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

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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. Previous reports indicate the presence of multiple 5A11/Basigin polypeptides within the retina. 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

Recent, 5A11/Basigin, a member of the immunoglobulin gene superfamily, has emerged as an important glycoprotein in the mouse retina. Mice in which the gene for 5A11/Basigin has been inactivated are visually impaired from the time of eye opening (2 weeks of age) through maturity, as indicated by ERG analyses. The architecture of the retina appears normal at eye opening, despite the electrophysiologic deficiency. However, by 3 weeks of age (visual maturity in the mouse), the photoreceptor outer segments in the 5A11/Basigin-null mouse retina appear less dense and shorter than those of control littersmates. Degeneration of the retina is first observed at 8 weeks of age and by 1 year, the entire photoreceptor cell layer is missing. The biological mechanism underlying the visual dysfunction and failed retinal maturity in 5A11/Basigin-null mice remains to be determined. Therefore, current efforts are directed at studying the biological relevance of this membrane glycoprotein.

The first description of 5A11 appeared in 1986 in a report by Linser et al., who used antibodies produced against embryonic day-7 chick tissues to identify a novel Müller-cell (MC)-specific protein. Subsequent work by Fadool and Linser identified the 5A11 antigen as a cell membrane glycoprotein found on MCs and mature retinal pigmented epithelia (RPE) of chicken retina. Characterization of the 5A11 antigen showed that it is identical with the HT7 antigen, an inducible marker on endothelial cells of the blood–brain barrier. Other homologous proteins in this family include M6/EMMPRIN in the human, which has been implicated in tumor metastasis and matrix metalloproteinase induction; OX-47 or CD-147, a lymphocyte activation marker in the rat; CE-9 or PE-2 in rat retina; and P7E in bovine retina.

A molecular function for 5A11/Basigin has yet to be defined; however, it is thought that this glycoprotein participates in cell–cell interactions through binding to an unknown ligand. Several recent publications have demonstrated the importance of 5A11/Basigin (CD147) in oocyte maturation, thymic development, and HIV-1 infection. The gene for mouse 5A11/Basigin, located on chromosome 10, centromeric (CM) position 42.2 (the short arm of human chromosome 19, region 13.3) has been cloned and characterized as having seven exons (Ref. 14, http://www.ncbi.nlm.nih.gov/genome/guide/mouse/ provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). A single 5A11/Basigin transcript of 1.2 kb has also been cloned from a mouse carcinoma cell line. The resultant nascent 5A11/Basigin polypeptide is 30 kDa. After glycosylation, the molecular mass increases to ~50 kDa. All the known members of this family share extensive amino acid sequence identity in the transmembrane and cytoplasmic regions, with less, but significant, similarity in the extracellular domains.

Previous work in this laboratory demonstrated the presence of an additional form of 5A11/Basigin in the chick neural
Molecular Diversity of 5A11/Basigin in the Retina

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. 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 ~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 alignment indicates that this sequence has significant identity to rat neuroplastin gp65, a synaptic membrane glycoprotein. 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. 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. Rat eyecups from albino and pigmented animals were a generous gift of Dietmar Kultz and Devulapalli Chakravarty of the Whitney Laboratory. Fertilized chicken eggs were obtained from Charles River Laboratories (North Franklin, CT) and incubated in a forced-draft incubator at 37°C and with saturated humidity at the Whitney Laboratory. Rabbits were obtained from Myrtle’s Rabbity, Inc. (Thompson Station, TN) and maintained at the Whitney Laboratory for the purpose of antibody production. Zebrafish (Ekwill strain) were reared at the Florida State University Animal Care Facility.

Care and handling of these animals was in accordance with the guidelines established by the University of Florida, Florida State University, and the Institutional Animal Care and Use Committee (IACUC). These experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Reagents

The 5A11/Basigin-specific polyclonal antiserum was generated by this laboratory with a 5A11/Basigin-GST fusion protein supplied by one of the authors (TM) and has been described previously. The neuroplastin polyclonal antiserum was a generous gift from Philip Bescoby (Royal Holloway University of London, UK).

