Deleted in Polyposis 1-like 1 Gene (*Dp1l1*): A Novel Gene Richly Expressed in Retinal Ganglion Cells

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Purpose. To characterize a novel gene, deleted in polyposis 1-like 1 (*Dp1l1*), which is expressed in the retina.

Methods. A systematic screening by subtraction hybridization of the cDNAs from mouse retina and mouse brain was performed to obtain novel genes expressed in the retina. In situ hybridization, immunohistochemistry, and intracellular localization analyses were performed to investigate the expression patterns of *Dp1l1*. The chromosomal location of *Dp1l1* was determined by radiation hybrid mapping. Bioinformatics was used for homology analysis.

Results. A novel gene, *Dp1l1*, was expressed abundantly in the retina. It encodes a 201-amino-acid protein, and the encoded protein is designated mouse TB2-like 1. It is highly homologous to the mouse TB2, which is encoded by deleted in polyposis 1 (*Dp1*). In situ hybridization and immunohistochemical analyses showed that *Dp1l1* mRNA and the TB2-like 1 were localized richly in retinal ganglion cells (RGCs). TB2-like 1 was present in the cytoplasm in a punctate pattern. *Dp1l1* was mapped to mouse chromosome 10 by radiation hybrid mapping.

Conclusions. TB2-like 1 is a membrane protein that belongs to the YOP1/TB2/DP1/HVA22 family, and it probably plays an important role in intracellular membrane trafficking in RGCs, based on the properties of other homologous proteins. (*Invest Ophthalmol Vis Sci.* 2005;46:791–796) DOI:10.1167/iovs.04-0867

Retinal ganglion cells (RGCs) are third-order retinal neurons that encode and transmit information from the eye to the brain. Their diverse physiological and anatomic properties have been intensively investigated,[1] and knowledge of ganglion cell-associated diseases, such as glaucoma and optic atrophy, has been rapidly accumulated. For example, the *OPA1* gene was identified as the gene responsible for autosomal dominant optic atrophy[2,3] and was recently reported to be specifically expressed in ganglion cells and neurons of the retina.[4] In contrast, evidence has been collected that suggests that damage to the optic nerve axons and RGCs in glaucoma may be initiated or sustained, not only by high intraocular pressure, but also by any number of factors including ischemia, excitotoxicity, neurotrophin insufficiency, peroxynitrite damage, and others not yet identified.[5,6] Therefore, there have been many studies of neuroprotective therapy and survival mechanisms for RGCs.[7,8]

The purpose of this study was to isolate and characterize genes expressed in the RGCs because these genes can contribute to the understanding of the physiological properties of RGCs and the pathogenesis of RGC-associated diseases.

We have cloned a novel gene, *Dp1l1*, which is richly expressed in RGCs. We present the cDNA structure, expression patterns, and chromosomal location of this gene and discuss its function, based on other homologous proteins.

Materials and Methods

Animals

Adult C57BL/6 and ICR mice and Sprague-Dawley (SD) rats were used. All studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

cDNA Subtraction Hybridization

Brain and retinas of adult C57BL/6 mice were used to prepare the cDNAs. The amplification and subtraction of the cDNA from retina and brain were performed as described by Kaneko-Ishino et al.[9] The subtracted cDNAs obtained after three separate subtractions and polymerase chain reaction (PCR) amplifications were used as probes for the screening.

cDNA Library Screening

A retinal cDNA library was constructed from adult C57BL/6 mice using a cDNA synthesis kit (Uni-ZAP XR and Gigapack III Gold Packaging Extract; Stratagene, Japan). Thirty thousand plaques were blotted (Colony/Plaque Screen; DuPont/NEN, Boston, MA). Two identical replicas of the membranes were hybridized with two different digoxigenin-labeled probes of the cDNA from the brain and the cDNA obtained after the third subtraction. The hybridization signals were revealed by chemiluminescence detection (Roche Molecular Biochemicals, Tokyo, Japan). The plaques that showed positive signals with the subtraction cDNA probes and negative signals with the brain cDNA probes were converted to pBluescript (Stratagene).

DNA Sequence Analysis

The DNA sequence was determined for both strands on an automated DNA sequencer (model 310, using a Big Dye Terminator Cycle Sequencing FS Ready Reaction kit; Applied Biosystems, Inc., Foster City, CA).

