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Molecular Cloning and Characterization of a New Neuron-Specific Homologue of Rat Polypyrimidine Tract Binding Protein[1]

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Cancer-associated retinopathy (CAR) is a rare form of retinal degeneration and also one of the paraneoplastic neurologic disorders. Sera of CAR patients usually contain high titers of antibodies against retinal proteins, and CAR is believed to be an autoimmune disease. Using serum from a CAR patient as a molecular probe, a homologue of the polypyrimidine tract–binding protein (PTB) was isolated from a cDNA library of rat neonatal retina. This homologue, named PTB-like protein (PTBLP), encodes a 532 amino acid residue protein and has 73.5 and 68.8% homology with PTB and with a regulator of differentiation 1, respectively. Functional domains in the PTB, such as nuclear localization signals and four RNA recognition motifs (RRMs), were highly conserved. The expression of PTBLP mRNA was observed in the retina and brain but not in liver, kidney, spleen, or lung. The expression of PTBLP protein in rat retina was distributed in most of the cells in the ganglion cell layer and some cells in the inner nuclear layer. The PTBLP protein was localized in the nuclei of these cells. These results suggest that PTBLP is a new member of the PTB gene family and a neuron-specific homologue.

Key words: cancer-associated retinopathy, neuron-specific protein, paraneoplastic syndromes, polypyrimidine tract binding protein, PTB-like protein.

Paraneoplastic syndromes affecting the nervous system are caused by the remote effect of cancer cells without direct invasion or metastasis and are associated with antibodies reacting against neuronal proteins (*1*, *2*). These antibodies are markers for the diagnosis of specific neurological disorders as well as for the early detection of the associated tumors (*3*, *4*). In some of the paraneoplastic neurologic disorders, the neuronal proteins that react with the sera of patients, such as CDR (*5–7*), HuD (*8*), NOVA (*9*, *10*), βNAP (*11*, *12*), and Ma1 (*13*), have been isolated. Although the exact physiological functions of these proteins are still unknown, they seem to play an important role in the development and maintenance of neurons. These earlier studies have demonstrated that sera of patients are useful molecular probes for the exploration of unknown neuron-specific proteins that may play significant roles in neuronal cells.

Cancer-associated retinopathy (CAR) is one of the paraneoplastic syndromes of the visual system, first described by Sawyer *et al.* (*14*). CAR is characterized by a progressive loss of vision, clinical signs of retinal degeneration, and reduced electroretinograms. Clinical, immunological, and pathological examinations strongly suggest that autoimmunological mechanisms play a role, although the mechanism of this disorder has not been fully elucidated (*15–19*). The sera of CAR patients usually contain high titers of antibodies against retinal proteins, and such antiretinal antibodies predominantly react with photoreceptors and ganglion cells in the retina (*20–31*).

In our previous studies, we showed that the serum from a CAR patient with endometrial carcinoma reacted with at least three retinal proteins of different molecular size (*32*), and two of these proteins were isolated from a human retinal cDNA library as recoverin and TULP1 (*33*). TULP1 is currently identified as a candidate gene for autosomal recessive retinitis pigmentosa (*34*, *35*). In this study, we further examined a retinal antigen that reacts with the serum of the patient using a cDNA library of rat neonatal retina. A new retinal and neuronal protein, a homologue of polypyrimidine tract–binding protein (PTB), was cloned. This protein was named polypyrimidine tract–binding protein–like protein (PTBLP). The tissue distribution of PTBLP was studied by reverse transcriptase–polymerase chain reaction (RT-PCR) and immunohistochemistry.

MATERIALS AND METHODS

Animals—Adult and one-week-old Sprague-Dawley rats were used throughout this study. The eyes and tissues were enucleated after euthanasia and further processed depending on the experimental protocol. New Zealand white rabbits were used for the generation of antibodies against

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Abbreviations: CAR, cancer-associated retinopathy; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RRM, RNA recognition motif; PTB, polypyrimidine tract–binding protein; RT-PCR, reverse transcriptase-polymerase chain reaction.