A 5A11/Basigin-2 specific antiserum was also generated by this laboratory. A potentially immunogenic region of the novel immunoglobulin loop was synthesized as a peptide (CRASSDPDRNH1LTP-PVRKW) by the University of Florida ICBR Protein Chemistry and Biomarkers Facility (Gainesville, FL), which was subsequently conjugated to keyhole limpet hemocyanin (KLH) by using a kit (EZ-Link 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 Bsgex7F (5′-AGGGCAATGGAGACTCACTGAAATGAC) and BsgendRV (5′-GGTGGGATTAGTCTTATTTTGGG). 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 32P-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/μL of 32P-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 (Phosphorimagier 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. 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 antigen (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...
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 (Iama Zap II; Stratagene, La Jolla, CA) was provided as a generous gift from Robert Nicells (currently at the University of Wisconsin) and Donald Zack (Johns Hopkins Medical School). PCR amplification was performed using Taq polymerase (Etaxq; Panvera/Takara) in conditions suggested by the manufacturer and 50 picomoles each of a universal T3 primer and a gene-specific primer. Bsgex2RV (5'-GCAGGTAACCTGTGTTTTGAGGGTGA) that anneals within exon 2 of the Basigin 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 reaction 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.0 A primer B6799 (5'-CTGGTTTCCCTAAGGCCACGTG) that 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 Bsgex1 (5'-CACGCCCTTGAGGGCCACATG) and BsgendRV (5'-GGTGGGGTAGTTGCCTTTATTTGGGG) primers through RT-PCR using polyolipidic 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 1769-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) Bsgex1 and Bsgex2RV 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 BsgendRV 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 Bsgex1 and BsgendRV by RT-PCR, using the polyolipidic 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, an 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 (5'-GAGGGGCGGAGATGGGCGGCGG) and avBsg3RV (5'-GCCCTGCTACTGCTGCTG) 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 chicken primers each of avBsg1 and avBsg2RV (5'-GGAAAGAAAAGG-GAGGAATCATTCATTTTAT) by RT-PCR using beads according to the protocol of the manufacturer and the cycling protocol that was used to obtain the full-length mouse and rat cDNAs.

cDNA Cloning of 5A11/Basigin-2 from Zebrafish

Partial nucleotide sequences in the zebrafish EST database (GenBank accession numbers BH281837, BH294477, and AI416365) provided information for designing oligonucleotide primers 5'-CAAGCGCAAGG-TATGACAGG-3' (Ig) and 5'-TCTGCTTGGGAGCACATGAT (5' untranslated region [UTR]) for cloning the partial 5A11 cDNA. Total RNA from adult zebrafish retina was obtained using extraction reagent (TRlzol; 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 Ig and 5'UTR primers. Sequence information from the resultant PCR product was used to design gene-specific oligonucleotide primers for RACE: 5'-GGATGATGTCACACTGCGGACT and 5'-GGTGCAAGTCGATGTCCTG. The 5' cDNA sequence was obtained by 5'RACE (GeneRacer Kit; Invitrogen). The full length cDNA was amplified from retinal cDNA using oligos 5'UTR (5'-AGTGAGCTCCTGAGTCGAGGAC) 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,25 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.25,26 Briefly, mRNA from human retina was used to create a cDNA library in a commercial vector (pSPor1; 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 (1:100), 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.
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 salt 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 1:1000 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 quantification 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: ABBASF 5′-CTGGGCGG-CCGGGCGGACCAT; ABBASR 5′-GGTGTTCACAAAGGACGTAAGCT; ABBAS2F 5′-TGGACCGGTGTTCACTCAT; ABBAS2R 5′-CCCACTG-AACAGAGGCGGAACCT; ABRNASF 5′-AGGCCCTGCCCCCTTGTACACA; ABRNASR 5′-CCCGGGCCTCCTACAAACC. 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 30 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 exon 7 region of the mouse 5A11/Basigin gene. Two transcripts of ~1.5 kb and ~1.8 kb are observed in normal mouse retina, but no signal is observed in the null mouse.

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 mouse 5A11/Basigin. 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 (R. Gupta, 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-

![Figure 2. Cloning strategy used to isolate mouse 5A11/Basigin-2. Previously identified exons within the mouse 5A11/Basigin gene are shown as filled boxes. The newly identified exon is shown as an open box. The general locations of pertinent primers are indicated. Arrows: orientation of the primers. A universal T3 primer and the ex2RV primer were used to obtain the initial fragment within exon 1A from a mouse retina cDNA library through PCR. The B6799 primer was then designed to elongate the sequence in the 3' direction with the use of a universal T7 primer. Bsgex1 and BsgendRV primers were used to obtain the contiguous 5A11/Basigin-2 cDNA from mouse retina RNA by RT-PCR.](http://iovs.arvojournals.org/)

![Figure 3. Nucleotide and deduced amino acid sequence of 5A11/Basigin and 5A11/Basigin-2. The insertion of the 5A11/Basigin-2-specific sequence is in phase with the reading frame of 5A11/Basigin. The region of the cDNA specific to 5A11/Basigin-2 is underscored. In the deduced amino acid sequence, the leader sequence and transmembrane domain are in boxes. Conserved cysteine of the Ig domains are in parentheses.)
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 AY155495).

