real-time RT-PCR.

RESULTS. Two 5A11/Basigin transcripts of ~1.5 kb and ~1.8 kb, which correspond to glycosylated proteins of ~45 and ~55 kDa, respectively, were identified in mouse retina. The shorter form was previously cloned. However, the longer form, a splice variant of mouse 5A11/Basigin, is a member of the immunoglobulin gene superfamily and has been named 5A11/Basigin-2. Homologous transcripts were also cloned from rat, chicken, zebrafish, and human retina. 5A11/Basigin-2 expression was limited to the retina, specifically to photoreceptor cells, where it appeared to be most concentrated in the inner segments.

CONCLUSIONS. The specific and limited expression of 5A11/Basigin-2 explicitly within photoreceptor cells implies that this glycoprotein plays a fundamental role within the retina. However, its role remains to be determined. (Invest Ophthalmol Vis Sci. 2003;44:4086–4096) DOI:10.1167/iovs.02-0995

From 1The Whitney Laboratory of the University of Florida, St. Augustine, Florida; the 2Department of Biological Science, Florida State University, Tallahassee, Florida; the 3Section on Molecular Structure and Function, National Eye Institute, National Institutes of Health, Bethesda, Maryland; and the 4Department of Biochemistry, Nagoya University School of Medicine, Nagoya, Japan.

Supported by NSF IBN0111567 (PJL), National Eye Institute F32EY13918 (JDO); and EY13020 (JMF).

Submitted for publication September 27, 2002; revised December 17, 2002; accepted January 27, 2003.

Disclosure: J.D. Ochrietor, None; T.P. Moroz, None; L. van Ekeris, None; M.F. Clamp, None; S.C. Jefferson, None; A.C. deCarvalho, None; J.M. Fadool, None; G. Wistow, None; T. Muramatsu, None; P.J. Linser, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Judith D. Ochrietor, Whitney Laboratory of the University of Florida, 9505 Ocean Shore Boulevard, St. Augustine, FL 32080; jdo@whitney.ufl.edu.
retina.\textsuperscript{5,18} It was shown that the second form is not attributable to differential glycosylation of the 5A11/Basigin polypeptide, as two forms were observed after deglycosylation.\textsuperscript{19} Presently, we report that a splice variation of 5A11/Basigin, found only in the retina, is encoded within the mouse gene. This second form, which we have named 5A11/Basigin-2 (GenBank accession number AY120888), is a transcript of $\approx$1.8 kb and is the result of additional sequence from the intron 1 region spliced into the 5’ end of the previously cloned 5A11/Basigin transcript. Prediction software indicates that the 5A11/Basigin-2 polypeptide is similar in structure to that of 5A11/Basigin, with an additional C2 Ig domain in the extracellular portion of the molecule. Expression of 5A11/Basigin-2 is specific to the retina, particularly to photoreceptor cells, as determined by immunoblot and real-time PCR analyses.

Analysis of the amino acid sequence of mouse 5A11/Basigin through a BLAST\textsuperscript{20} alignment indicates that this sequence has significant identity to rat neuroplastin gp65, a synaptic membrane glycoprotein.\textsuperscript{21} Therefore, 5A11/Basigin-2 was cloned from rat retina RNA (GenBank accession number AY120888) to ensure that they were indeed distinct transcripts, rather than species-specific homologues. 5A11/Basigin-2 was also cloned from chicken and zebrafish retina cDNA. In addition, the human version of this transcript was identified and subsequently cloned using expressed sequence tags (ESTs) generated from human retina at the National Eye Institute.\textsuperscript{22} Significant sequence identity exists within the amino-terminal Ig loops of 5A11/Basigin-2 from these species. Analyses of the amino acid sequences of the 5A11/Basigin and neuroplastin family members suggest that they are indeed closely related yet distinct membrane glycoproteins that should be grouped within the same subset of the IGSF.

**MATERIALS AND METHODS**

**Animals**

Tissue samples were obtained from wild-type (+/+) for 5A11/Basigin) C57/129 hybrid (the background of the 5A11/Basigin knockout) mice.\textsuperscript{23} Rat eyecups from albino and pigmented animals were collected from chickens by cannulating to keyhole limpet hemocyanin (KLH) by using a kit (EZ Anti-Body Production and Purification; Pierce, Rockford, IL). The immunization protocol followed that specified for Pierce for this conjugation protocol.

All bleeds were allowed to clot overnight at 4°C, and the serum was isolated by centrifugation at 3000 rpm for 15 minutes (Damon IEC PR-6000 centrifuge; GMI, Albertville, MN). Antibodies specific for the 5A11/Basigin-2 peptide were affinity purified using the peptide listed, earlier conjugated to a gel column (SulfoLink; Pierce) according to the protocol of the manufacturer. Antibody specificity for 5A11/Basigin-2 within mouse retina was evaluated by immunoblot analyses. Serial dilutions of the purified antibody were used to determine the proper working dilution. Preabsorption of peptide with antibody before probing the membrane was also performed to verify antibody specificity for mouse 5A11/Basigin-2.