Northern Blot Analysis

A total of 1.5 μg of each mRNA from different mouse organs was electrophoresed on 1.0% agarose formaldehyde gel and transferred onto a membrane (Hybond N^+; Amersham Biosciences, Tokyo, Japan). A 234-bp fragment corresponding to nucleotides (nt) 1515-1748 of *Dp1l1* cDNA was digoxigenin-labeled (PCR DIG Probe Synthesis kit; Roche). Hybridization was performed as described.[10]

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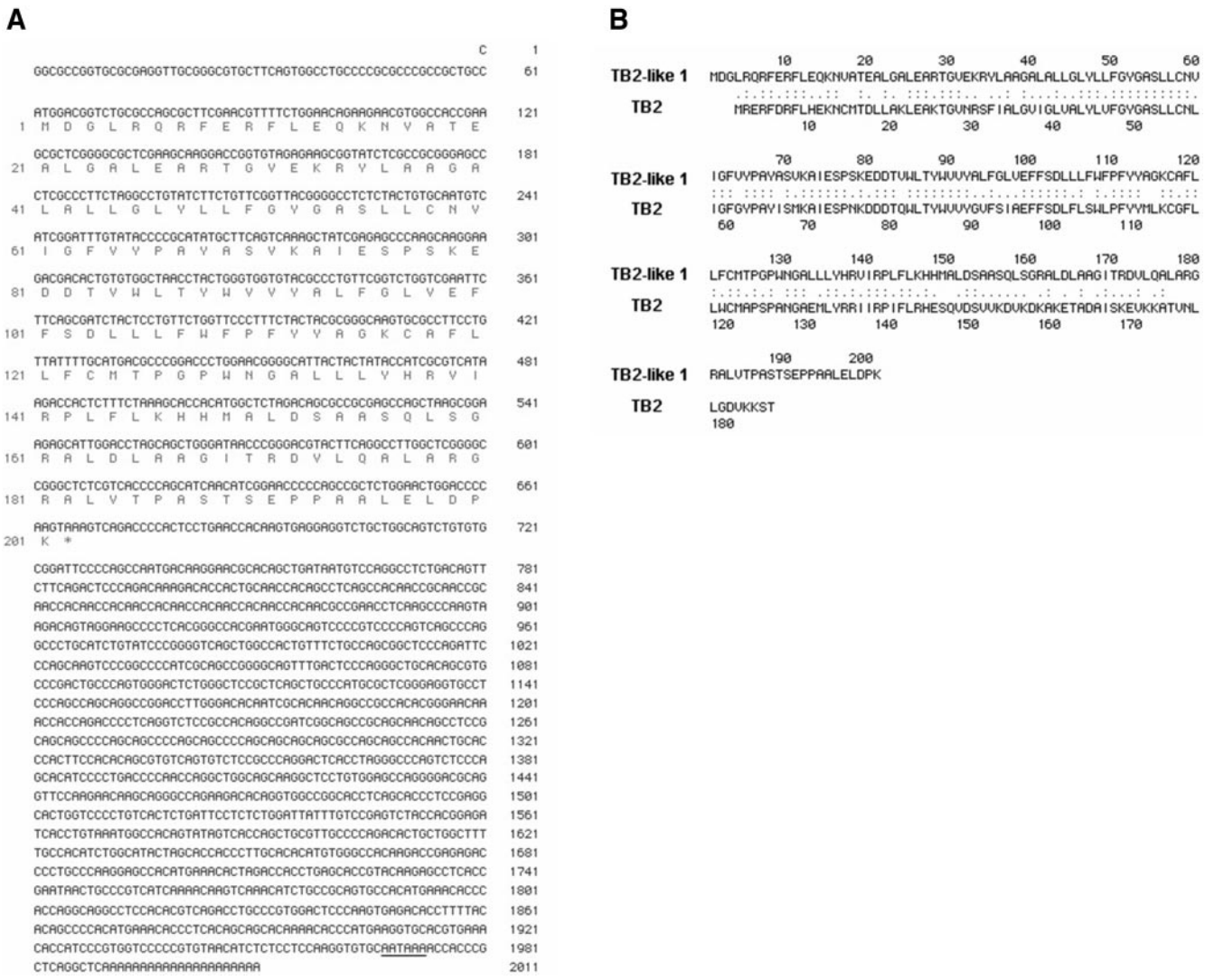

FIGURE 1. (**A**) Nucleotide and deduced amino acid sequence of the mouse gene deleted in polyposis 1-like 1. *Asterisk*: translation termination codon; *underscore*: the putative polyadenylation signal. Nucleotides are numbered on the *right*, and the deduced amino acids on the *left*. (**B**) Comparison of the amino acid sequences of TB2-like 1 and TB2. (:) Identical and (.) similar amino acid residues. Sequences were aligned using the FASTA program (http://fasta.genome.jp/; provided in the public domain by Bioinformatics Center Institutes for Chemical Research, Kyoto University, Kyoto, Japan). Note that the homology between them is approximately 88%.