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 shown share ~60% 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 neuroplasins 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 neuroplasins 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 eyecup were incubated with the antiserum 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 eyecup 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). 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
present in the 5A11/Basigin null mouse retina. cDNA cloning, cell layer. The exact biological function of 5A11/Basigin is not known; however, this laboratory proposes that it is critical for avian retina. The mouse retina also contains two 5A11/Basigin polypeptides, whereas the other tissues examined had only one. A duplicate blot was probed with the affinity-purified antibody specific for 5A11/Basigin-2. A signal is only observed in the retina protein extract. (C) Real-time RT-PCR analyses of the various mouse tissues were performed. All runs were performed in triplicate and expression was normalized to that of the mouse 18S ribosomal RNA. The value obtained for 5A11/Basigin in the retina was set at one, and the expression in other tissues, as well as the values obtained for 5A11/Basigin-2, were determined relative to that value. (□) 5A11/Basigin; (□) 5A11/Basigin-2.

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

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 neuroplastins 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. Neuroplastin gp55 is ubiquitously expressed, whereas gp65 expression is primarily limited to the brain and 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 expressed from neural 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.

FIGURE 9. Expression of 5A11/Basigin-2 in mouse tissues. The presence of 5A11/Basigin and 5A11/Basigin-2 in mouse retina (R), brain (B), lung (Lu), liver (Li), kidney (K), heart (H), spleen (S), uterus (U), testes (T), and skeletal muscle (SM), was investigated by immunoblot (A, B) and real-time RT-PCR (C) analyses. (A) Proteins isolated from the various mouse tissues were probed with the polyclonal antiserum against 5A11/Basigin. The amount of protein in each lane varied, to help show 5A11/Basigin expression within each tissue. The results showed the mouse retina proteins to contain two 5A11/Basigin polypeptides, whereas the other tissues examined had only one. (B) A duplicate blot was probed with the affinity-purified antibody specific for 5A11/Basigin-2. A signal is only observed in the retina protein extract. (C) Real-time RT-PCR analyses of the various mouse tissues were performed. All runs were performed in triplicate and expression was normalized to that of the mouse 18S ribosomal RNA. The value obtained for 5A11/Basigin in the retina was set at one, and the expression in other tissues, as well as the values obtained for 5A11/Basigin-2, were determined relative to that value. (□) 5A11/Basigin; (□) 5A11/Basigin-2.
neuronal development and maturation. Perhaps it is necessary for the depressed ERGs observed in 5A11/Basigin-null mice. It is reasonable to postulate, however, that 5A11/Basigin-2 interacts in trans with the 5A11/Basigin-2 homologues possess 79.5% sequence identity across species and possess a putative leucine zipper motif and a glutamic acid residue within the hydrophobic domain. Neuroplastin gp65 shares this transmembrane motif, specific to 5A11/Basigin family members (Fig. 5). It can therefore be argued that these two 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, thymocyte development, and erythrocyte trapping in the spleen. It is clear that 5A11/Basigin is a cell recognition molecule that interacts with other cell surface molecules, presumably through the carbohydrate moieties. 5A11/Basigin is glycosylated differentially, depending on the tissue in which it is expressed. It is, therefore, reasonable to expect 5A11/Basigin to have numerous binding partners, depending on the tissue or cell type in which it is expressed. However, the high amino acid sequence identity of the amino-terminal Ig loop across the species from which 5A11/Basigin-2 was cloned suggests that the polypeptide itself interacts with a binding partner, rather than through associated carbohydrates.

At this time, the relationship between the two forms of 5A11/Basigin within the mouse retina is unknown. In a recent paper from this laboratory, it was demonstrated that cell membrane expression of the lactose transporters MCT-1 and -4 is dependent on 5A11/Basigin expression. MCT-1 is expressed on the surface of MCs, PC bodies, and inner segments and on the apical membrane of the RPE. The results of the localization of 5A11/Basigin family members presented herein suggest that both forms of 5A11/Basigin interact with MCT-1. 5A11/Basigin and MCT-1 interact on MCs and the RPE, whereas 5A11/Basigin-2 and MCT-1 interact on PCs. It has been shown that 5A11/Basigin and MCT-1 interact in cis in the plasma membrane. Perhaps the 5A11/Basigin-MCT-1 complex on MCs interacts in trans with the 5A11/Basigin-2-MCT-1 complex on PCs to establish the glial/neuronal lactose transport system within the retina. As we hypothesized in a publication by Phlp et al., failure of this complex to form could be responsible for the depressed ERGs observed in 5A11/Basigin-null mice. It is reasonable to postulate, however, that 5A11/Basigin-2 interacts with other protein(s) within the developing retina, and that 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.

Molecular Diversity of 5A11/Basigin in the Retina

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