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

Hajime Sato,1 Hirosi Tomita,1 Toru Nakazawa,1 Shigeharu Wakana,2 and Makoto Tamai1

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 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 retina.

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 atrophy2–5 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.7 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 (UniZAP XR and Gigapack III Gold Packaging Extract; Stratagene, Japan). Thirty thousand plaques were blotted (Colon/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

From the 1Department of Ophthalmology, Tohoku University Graduate School of Medicine, Sendai, Japan, and the 2Mouse Functional Genomics Research Group, RIKEN Genomic Sciences Center, Yokohama, Japan.

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Corresponding author: Hajime Sato, Department of Ophthalmology, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan; hasato@oph.med.tohoku.ac.jp.
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'-AGTCAAAGCACTATCGAGAGCC-3' and reverse (R)1, 5'-TGCTAGGTCCAATGCTCTTC-3'; and 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

An EcorI fragment corresponding to nt 220-357 of Dp1l1 cDNA was ligated into EcorI, 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 DraI and EcoRI (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 Kit; Stratagene).

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, Tokyo, Japan). Note that the homology between them is approximately 88%.
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 \( \text{m}-\text{maleimidobenzoyl-N-hydroxysuccinimide ester} \) 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 \( \times \) g for 5 minutes at 4°C. The pellet was used as the nuclear fraction, and the supernatants were centrifuged at 10,000 \( \times \) g for 15 minutes at 4°C. This pellet was used for the mitochondrial fraction, and the supernatants were centrifuged at 100,000 \( \times \) 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
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 Dp1l1 cDNA was subcloned at the NheI 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 MgCl2, 0.01% gelatin, 0.2 mM each dNTP, and 1.0 U Taq DNA polymerase. The localization of Dp1l1 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 Dp1l1 were Dp1l1 F2, 5'-TCACTC-TGATTCCTCTGG-3' and Dp1l1 R2, 5'-GTTATTCGGTGAGGCTTTG-3'.

Sequence data of Dp1l1 have been deposited with the DDBJ/EMBL/GenBank data libraries under accession number AB039933.

RESULTS
Isolation of Dp1l1
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 (Dp1l1), and the encoded protein as mouse TB2-like 1.

The sequence of Dp1l1 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 Dp1l1
Northern blot analysis revealed the presence of a 2.0-kb transcript in the mouse retina. The expression of Dp1l1 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
very weak in spleen and lung, RT-PCR was performed. DpIII 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 DpIII 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 DpIII 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. DpIII 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 DpIII, 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 DpIII.

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
protein in 293 cells that showed 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 DpIII. DpIII was mapped between microsatellite marker D10Mit207 and D10Mit140 on mouse chromosome 10 with an lod score >3.0.

Discussion

DpIII is probably a full-length cDNA, not only because the DpIII 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 (i1-63, 86-108, 116-138). Computer analysis using the PROSITE program (http://www.ncbi.nlm.nih.gov/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 vesicular 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

DpIII 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 Yip1p in Rab-mediated membrane transport in Saccharomyces cerevisiae.18 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 vesicular 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

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

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References