Reverse Transcription–PCR

First-strand cDNA was synthesized from total RNA of mouse spleen, lung, and retina (First-Strand cDNA Synthesis kit; Amersham Biosciences). One nanogram of cDNA was amplified by PCR for 35 cycles at 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute. The oligonucleotide primer sets were *Dp1l1*-forward (F)1, 5′-AGTCAAAGCTATCGAGAGCC-3′, and reverse (R)1, 5′-TGCTAGGTCCAATGCTCTTC-3′; and β-actin F, 5′-AAGTGTGACGTTGACATCCG-3′, and R, 5′-GATCCACATCTGCTGGAAGG-3′. The PCR products were made visible by staining with ethidium bromide on 2% gel electrophoresis.

In Situ Hybridization

A *Dra*II-*Eco*RI fragment corresponding to nt 220-357 of *Dp1l1* cDNA was ligated into *Dra*II-*Eco*RI, the site of the predigested phagemid vector (pBluescriptII SK+; Stratagene). Digoxigenin-labeled antisense and sense RNA probes were generated by transcribing from the T3 and T7 promoters, respectively, after linearizing by digesting with *Dra*II and *Eco*RI (Roche Molecular Biochemicals).

Adult ICR mice were used. The excised mouse eyes were fixed with 4% paraformaldehyde in 0.1% diethylpyrocarbonate-treated phosphate buffer (PB; pH 7.4) at 4°C for 8 hours. The samples were rinsed with 0.1 M PB, immersed in 10%, 20%, and 30% sucrose in 0.1 M PB for several hours, embedded in optimal cutting temperature (OCT) compound (Sakura, Tokyo, Japan) and frozen in dry ice and acetone. The tissue blocks were sliced into 5-μm sections by a cryostat, mounted on aminopropyl silane–coated slides, and air-dried at room temperature (RT). The slides were treated for in situ hybridization (In Situ Hybridization Reagent kit; Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. Digoxigenin-labeled RNA was detected with anti-digoxigenin antibody coupled to alkaline phosphatase and made visible by reaction with nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate (NBT/BCIP), using a digoxigenin nucleic acid detection kit (Roche Molecular Biochemicals).

Laser Microdissection

Retinal sections (8 μm thick) were treated as described earlier. Selected areas of the retina were captured into the microfuge cap with laser scissors (model CRI-337; Cell Robotics, Albuquerque, NM), and mRNA was prepared (QuickPrep Micro mRNA Purification

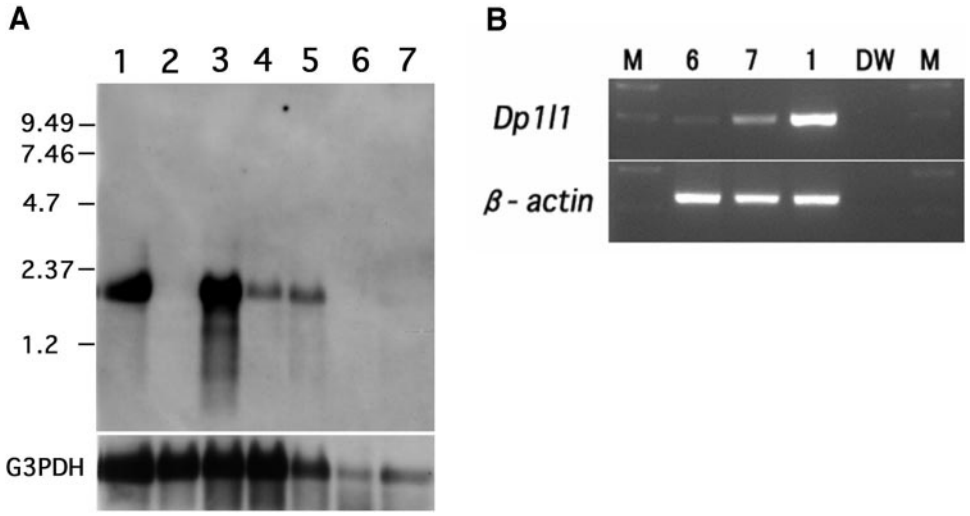

FIGURE 2. (**A**) Northern blot of *Dp111*. The *Dp111* probe detected a strong signal in the retina and liver and a weak signal in kidney and testis. The transcript was approximately 2.0 kb. G3PDH is an internal control. *Lane 1*: retina; *lane 2*: brain; *lane 3*: liver; *lane 4*: kidney; *lane 5*: testis; *lane 6*: spleen; and *lane 7*: lung. Molecular sizes (in kilobases) are indicated on the *left*. Please note that due to the quality of RNA from spleen and lung tissues, a weak signal was observed for G3PDH. (**B**) RT-PCR for *Dp111*. *Dp111* was weakly expressed in spleen (*lane 6*) and lung (*lane 7*) tissues. β-Actin is an internal control. M, DNA size marker; DW, distilled water.

kit; Amersham Biosciences). RT-PCR was performed as described earlier.