**RNA and Protein Isolation**

Wildtype (+/+) for 5A11/Basigin) mice, rats, or hatching chicks were killed according to accepted protocols, the eyes were enucleated immediately, and the neural retinas were isolated. Other mouse tissues, including brain, liver, lung, heart, skeletal muscle, spleen, testes, and uterus, were also isolated. The tissues were homogenized in extraction reagent (TRI; MRC, Cincinnati, OH), and total RNA was extracted. Proteins were then isolated from the organic phase and solubilized in 1% SDS. Both procedures were performed according to the protocol of the manufacturer.

**Northern Blot Analysis**

The sequence corresponding to exon 7 of the mouse 5A11/Basigin gene was amplified by PCR using primers BsgexF (5'-AGGCCAGTG-GAACTCACATGAAATGAC) and BsgendRV (5'-GGTGGGGTAGCTTT-TATTTGGG). Taq polymerase (Ex Taq; Panvera/Takara, Madison, WI), and the following cycling parameters: 96°C for 5 minutes, followed by 30 cycles of 96°C for 1 minute, 55°C for 1 minute, and 72°C for 3 minutes. The resultant product was then radiolabeled with $^{32}P$dCTP and the Klenow fragment (Oligolabeling kit; Amersham Biosciences, Piscataway, NJ).

Total RNA from normal and null mice was separated on a glyoxyl/dimethyl sulfoxide (DMSO) agarose gel and transferred to a nitrocellulose membrane using a commercial system (Turboblotter; Schleicher & Schuell, Keene, NH). The blot was incubated in prehybridization buffer (50% formamide, 5× SSPE, 0.5% SDS, 5× Denhardt’s solution, 100 mg/mL herring sperm DNA) for 1 hour at 42°C, followed by an overnight incubation in hybridization buffer (prehybridization buffer containing 100 ng/mL of $^{32}P$-labeled exon 7 probe) at 42°C. The blot was subjected to several rounds of washes in 1× SSPE and 0.1% SDS; followed by 0.1× SSPE, 0.1% SDS at 60°C. The blot was placed in a phosphor screen cassette (Amersham Biosciences, Sunnyvale, CA) for visualization using a phosphorescence imager (Phosphorimager SI; Amersham Biosciences) and accompanying software (Image Quant; Amersham Biosciences). Figure 1 was assembled on computer (CorelDraw 9.0; Corel Corp., Ottawa, Ontario, Canada).

**Immunoblot Analysis**

Immunoblot analysis was performed as previously described.\textsuperscript{3} Briefly, retina protein lysates (6 µg total protein) or mouse tissue proteins were separated by SDS-PAGE using 4% to 12% gradient gels in 2-(N-morpholino)ethane sulfonic acid (MES) buffer (Novex, San Diego, CA) and transferred to nitrocellulose membranes (NitroPure; MSI, Westborough, MA). Blots were stained with 0.1% fast green in methanol, acetic acid, and H2O (5:1:5), destained, documented, and blocked with a 2% solution of nonfat dry milk in TTBS (TBS containing 0.1% Tween-20) for 1 hour at room temperature. After incubation in blocking buffer, the blots were incubated in anti-5A11/Basigin antibody (1:100 dilution), affinity-purified anti-5A11/Basigin-2 antibody (1:10 dilution), affinity-purified anti-5A11/Basigin-2 antibody (1:10 dilution) with 100 µg/mL 5A11/Basigin-2 peptide, or anti-rat neuroplastin polyclonal antiserum (1:500) for 1 hour at 37°C. Blots were washed and incubated

---

**Molecular Diversity of 5A11/Basigin in the Retina**

**Materials and Methods**

**Animals**

Tissue samples were obtained from wild-type (+/+) for 5A11/Basigin) C57/129 hybrid (the background of the 5A11/Basigin knockout) mice.\textsuperscript{23} Rat eyecups from albino and pigmented animals were collected from chickens by cannulating.
in the appropriate alkaline phosphatase (AP)-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1:500 for 1 hour at 37°C. The blots were then incubated in AP substrate (Bio-Rad, Hercules, CA). Protein expression was documented using a scanner (Scanjet 6100C; Hewlett Packard, Palo Alto, CA), and the figures were assembled on computer (CorelDraw 9.0).