PTBLP. The experiment and animal care conformed to the NIH guidelines for animal research.

Materials—The serum used in this study was obtained from a 60-year-old woman with endometrial cancer. A detailed clinical picture of this patient has been presented elsewhere (*32*). The FastTrack mRNA isolation kit was purchased from Invitrogen; the FirstStrand cDNA synthesis kit was a product of Pharmacia-Biotech; the Taq Dye Terminator Cycle Sequencing kit was obtained from Perkin-Elmer. The horseradish peroxidase (HRP)–conjugated and the alkaline phosphatase–conjugated goat anti–human IgG, used as secondary antibody for immunoblot analysis, were purchased from Jackson Immunolaboratories, and the FITC-conjugated goat affinity purified IgG to rabbit IgG for immunohistochemistry was obtained from DAKO A/S. The digoxigenin-labeled RNA molecular weight marker for Northern blot analysis and its detection kit were obtained from Boehringer. Other chemicals were purchased from Sigma or Wako Pure Chemicals, unless otherwise specified.

Cloning and Sequencing of PTBLP cDNAs—Poly(A)$^+$ mRNA was isolated from the retina of 8 to 10-day-old rats using a FastTrack mRNA isolation kit. The mRNA was further purified through an oligo(dT) cellulose column. cDNA was prepared from the mRNA according to the manufacturer's protocol using oligo(dT) primers containing the *Xho*I restriction enzyme recognition site downstream to a 18-base poly(dT) sequence and cloned into the lambda ZAP II XR vector (Stratagene).

Expression screening of the library, using the serum from the patient, was carried out according to established protocols. Briefly, 2.0×10^6 phages were plated, grown, and transferred to a nitrocellulose membrane presoaked with 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Filters were blocked in 5% dry skim milk and 0.05% sodium azide in 0.05% Tween 20 and Tris-buffered saline (TBST), then incubated with 1:1,000 dilution of the patient's serum in 1% bovine serum albumin in TBST for 2 h at room temperature. After being washed in TBST, the filters were incubated for 1 h with HRP-conjugated anti–human IgG, the secondary antibody. Positive plaques were visualized with diaminobenzidine as the substrate. For *in vivo* excision, the phages were coinfected with helper phage ExAssist (Stratagene, titer $> 10^{10}$ pfu/ml) into *Escherichia coli* XL1-Blue MRF′, and after incubation for 4 to 5 h, the supernatants containing the recombinant M13 phages were incubated with *E. coli* SOLR to convert them to a plasmid form. Cloned cDNAs were subjected to double-strand DNA sequencing using a Taq Dye Terminator Cycle Sequencing kit and an autosequencer ABI Prism 310 (Perkin Elmer). The nucleotide sequences and deduced amino acid sequences were analyzed for homology with published sequences using databases. Alignments were performed using FASTA (*36*) and BLAST (*37*) programs in the GenomeNet. The cDNA sequence of PTBLP appears in EMBL data bank under accession number AJ010585.

Fusion Proteins and Epitope Analysis—PTBLP cDNA clones were digested with restriction enzymes and four different-size fragments were purified by agarose gel electrophoresis. The restriction enzymes and the nucleotide numbers of fragments based on the nucleotide sequence of PTBLP cDNA in Fig. 1 were as follows. Construct 1, *Hin*dIII and *Pvu*II, 35–517 containing translation initiation site of PTBLP; construct 2, *Eco*RI and *Xho*I, 469–1306; construct 3, *Bam*HI, 1042–1380; and construct 4, *Bcl*I and *Bgl*II, 1322–2715 containing translational termination site of PTBLP. The fragments were inserted into pET-32 prokaryotic expression vector. All constructs were sequenced to ensure that they were cloned in the correct reading frame.