Antibody Production

A polyclonal antibody was prepared against the rat TB2-like 1 protein (Sigma Genosys, Ishikari, Japan). Two Japanese white rabbits were injected intradermally with 200 μg of the synthetic peptides CSASESPAALEPDPK, emulsified with complete Freund's adjuvant. Keyhole limpet hemocyanin (KLH) was used as a carrier protein, and synthetic peptides were conjugated to it by the *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester method. The rabbits received five booster injections of 100 μg of each of the synthetic peptides emulsified with incomplete Freund's adjuvant on days 7, 14, 21, 28, and 42. The antibody titer was measured by ELISA on days 35 and 49. After blood was collected on day 49, the serum was purified through a KLH column.

Preparation of Protein Fractions

Retinal proteins of SD rat were fractionated as described.[11] Briefly, retinas were homogenized in lysis buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EGTA, 0.05% bovine serum albumin, and protease inhibitor mixture [pH 7.2]). The homogenates were centrifuged at 1000*g* for 5 minutes at 4°C. The pellet was used as the nuclear fraction, and the supernatants were centrifuged at 10,000*g* for 15 minutes at 4°C. This pellet was used for the mitochondrial fraction, and the supernatants were centrifuged at 100,000*g* for 60 minutes at 4°C. This pellet was used for the lysosomal fraction, and the supernatants were used as the cytosolic fraction.

Western Blot Analysis

For Western blot analysis, 30 μg of each fraction was electrophoresed on 4% to 12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). The membranes were hybridized with anti-TB2-like 1 antibody and then washed with TBST (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, and 0.1% Tween 20) three times. Alkaline phosphatase–conjugated donkey anti-rabbit IgG (Promega, Madison, WI) was used as a secondary antibody. Protein bands were developed on film (CDP-Star detection reagent; Amersham Biosciences) according to the manufacturer's instructions.

Immunohistochemistry and Retrograde Labeling

SD rats were used. The sections (10 μm) were treated with 3% goat normal serum at RT for 1 hour and incubated with rabbit anti-TB2-like 1 antibody (4 μg/mL) in a moist chamber at 4°C overnight. After the sections were washed three times with phosphate-buffered saline (PBS) for 5 minutes, they were incubated in Alexa Fluor 488–conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) at RT for 1 hour and washed with PBS. The sections were examined with a fluorescence microscope (model Q550; Leica, Deerfield, IL) with a L5 filter (excitation, 480 ± 20 nm; emission, 527 ± 15 nm). Normal rabbit IgG was used at the same concentration for control experiments.

In other animals, the RGCs were retrogradely labeled with gold fluorescent tracer (Fluoro-Gold; Fluorochrome, Denver, CO) as described.[12] A Cy3-conjugated anti-rabbit IgG (dilution 1:400; Amersham Biosciences) was used as a secondary antibody. Photomicrographs

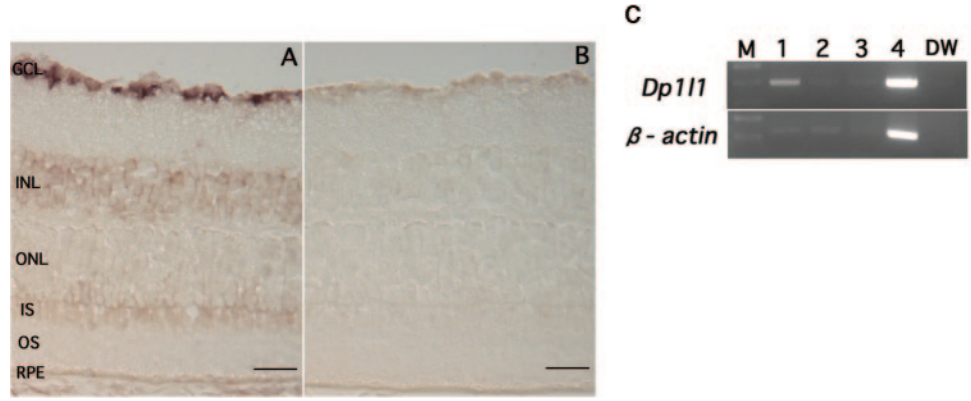

FIGURE 3. Expression of *Dp111* mRNA in the mouse retina. (**A**) Intense hybridization signals were detected in the ganglion cell layer with an antisense cRNA probe. (**B**) No significant signal was detected with a sense probe. Bar, 30 μm. (**C**) *Dp111* was also expressed in the INL and ONL/IS/OS, but its expression was very weak. *Lane 1*: GCL; *lane 2*: INL; *lane 3*: ONL/IS/OS; *lane 4*: retina.