cDNA Cloning of Mouse, Rat, and Chicken 5A11/Basigin-2

A mouse retina cDNA library prepared by Stratagene (Imanda Zap II; Stratagene, La Jolla, CA) was provided as a generous gift from Robert Nickells (presently at the University of Wisconsin) and Donald Zack (Johns Hopkins Medical School). PCR amplification was performed using Taq polymerase (ExTaq; Panvera/Takara) in conditions suggested by the manufacturer and 50 picomoles each of a universal T3 primer and a gene-specific primer. Bsgex2RV (5′-GCGGTAAAGACTGGTTTTGGAGTTGAC), that anneals within exon 2 of the Basigin gene in the antisense direction. PCR conditions were as follows: 96°C for 5 minutes, followed by 30 cycles of 96°C for 1 minute, 55°C for 1 minute, and 72°C for 3 minutes. All specific products obtained were subcloned into a commercial vector (pCR-4-TOPO; Invitrogen, Carlsbad, CA) for sequencing using a commercial system (Big Dye terminator reagent and model 310 automated sequencer; Applied Biosystems, Inc., Foster City, CA). A PCR colony screen using M13Rev and M13F universal primers, a PCR master mix (Promega, Madison, WI), and the cycling parameters listed earlier, found a clone containing a 500-bp insert that aligned within the exon 1 region, as well as the intron 1 region of the mouse Basigin gene after a BLAST search. A primer B6799 (5′-CTGGTTTCTCCAAAGCCCATGTG), which aligns within intron 1 starting at base 6799 of the mouse gene, and a universal T7 primer were used for rapid amplification of cDNA ends (3′-RACE). The PCR conditions were the same as those just indicated.

The contiguous 5A11/Basigin-2 transcript was generated from mouse retina total RNA using 50 picomoles each of BsgeRx1 (5′-CACCTCTGGAAGGACCACATG) and BsgeVdRV (5′-GGTTGGGTTACTGCTTTATTTGAGG) primers through RT-PCR using polyolivated beads (Ready-To-Go RT-PCR beads; Amersham Biosciences), according to the protocol of the manufacturer. The same PCR cycling protocol was used as described earlier; however, 60 cycles were necessary to observe a product at ~1800 bp. A diffuse product was observed and excised for use in a second round of PCR, using the same primer set. This procedure was repeated a second time, with the same primers, before the desired product was visualized. The 1760-bp contig was then subcloned into the pCR-4-TOPO vector (Invitrogen), and colonies containing appropriately sized inserts, as determined by the PCR colony screening method described herein, were sequenced as described earlier and compared with the published mouse 5A11/Basigin gene sequence.

For cDNA cloning from rat retina RNA, RT-PCR was performed using the beads (Ready-To-Go RT-PCR; Amersham BioSciences), according to the protocol of the manufacturer. PCR conditions were as described earlier. PCR primers (50 picomoles each) BsgeRx1 and BsgeRx2V were used to obtain an initial product of ~400 bp. A primer was subsequently generated to anneal within this sequence in the sense direction. RT-PCR using the B6799 and BsgeVdRV primers was performed as described to obtain the 5′ end of the transcript. The contiguous rat 5A11/Basigin-2 transcript was generated with 50 picomoles each of BsgeRx1 and BsgeVdRV by RT-PCR, using the polyolivated beads according to the protocol of the manufacturer and the cycling protocol that was used to obtain the full-length mouse cDNA.

For cDNA cloning from chicken retina, a alignment of the mouse and chicken 5A11/Basigin (short form) amino acid sequences provided information for designing oligonucleotide primers. RT-PCR was performed using the beads (Amersham BioSciences), according to the protocol of the manufacturer, and the cycling parameters described earlier. PCR primers (50 picomoles each) avBsg2 and avBsg3RV (5′-GGTTGGGGTTACTGCTTTATTTGAGG) were used to obtain a partial sequence of the transcript. Various primers were designed, based on the chicken 5A11/Basigin sequence (accession number A31001) to obtain the remainder of the sequence using a chicken retina cDNA library (Stratagene) received as a generous gift from Susan Semple-Rowland of the University of Florida. The contiguous chicken 5A11/Basigin-2 transcript was generated using 50 picomoles each for 1 hour at 37°C, followed by an overnight incubation at 4°C. The sections were washed in TBS, followed by incubation for 1 hour in TBS containing 2% normal goat serum with FITC-conjugated goat anti-rabbit secondary antibody (1:50, Jackson ImmunoResearch Laboratories). Coverslips were mounted with TBS/glycerol (1:1) containing p-phenylenediamine (Sigma-Aldrich, St. Louis, MO) and viewed with a confocal microscope (model SP2; Leica, Deerfield, IL). Images were gathered digitally with the confocal software

**cDNA Cloning of 5A11/Basigin-2 from Zebrafish**

Partial nucleotide sequences in the zebrafish EST database (GenBank accession numbers BH281357, BH29447, and AI416365) provided information for designing oligonucleotide primers 5′-CAACGCCGAACG-TATGACAGG-3′ (lg) and 5′-CTGTCTTGGACGACAGTGATG (5′ untranslated region [UTR]) for cloning the partial z5A11 cDNA. Total RNA from adult zebrafish retina was obtained using extraction reagent (TRIzol; Invitrogen-Gibco, Rockville, MD). Retinal cDNA, obtained by reverse transcription using oligo dT primer (Advantage RT-for-PCR Kit; Clontech, Palo Alto, CA), served as template for PCR, using the lg and 5′UTR primers. Sequence information from the resultant PCR product was used to design gene-specific oligonucleotide primers for RACE: 5′-GGATTTGCTCACTGGGACTT and 5′-GGGT-CAGGTCTAGC-3′ (GeneRacer Kit; Invitrogen). The full-length cDNA was amplified from retinal cDNA using oligos 5′UTR (5′-AGTGAATCTCCTGAGTCCAGAACG) and 3′UTR and a commercial system (Expand High Fidelity PCR System; Roche Diagnostics, Indianapolis, IN).

**cDNA Cloning of Human 5A11/Basigin-2**

As part of the NEIBank project, several thousand human retina-expressed transcripts have been identified by EST analysis (Ref. 22 and unpublished; http://neibank.nei.nih.gov/ provided in the public domain by the National Institutes of Health, Bethesda, MD). Details of library construction and sequencing are described elsewhere. Briefly, mRNA from human retina was used to create a cDNA library in a commercial vector (pSport1; Invitrogen), and this library was subjected to sequencing at the NIH Intramural Sequencing Center, using automated fluorescence sequencers (model 3700; Applied Biosystems).

**Immunohistochemistry**

Primary fixation of mouse retinas was by perfusion with 4% paraformaldehyde in 0.1 M cacodylate (pH 7.4) followed by enucleation and immersion in the same solution for 1 hour at 4°C. The tissue was transferred to a 30% sucrose solution and incubated overnight at 4°C. The tissue was then transferred to optimal cutting temperature (OCT) compound (Sakura Finetek USA, Inc., Torrance, CA) and frozen. Tissues were sectioned 12 μm thick, mounted on gelatin-coated slides, and transferred to Tris-buffered saline (TBS: 0.01 M Tris, 0.15 M NaCl, 1.3 mM CaCl2; pH 7.4). The tissues were labeled for indirect immunofluorescence localization of both forms of 5A11/Basigin or of 5A11/Basigin-2 specifically. Briefly, the tissues were incubated in TBS (TBS containing 0.1% Tween 20), containing 2% normal goat serum in a humid environment for 1 hour at 37°C. The tissues were then incubated in TBS containing 2% normal goat serum with rabbit anti-5A11/Basigin-1 (1:100), affinity-purified rabbit anti-5A11/Basigin-2 (1:10), or affinity-purified rabbit anti-5A11/Basigin-2 (1:100) with 100 μg/mL 5A11/Basigin-2 peptide for 1 hour at 37°C, followed by an overnight incubation at 4°C. The sections were washed in TBS, followed by incubation for 1 hour in TBS containing 2% normal goat serum with FITC-conjugated goat anti-rabbit secondary antibody (1:50, Jackson ImmunoResearch Laboratories). Coverslips were mounted with TBS/glycerol (1:1) containing p-phenylenediamine (Sigma-Aldrich, St. Louis, MO) and viewed with a confocal microscope (model SP2; Leica, Deerfield, IL). Images were gathered digitally with the confocal software.
and assembled for publication on computer (CorelDraw 9.0; Corel Corp.).

Single Cell-Type Isolation and cDNA Synthesis
A wild-type mouse was killed according to accepted protocols, and the eyes were removed immediately. The neural retina was separated from the RPE and dissociated with papain, as previously described. Briefly, the neural retinas were incubated in calcium- and magnesium-free saline solution containing 500 μg/mL cysteine and 500 μg/mL papain for 1 hour at 37°C. Digestion was stopped by the addition of soybean trypsin inhibitor and 10% fetal bovine serum. The tissue was disrupted by trituration with a glass Pasteur pipette. The cells were diluted 1:1,000 in Medium 199 (Sigma-Aldrich) containing 10% FBS, placed on a depression slide, and viewed with a phase-contrast microscope (Nikon, Melville, NY). MCs and photoreceptor cells (PCs), 100 of each, were selected separately based on the appearance of dissociated retina cells from a previous publication from this laboratory, using a glass micropipette mounted on a microinjection apparatus (Nanoject; Drummond Scientific Co., Broomall, PA) and immediately placed in 100% ethanol. Reverse transcription without RNA isolation was performed with a kit (Cells-to-cDNA; Ambion, Austin, TX), according to the instructions of the manufacturer. The cDNA was then used as a template for relative quantitation of gene expression using real-time PCR.

Relative Quantitation of Gene Expression Using Real-Time PCR
Relative amounts of 5A11/Basigin and 5A11/Basigin-2 were determined with green nucleic acid gel stain on a sequence-detection system (SYBR Green and Prism 7000; Applied Biosystems, Inc.). Primers specific for 5A11/Basigin, 5A11/Basigin-2, and mouse 18S ribosomal RNA were designed on computer (Primer Express; Applied Biosystems, Inc.). Primer sets were as follows: ABIBASF 5’-CAGCTCGGG-CCGGGACCAT; ABIBASR 5’-GCTGTTCAAAGAGCAGGTAAGCT; ABIBAS2F 5’-TGAGACGGTGTTCCATCATCA; ABIBAS2R 5’-CCACATGAAAGAGGAGGCAACT; 18SRNAF 5’-AGTCCTGGCCCTTGTACACA; 18SRNAR 5’-CCGAGGCCCTCACTAAACC. The 5A11/Basigin sense primer spans the exon 1-exon 2 boundary so that this transcript would be specifically recognized. All runs were performed in triplicate according to the default PCR protocol (50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute) or the default one-step RT-PCR protocol (42°C for 50 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute). Relative standard curves were generated for each primer set so that the input amount from unknown samples could be calculated. Expression of 5A11/Basigin and 5A11/Basigin-2 was normalized with 18S ribosomal RNA expression within the corresponding tissue or cell type. Expression within each tissue or cell type, relative to that found in the retina (or MCs), was compared and expressed in graph format (Excel; Microsoft, Redmond, WA).