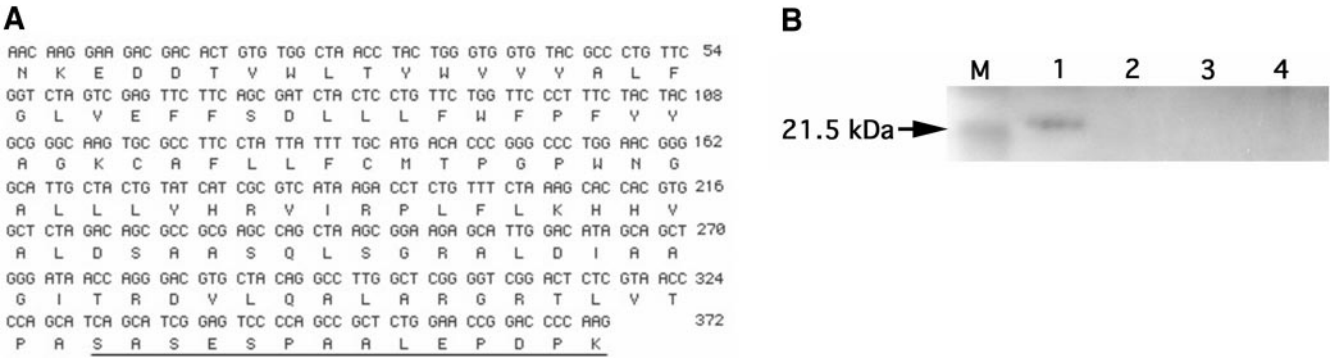

FIGURE 4. (**A**) Partial nucleotide and deduced amino acid sequence of rat *Dp111*. This fragment corresponds to the amino acids (78-201) of mouse TB2-like 1, and the amino acid identity between them is 94%. The amino acid sequences used for antibody production are *underscored*. (**B**) Western blot analysis. The antibody detected a protein of approximately 22 kDa in the cytosolic fraction of retinal extract. M, molecular standard; *lane 1*: cytosolic fraction; *lane 2*: mitochondrial fraction; *lane 3*: lysosomal fraction; *lane 4*: nuclear fraction.

were taken with a confocal microscope with a laser system (MZA PO; Leica) and an FITC filter (for the fluorescent gold tracer) and a rhodamine filter (TB2-like 1).

Intracellular Localization of TB2-like 1

The coding region of *Dp111* cDNA was subcloned at the *Nhe*I site of a BFP-expression vector, pQBI 50 (Takara Biomedicals, Tokyo, Japan) in-frame to produce a BFP fusion protein. The construct was prepared (EndoFree Plasmid Maxi kit; Qiagen, Tokyo, Japan) and transfected into 293 cells (Effectene Transfection Reagent; Qiagen). Transfected cells were observed under a fluorescence microscope (model Q550; Leica) with a 4′,6′-diamino-2-phenylindole (DAPI) filter 24 hours after transfection.

Radiation Hybrid Mapping

Radiation hybrid mapping was performed (T31 Radiation Hybrid Panel; Research Genetics, Huntsville, AL), and PCR was performed in a volume of 25 μL using 50 ng DNA of each hamster/mouse hybrid cell line, 5 picomoles of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.0 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each dNTP, and 1.0 U *Taq* DNA polymerase. The localization of *Dp111* in the radiation hybrid map was determined at the Whitehead Institute for Biomedical Research Web site (http://www.genome.wi.mit.edu; provided in the public domain by the Massachusetts Institute of Technology, Cambridge, MA). The oligonucleotide primers set for *Dp111* were *Dp111* F2, 5′-TCACTCTGATTCCTCTCTGG-3′, and *Dp111* R2, 5′-GTTATTCGGTGAGGCTCTTG-3′.

Sequence data of *Dp111* have been deposited with the DDBJ/EMBL/GenBank data libraries under accession number AB039933.

RESULTS

Isolation of *Dp111*

A novel cDNA was isolated that was highly homologous to a mouse homologue (*Dp1*) of the human gene *TB2/DP1* (deleted in polyposis 1; Fig. 1A). The deduced amino acid sequence was homologous to the mouse TB2 in which 57.2% of the amino acids (99/173) were identical and an additional 53 amino acids (53/173) were conservative substitutions, yielding a homology of 87.9% (Fig. 1B). This gene was designated deleted in polyposis 1-like 1 (*Dp111*), and the encoded protein as mouse TB2-like 1.

The sequence of *Dp111* has a large open reading frame of 603 bp (nt 62-664) with a long 3′ untranslated region and encodes a 201-amino-acid protein with a calculated molecular weight of 22,202.

Expression Patterns of *Dp111*

Northern blot analysis revealed the presence of a 2.0-kb transcript in the mouse retina. The expression of *Dp111* mRNA was not detected in the brain, but was present in other tissues, such as liver, kidney, and testis. The signal in the liver was as strong as that in the retina (Fig. 2A). Because the signal of G3PDH was

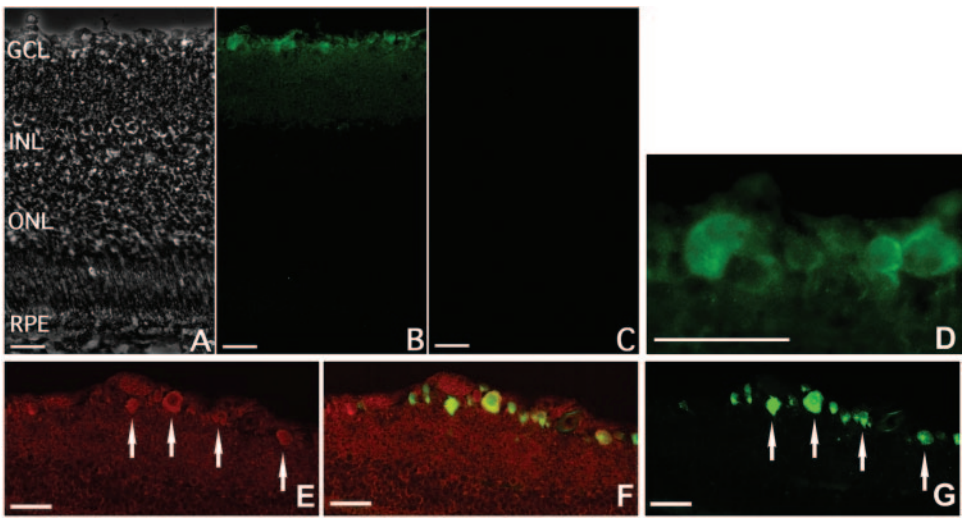

FIGURE 5. Localization of TB2-like 1 in the rat retina. (**A**) Bright-field micrograph of the section. (**B**) Signals were visible in the GCL using the polyclonal antibody. (**C**) Normal rabbit IgG was used for the control, and no significant signals were visible. (**D**) High magnification of the section showing signals in the cytoplasm. (**E**) The signals show the cells with immunostaining of TB2-like 1. (**G**) Retrograde labeling of RGCs with fluorescent gold. (**F**) Merged images in (**E**) and (**G**). *Arrows*: RGCs with immunostaining of TB2-like 1. Bars, 30 μm.

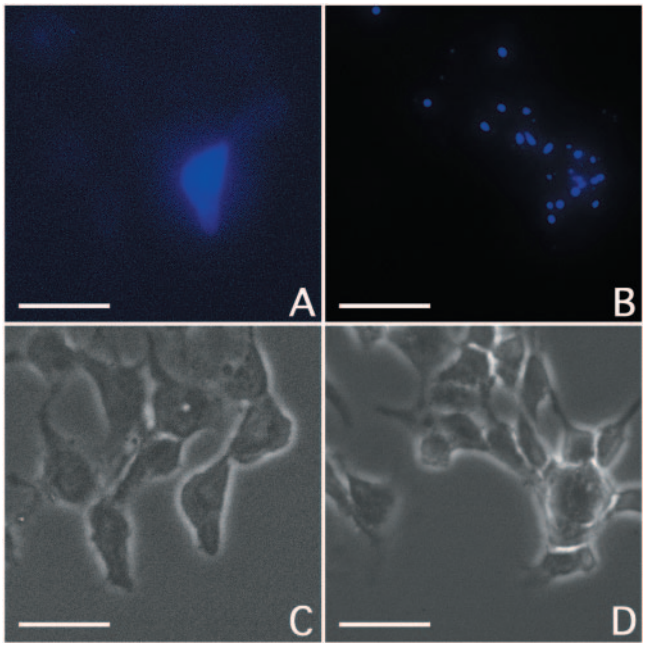

FIGURE 6. Intracellular localization of the TB2-like 1–BFP. (**A**) Only the pBQI50 vector was transfected as a control. BFP is diffusely expressed in the cytoplasm. (**B**) TB2-like 1–BFP is detected in the cytoplasm in a punctate pattern. (**C**, **D**) Bright-field micrographs of (**A**) and (**B**), respectively. Bars, 30 μm.

very weak in spleen and lung, RT-PCR was performed. *Dp111* was also expressed in these tissues, and its expression was very weak (Fig. 2B).