RESULTS
5A11/Basigin-2 cDNA Cloning
Previous studies by this laboratory indicated that two forms of 5A11/Basigin exist within the avian retina, but not in any other avian tissue examined. Fadool and Linser demonstrated that these are two distinct polypeptides and not simply the products of differential glycosylation events. This unique expression pattern of 5A11/Basigin in the retina was observed in the mouse as well. Figure 1 shows analyses of 5A11/Basigin mRNA and protein in the mouse neural retina. Total RNA from normal and 5A11/Basigin null mouse retinas were probed with a radiolabeled oligonucleotide corresponding to the 3’ end of 5A11/Basigin. Two transcripts of ~1.5 and ~1.8 kb were observed in the RNA from the normal mouse, but no signal was found in the null mouse retina. Similarly, two polypeptides of ~45 and ~55 kDa were observed on immunoblots of normal mouse retina proteins probed with a polyclonal antibody specific for 5A11/Basigin, but again no signal was detected in null mouse retina. Because both forms of 5A11/Basigin are missing in the null mouse, which was generated by insertion deletion, it suggests that they are splice variants of a single gene.

The mouse gene (11.8 kb) was cloned from a mouse liver genomic library and was characterized as containing seven exons. The intron 1 region between exons 1 and 2 is large (~5 kb) and therefore served as the starting point for the investigation of 5A11/Basigin splice variations. Our hypothesis was that the two forms differed only in the number of C2 Ig loops in the extracellular domain. Precedence for this type of splice variation in the IGSF was recently demonstrated for neuroplasmin gp55 and gp65. These two polypeptides differ only in the extracellular domain, so that gp55 possesses two Ig-like loops and gp65 has three Ig-like loops.

A diagram of the cloning strategy is shown in Figure 2. A primer was designed to anneal within exon 2 of the mouse 5A11/Basigin gene in the antisense direction (ex2RV). A mouse retina cDNA library served as the template for PCR amplification using the ex2RV primer and a universal T3 primer, which anneals to the 5’ end of the cDNA library. One clone contained a sequence that aligned within intron 1 of the mouse 5A11/Basigin gene. Additional primers were designed to extend the cDNA using the initial sequence obtained and 5’/3’ RACE was performed to get the full sequence of 5A11/Basigin-2. The complete cDNA sequence and the deduced amino acid se-
quence for 5A11/Basigin-2 are shown in Figure 3. RT-PCR, using primers at the extreme 5’ and 3’ ends of the transcript (Bsgex1 and BsgendRV, respectively), was also performed on C57/129 hybrid mouse retina RNA to ensure that the cloned cDNA was biologically relevant. The newly identified exon, between bases 6799 and 7146 of the mouse gene, has been named 1A to avoid confusion in the literature.

Structure prediction software analyses of the 5A11/Basigin-2 sequence using the Pfam database suggest that the spliced sequence forms a third extracellular C2 Ig loop in the predicted protein. Figure 4 shows the predicted structure of 5A11/Basigin and 5A11/Basigin-2. It has been determined that 5A11/Basigin is present in the cell membrane as a dimer; however, the stoichiometry of 5A11/Basigin-2 has not yet been determined. 5A11/Basigin possesses three N-linked glycosylations, as well as two O-linked glycosylations per polypeptide. Prediction software analyses of the 5A11/Basigin-2 amino acid sequence using the Technical University of Denmark Center for Biological Sequence Analysis website indicate that several N-linked and O-linked glycosylations are present in this form as well (Gupta, R, et al. manuscript in preparation). Our future studies will be aimed at identifying the carbohydrate moieties associated with 5A11/Basigin-2.

A BLAST search, using the cDNA sequence encoding the novel Ig loop within mouse 5A11/Basigin-2, indicated that this sequence is 68% identical with rat neuroplastin gp65. It was then necessary to determine whether these are species-specific homologues of the same proteins or distinct membrane glyco-
Molecular Diversity of 5A11/Basigin in the Retina

5A11/Basigin-2 is a member of the IGSF, based on structure prediction analyses. The tertiary structures of 5A11/Basigin and 5A11/Basigin-2 are shown as simple schematics. The Ig domains within each peptide are represented by circles. The two peptides differ in structure only by the presence of a third Ig loop in the extracellular domain of 5A11/Basigin-2. 5A11/Basigin forms a homodimer within the cell membrane; however, the stoichiometry of 5A11/Basigin-2 association is not yet known. N-linked glycosylations are represented by shaded icons and O-linked glycosylations are represented by open icons. Glycosylation sites on the 5A11/Basigin molecule are known, whereas those shown for 5A11/Basigin-2 are predicted.