In situ hybridization was performed with a digoxigenin-labeled cRNA probe to determine the distribution of *Dp111* mRNA in the mouse retina. Intense hybridization signals were detected in the ganglion cell layer (GCL), with additional weak hybridization signals in the inner nuclear layer (INL) and the inner segment (IS) of the photoreceptors (Fig. 3A). There was no significant hybridized signal in the retina with the sense probe (Fig. 3B).

To refine the spatial expression of *Dp111* mRNA, the cells from the GCL, INL, and the outer nuclear layer (ONL), IS, and outer segments (OS) were captured (Laser Scissors; Cell Robotics) and RT-PCR was performed. *Dp111* was weakly expressed in the INL and in the ONL/IS/OS (Fig. 3C).

Localization of TB2-like 1 Protein

Because the rat eye is more convenient for manipulations, such as crushing the optic nerve, RT-PCR was performed to obtain the rat homologue of *Dp111*, and a partial cDNA was cloned (Fig. 4A). The cDNA had about a 94% amino acid identity with that of mouse, indicating that this clone is a partial fragment of the rat homologue of *Dp111*.

Western blot analysis revealed that the antibody produced was immunoreactive to TB2-like 1, in that a single band of approximately 22 kDa was detected in the cytosolic fraction of retinal extract (Fig. 4B).

Immunohistochemical analysis with a polyclonal antibody against rat TB2-like 1 demonstrated that signals were present in the GCL (Figs. 5A, 5B, 5C). At high magnification, the signals were detected in the cytoplasm, which is consistent with the results of Western blot analysis (Fig. 5D). Retrograde labeling of RGCs with fluorescent gold (Fluoro-Gold; Fluorochrome) confirmed that rat TB2-like 1 was located in RGCs (Figs. 5E, 5F, 5G). In addition, assays to express a TB2-like 1–BFP fusion

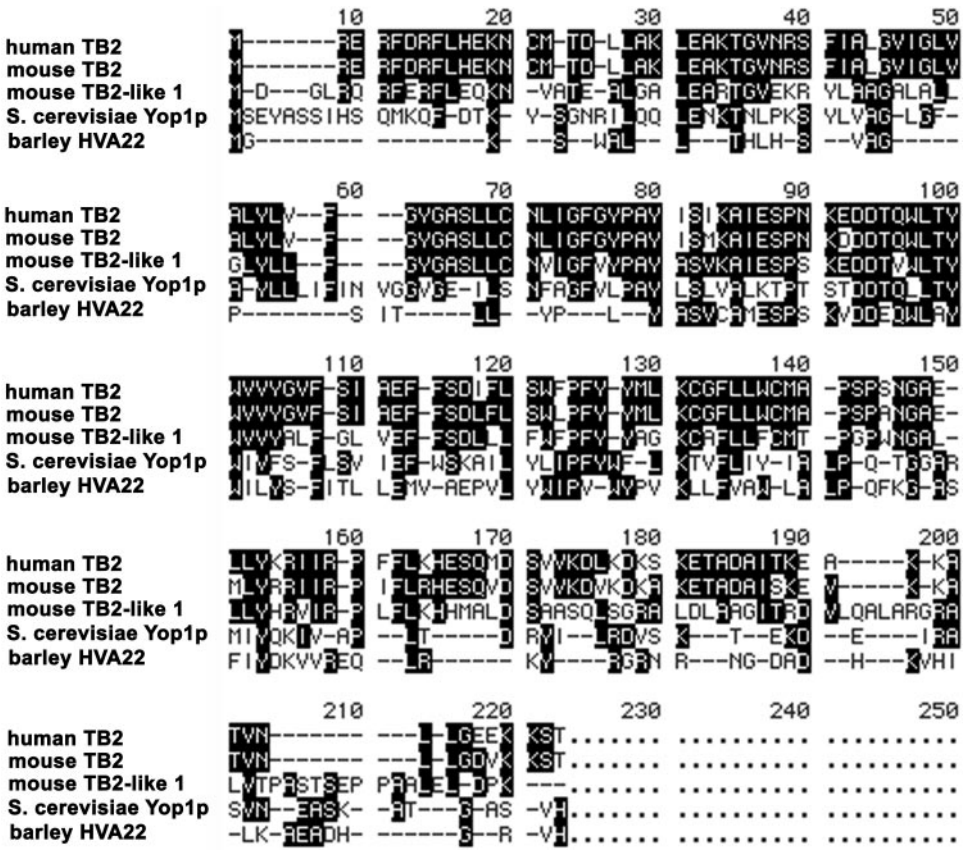

FIGURE 7. Alignment of TB2-like 1 with database homologues. *Dashes*: deletions introduced to obtain optimal alignment; *shaded residues*: represent identical amino acid residues. TB2-like 1 has approximately 57% identity at the amino acid level with human and mouse TB2, and approximately 33% identity with Yop1p and HVA22. Accession numbers were M73547 (human TB2), U28168 (mouse TB2), NP015353 (Yop1p), and L19119 (HVA22).