Proteins. Therefore, 5A11/Basigin-2 was cloned from rat retina RNA, using a strategy similar to that used for the mouse homologue. A BLAST comparison of these two rat cDNA sequences indicates that they are indeed two distinct transcripts. The cDNA sequence for 5A11/Basigin-2 was similarly obtained from hatching chicken retina (in this laboratory) and from the zebrafish (Ekwill strain) retina, by the James Fadool laboratory at Florida State University (GenBank Accession number AB155495).

5A11/Basigin-2 was also identified through the NEIBank project. Twelve cDNA clones for 5A11/Basigin were identified in the human retina collection (22 and unpublished; http://neibank.nei.nih.gov). Seven clones were essentially full length, and of these, five contained the inserted exon sequence. A 1730-bp sequence was assembled by independent readings from 20 cDNA clones derived from different human eye tissues (GenBank accession number AF548371). The sequence was unambiguous, with two possible polymorphic sites, at position 735 (C/T) and at 1249 (A/G). Both these variants are silent with respect to predicted amino acid sequence.

Figure 5 shows an alignment of the 5A11/Basigin-2 amino acid sequences from mouse, rat, human, chicken, and zebrafish, as well as rat neuroplastin gp65. The 5A11/Basigin-2 amino acid sequences shared 80% identity. Within the amino-terminal Ig loop, these same sequences share 79.5% identity. These sequences and the rat neuroplastin gp65 amino acid sequence are 74.5% identical and 67% homologous in their amino-terminal Ig loops, based on BLAST database searches.

It was also striking to note the high sequence conservation within the transmembrane domains of these proteins. The transmembrane sequences of 5A11/Basigin family members and the neuroplastins are 67% identical. The neuroplastin gp65 sequence also contains the characteristic and yet peculiar glutamate residue present within the hydrophobic transmembrane domain of all 5A11/Basigin family sequences.

Generation of 5A11/Basigin-2–Specific Antiserum

The deduced amino acid sequence and predicted tertiary structure of mouse 5A11/Basigin-2 was used to identify a potentially immunogenic region specific to that form. A peptide was generated and conjugated to a carrier protein for antibody production in rabbits. Figure 6A shows immunoblots of total mouse retina proteins probed with the anti-5A11/Basigin-2 affinity-purified polyclonal antibody (lane 1) or the general 5A11/Basigin antiserum (lane 3). A single polypeptide of 55 kDa was observed using the 5A11/Basigin-2-specific antibody whereas the general antiserum recognized both the 55- and 45-kDa forms. The amino acid sequence chosen is identical with the rat sequence, which suggests that the antibody would recognize 5A11/Basigin-2 within rat retina lysates. Indeed, the 5A11/Basigin-2–specific antibody recognizes a single polypeptide of 50 kDa, which is a slightly smaller molecular mass than the mouse homologue (Fig. 6A, lane 2). The rat 5A11/Basigin-2 polypeptide is one amino acid shorter than the mouse polypeptide. Perhaps different sugar moieties are associated with 5A11/Basigin-2 in mouse and rat retina, which would contribute to the difference in size between the two species. Both forms are recognized by using the general 5A11/Basigin antiserum (50 and 40 kDa in rat, Fig. 6A, lane 4). This antibody did not, however, detect 5A11/Basigin-2 within retina lysates prepared from chicken or human retinas (data not shown), most likely because of the few amino acid differences between these species and mouse within the peptide used to generate the antibody. The presence of neuroplastins within rat retina lysates is also demonstrated (Fig. 6B, lane 2) and is consistent with the results obtained by Kreutz et al. No neuroplastin signal was detected using mouse retina lysates (data not shown).

Localization of 5A11/Basigin-2 Expression within the Mouse Retina

The distribution of the two forms of 5A11/Basigin in the mouse eye was evaluated by immunohistochemical and quantitative real-time PCR analyses. Immunohistochemistry, in which cryosections of a mature mouse eyecups were incubated with the antibody that recognizes both forms of 5A11/Basigin, produced a signal on the apical and basal surfaces of the RPE, the surface of MCs, PC bodies and the inner segments, and blood vessels (Refs. 2, 3, Fig. 7A). Incubation of the cryosections with the 5A11/Basigin-2–specific affinity-purified antibody indicates that 5A11/Basigin-2 is present on the surface of photoreceptor cells and the inner segments (Fig. 7B). The 5A11/Basigin-2–specific antibody also faintly labeled the outer plexiform layer; however, no signal was detected on the RPE or blood vessels (Fig. 7B). This signal is considered specific for 5A11/Basigin-2, because incubation of peptide with the antibody before immunostaining blocked the signal completely (data not shown). Also, incubation of mouse eye cup cryosections with the secondary antibody alone produced no significant signal (data not shown).