protein in 293 cells showed that TB2-like 1 was located in the cytoplasm in a punctate pattern (Fig. 6).

Gene Mapping

Mouse radiation hybrid mapping was performed to determine the chromosomal localization of *Dp1l1*. *Dp1l1* was mapped between microsatellite marker *D10Mit207* and *D10Mit140* on mouse chromosome 10 with an lod score >3.0.

Discussion

Dp1l1 is probably a full-length cDNA, not only because the *Dp1l1* transcript was approximately 2.0 kb, but also because the sequence, GCCGCTGCCATGG, flanking the AUG initiator codon, is highly homologous to the Kozak consensus sequence, (GCC)GCC(A/G)CCATGG.[13,14] Hydropathic analysis, using the SOSUI program (http://www.sosui.proteome.bio.tuat.ac.jp; provided in the public domain by Tokyo University of Agriculture and Technology, Tokyo, Japan) of the primary amino acid sequence, showed that there were three potential transmembrane domains (41-63, 86-108, 116-138). Computer analysis using the PROSITE program (http://www.nhri.org.tw/prosite/ provided in the public domain by the Swiss Institute of Bioinformatics, Geneva, Switzerland) demonstrated that there were two potential sites for protein kinase C phosphorylation (Ser-70, Ser-159), three potential sites for casein kinase II phosphorylation (Thr-29, Ser-78, Ser-188), and a site for tyrosine kinase phosphorylation (Tyr-35). These results suggest that TB2-like 1 is a membrane protein and acts as a member of a signaling pathway.

A National Center for Biotechnology Information (NCBI, Bethesda, MD) conserved-domain search showed that mouse TB2-like 1 was homologous with Yop1p and HVA22 as well as mouse and human TB2 (Fig. 7). Human *TB2/DP1* was one of six genes deleted in severe cases of familial adenomatous polyposis disease, although its function is still undetermined.[15,16] Prieschl et al.[17] demonstrated that mouse TB2 regulates allergic effector cells and suggested that it functions as a member of a signaling pathway. Yop1p interacts with Yip1p in Rab-mediated membrane transport in *Saccharomyces cerevisiae*. It has been shown that disruption of *YOP1* had no apparent effect on cell viability, but overexpression resulted in cell death, accumulation of internal cell membranes, and blockage of membrane traffic.[18] In addition, it was suggested that Yop1p/HVA22 regulated vesicular traffic in stressed cells, either to facilitate membrane turnover or to decrease unnecessary secretion.[19] Thus, TB2-like 1 probably belongs to the YOP1/TB2/DP1/HVA22 family and may be involved in membrane traffic—for example, intracellular trafficking, secretion, and vesicular transport. The intracellular localization pattern of TB2-like 1 (Fig. 6B) was consistent with this functional role. It would be interesting to determine whether TB2-like 1 is associated with axonal protein transport in RGCs.[20]

Dp1l1 was mapped to a region between *D10Mit207* and *D10Mit140* on mouse chromosome 10. This region is considered syntenic to human chromosome 19 at p13.3 or 21q22.3. A human cDNA (GenBank accession number AK058112, BC008201; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by NCBI) isolated from the testis and eye encodes a protein that is approximately 82% identical with mouse TB2-like 1, and the sequence of this cDNA is in a genomic region of human chromosome 19 at p13.3. The results of Northern blot and mapping data strongly suggest that this gene is the human orthologue of *Dp111*. Although linkage analysis using sib pairs for adult-onset primary open-angle glaucoma (POAG) showed high lod scores on chromosome 19, the critical interval is between *D19S414* and *D19S246* on chromosome 19, q13.1-q13.4.[21] Therefore, the human orthologue of *Dp1l1* is excluded as a candidate gene for POAG in that study.

Overexpression or knockdown studies may elucidate the proposed role of *Dp1l1* in intracellular membrane trafficking. In this context, the newly generated TB2-like 1 antibody will serve as a valuable tool for further investigation.

Acknowledgments

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