The results of the immunohistochemistry, using the newly generated antibody indicates that 5A11/Basigin-2 expression is restricted to the PCs. The general 5A11/Basigin antiserum also labels the PCs; however, it is not known whether both forms contribute to the PC immunoreactivity, or just the long form. Because the entire sequence of the short form of 5A11/Basigin is contained within the long form, the ability to generate an antibody specific for the short form is greatly hindered. Therefore, analyses of the expression of 5A11/Basigin and 5A11/Basigin-2 were performed by quantitative real-time PCR with cDNA isolated from MCs and PCs of dissociated mouse retinas.
and green nucleic acid staining technology (SYBR Green; Applied Biosystems, Inc.). MCs and PCs were isolated based on their appearances after dissociation, using a protocol previously published by this laboratory. Primer sets were designed to specifically amplify 5A11/Basigin (spanning the exon 1 to 2 junction) or 5A11/Basigin-2 (within exon 1A). Figure 8A indicates that the shorter form is predominantly expressed by MCs, although this difference in expression was not statistically significant in a Student’s t-test. 5A11/Basigin-2 expression was observed in MC cDNA as well; however, it is predominantly expressed by PCs (Fig. 8B). The difference in 5A11/Basigin-2 expression between MCs and PCs is statistically significant by Student’s t-test (P < 0.01).

The ratio of 5A11/Basigin to 5A11/Basigin-2 expression within the mouse neural retina was also evaluated by real-time RT-PCR. RNA isolated from the neural retina of a 3-week-old mouse was analyzed, using the same primer sets described earlier. The results of such analyses are presented as a graph in Figure 8C. At this age, 5A11/Basigin-2 expression is approximately twice that of the short form (Fig. 8C).

Expression of 5A11/Basigin-2 in Other Mouse Tissues

The expression of 5A11/Basigin and 5A11/Basigin-2 in other mouse tissues was evaluated by immunoblot and quantitative
real-time RT-PCR analyses. Figure 9A shows an immunoblot of proteins isolated from mouse retina, brain, lung, liver, kidney, heart, spleen, uterus, and skeletal muscle. The amount of protein loaded in each lane varies to show 5A11/Basigin expression within each tissue. Therefore, this analysis does not represent a true quantitation of 5A11/Basigin expression. 5A11/Basigin was observed in all tissues examined; however, 5A11/Basigin-2 was observed only in the proteins isolated from mouse retina using the 5A11/Basigin-2 antibody, whereas the general antiserum recognizes both forms (45 and 55 kDa in mouse). A single polypeptide of \(-55\) kDa was observed in mouse retina using the 5A11/Basigin-2 antibody, whereas the general antiserum recognizes both forms (45 and 55 kDa in mouse). A single polypeptide of \(-55\) kDa was observed in mouse retina using the 5A11/Basigin-2 antibody, whereas the general antiserum recognizes both forms (45 and 55 kDa in mouse). To further illustrate the point that 5A11/Basigin-2 is present only within the retina but not the other tissues examined, a duplicate blot was probed with the affinity-purified antibody specific for 5A11/Basigin-2. Figure 9B shows this immunoblot, on which the 5A11/Basigin-2 signal was observed only in the retina.

The relative levels of transcription for 5A11/Basigin and 5A11/Basigin-2 in mouse tissues were evaluated by quantitative real-time RT-PCR. The results of these analyses are presented as a graph in Figure 9C. The same tissues analyzed by immunoblot were subjected to quantitative real-time RT-PCR using a nucleic acid staining technique (SYBR Green; Applied Biosystems, Inc.) with the primer sets described earlier. Expression of 5A11/Basigin was detected within all tissues examined (Fig. 9C). The greatest expression was observed in the retina and testes (eight times greater than that within retina; Fig. 9C). By
contrast, 5A11/Basigin-2 expression was limited to the retina (Fig. 9C).

**DISCUSSION**

5A11/Basigin is an important glycoprotein found within the mouse retina. Mice without 5A11/Basigin are blind from the time of eye opening, although the architecture appears normal until the time of normal visual maturity, when photoreceptor outer segments do not appear to have matured properly. Later, retinal degeneration destroys the entire photoreceptor cell layer. The exact biological function of 5A11/Basigin is not known; however, this laboratory proposes that it is critical for proper development of the retina.

In an effort to understand the function of 5A11/Basigin, we sought to determine the molecular diversity of this glycoprotein within the mouse retina. It has been shown by this laboratory that several forms of 5A11/Basigin are present within the avian retina. The mouse retina also contains two 5A11/Basigin polypeptides of ~45 and ~55 kDa, which are not present in the 5A11/Basigin null mouse retina. cDNA cloning, using a mouse retina library, allowed the sequencing of the longer transcript. Characterization of the sequence of the 1.8 kb transcript indicates that it is a splice variant produced from the mouse 5A11/Basigin gene. The resultant polypeptide is Ig-like in structure and can therefore be included as a new member of the IgSF. Structure prediction software suggests that 5A11/Basigin-2 possesses three extracellular Ig-like loops, a single hydrophobic transmembrane domain, and a short cytoplasmic tail.

Immunocytochemical analyses have indicated that 5A11/Basigin family members are expressed on the surface of MCs, PCs (inner segments), and blood vessels of the mouse retina, as well as on the RPE. The studies presented herein suggest that 5A11/Basigin (short form) is present on the surface of MCs, blood vessels, and the RPE, whereas 5A11/Basigin-2 is predominantly found on the PCs and inner segments. No labeling of the RPE was observed using the 5A11/Basigin-2-specific antibody. Similarly, no 5A11/Basigin-2 transcript was detected in cDNA isolated from the RPE (data not shown). This is consistent with observations from the NEIBank human EST project, in which the long form was found to be the predominant form in the human retina, but not the RPE (http://neibank.nei.nih.gov/) and suggests that 5A11/Basigin-2 expression is limited to the neural retina. Because 5A11/Basigin-2 expression was not observed in any of the other mouse tissues examined, and no full-length sequences of the amino terminal C2 Ig loop were found within nonretina human EST data bases (data not shown), it can be argued that 5A11/Basigin-2 has a very specialized, yet unknown, role within the neural retina.

Although previously detected as a rare transcript by immunoblot and Northern blot techniques, the real-time RT-PCR analyses presented herein indicate that 5A11/Basigin-2 is actually more abundant than the short form at the age examined. This present result is consistent with observations from the NEIBank human retina EST project. It was determined that 5A11/Basigin is one of the more abundant cDNAs in a human retina library, with 5A11/Basigin-2 as the predominant form. However, these results differ from those obtained by Northern blot techniques (Fig. 1A). Unpublished observations in this laboratory indicate that the ratio of short-form to long-form transcripts is not constant during mouse retina development. Because the RNA used for real-time RT-PCR studies was from a single time point or age, rather than a pool of ages, we consider this to be the more accurate assessment of 5A11/Basigin to 5A11/Basigin-2 expression in the mouse retina. Studies designed to increase understanding of the regulation of 5A11/Basigin family member transcript expression are currently being pursued to resolve this issue.

Now that a second 5A11/Basigin transcript has been identified, it is necessary to reevaluate the mouse 5A11/Basigin gene. Initially, this gene was characterized as containing seven exons. However, the data presented herein show that the gene actually possesses eight exons. We propose that this new exon be named exon 1A to minimize inconsistencies in the literature. Similar to the other exons present within the mouse 5A11/Basigin gene, exon 1A possesses conventional intron/exon boundaries (the exon is flanked by bases AG at the 5’ end and GT at the 3’ end). Splicing of exon 1A into the ubiquitously expressed transcript appears to occur only in the retina. However, the molecular mechanism driving the usage of exon 1A remains to be determined.

The concept of splice variation is not novel to the IgSF. The synaptic membrane proteins neurolamins gp55 and gp65, which are also members of the IgSF, are splice variants from a single gene, as well. Like 5A11/Basigin and 5A11/Basigin-2, these two glycoproteins have different patterns of expression. Neurolamin gp55 is ubiquitously expressed, whereas gp65 expression is primarily limited to the brain and retina.
Molecular Diversity of 5A11/Basigin in the Retina

The amino acid sequences of the recently cloned 5A11/Basigin-2 homologues with the rat neuropsinin gp65 indicates high identity, especially within the amino-terminal Ig loops and the transmembrane domains of these polypeptides. Typically, 5A11/Basigin homologues are only 40% to 50% identical in the extracellular domains; however, the 5A11/Basigin-2 homologues possess 79.5% sequence identity in the amino-terminal Ig loop. In addition, when compared with the amino-terminal Ig loop of neuropsinin gp65, there is 74.5% sequence identity. This suggests the potential for protein–protein interactions through these domains and a role in neuronal cell adhesion within the retina. The transmembrane domains of 5A11/Basigin homologues are highly identical across species and possess a putative leucine zipper motif and a glutamic acid residue within the hydrophobic domain. Neuropsinin gp65 shares this transmembrane motif, specific to 5A11/Basigin family members (Fig. 5). It can therefore be argued that these glycoprotein families should be grouped within the same subset of the IGSF.

5A11/Basigin is an important molecule in the retina and other tissues. A biological role for 5A11/Basigin has yet to be assigned; however, several recent publications have implicated 5A11/Basigin in HIV-1 infection,1 thymocyte development,16 and erythrocyte trapping in the spleen.34 It is clear that 5A11/Basigin and MCT-1 interact on PCs. It has been shown that the 5A11/Basigin-MCT-1 complex on PCs interacts in trans with the 5A11/Basigin null mice. Thus, the 5A11/Basigin-2 interacts with other protein(s) within the developing retina, and such interactions are necessary for proper neuronal development and maturation. Perhaps it is necessary to generate another null mouse, in which the 5A11/Basigin-2 transcript is specifically inactivated, to gain full understanding of its role in the neural retina.

References

21. Miyazaki T, Masuzawa Y, Muramatsu T. The basigin group of the immunoglobulin gene superfamily: complete conservation of a