E. coli cells carrying each constructed DNA were grown to an optical density of 0.6 and induced by 1 mM IPTG for 3 h at 37°C. Cells were isolated by a centrifugation and lysed by resuspension in SDS sample buffer containing 2% SDS. The lysates were resolved by 12% SDS-PAGE and transferred to nitrocellulose membrane. After blocking with 5% dry skim milk, the membrane was sequentially incubated with the patient's serum (1:2,000 dilution) overnight at 4°C and goat anti–human alkaline phosphatase-labeled IgG diluted 1:5,000, for 1 h at room temperature. Between steps, the membrane was washed with TBST. Antigen-antibody complexes were detected with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT).

Northern Blot Analysis—An 860-bp cDNA fragment containing 3′-noncoding sequence of rat PTBLP was amplified by PCR using PTBLP-specific primers (5′-TGACATACGTGCCTTACAAAGGAA-3′ and 5′-GTAGATTCTGAGCCATGTT-3′). Poly(A)$^+$ RNA was isolated from adult rat retina with reagents obtained from Invitrogen. Poly(A)$^+$ RNA (approximately 5 μg) was loaded and resolved on a 1% agarose gel containing 0.66 M formaldehyde. The RNA was transferred to a nylon membrane by use of a vacuum blot system from Pharmacia. The membrane was prehybridized and hybridized to the radiolabeled probe at 65°C for 16 h in DIG Easy Hyb solution from Boehringer. Following hybridization, the membrane was rinsed at room temperature in $2 \times$ SSC, 0.1% SDS, and washed twice at 60°C for 15 min in $0.1 \times$ SSC, 0.1% SDS. The blot was exposed to a phosphoimaging plate for 10 days, then analyzed with a Fuji BAS-1500 bio-image analyzer.

RT-PCR—Poly(A)$^+$ RNA was prepared from adult rat retina, brain, liver, kidney, spleen, and lung, as described above. About 0.15 μg of poly(A)$^+$ RNA was used to synthesize first-strand cDNA. Amplification of the target genes was carried out by a step cycle (95°C, 30 s; 55°C, 30 s; 72°C, 120 s) for 32 cycles. The primers used in this experiment were as follows: PTBLP, 5′-GCTGTGACACCTCATCTTCGTAAC-3′ (sense) and 5′-AGCATTAGGAATAGCCAAAGGACTG-3′ (antisense), which amplified a 600-bp region of the rat PTBLP cDNA species (399–998 in Fig. 1); PTB, 5′-AGTTCAAAGGTGATAACAGGAGCAC-3′ (sense) and 5′-GTTGTATTTGACGTTGAGACTGGTG-3′ (antisense), which amplified a 668-bp region of the rat PTB cDNA species (161–828 of GenBank: X60789); and GAPDH, 5′-TGAAGGTCGGTGTCAACGGATTTGGC-3′ (sense) and 5′-CATGTAGGCCATGAGGTCCACCAC-3′ (antisense), which amplified a 983-bp region of the rat GAPDH cDNA species (35–1017 of GenBank: M17701). The nucleotide sequence of the PCR products was determined by direct sequencing.

Generation of Antibodies against PTBLP Peptide—To generate antibodies specific to PTBLP, we selected a 19-amino-acid peptide of the N-terminal sequence of PTBLP (SSPNSNMSGMVVTANGNDS, 26–44). The peptide, containing additional cysteine in the N-terminus, and conjugated to keyhole limpet hemocyanin (KLH) (Sigma) with an *m*-maleimidobenzoyl-*N*-hydoxysuccinimide ester, was used as the antigen to immunize rabbits. After collecting blood for preparation of preimmune serum, the peptide/

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   1 CTCGCTGGCTGCGTGGCTCGGTTCTTGGGAGCGAAGCTTTGTCCGGTTCGGCAATGGACGGAATTGTCACTGAGGTTGCTGTTGGTGTGA
                                                     M  D  G  I  V  T  E  V  A  V  G  V  K  13
  91 AGAGAGGATCTGACGAGCTACTCTCAGGCAGTGTTCTCAGTAGTCCCAACTCTAATATGAGTGGCATGGTAGTTACAGCCAATGGTAACG
      R  G  S  D  E  L  L  S  G  S  V  L  S  S  P  N  S  N  M  S  G  M  V  V  T  A  N  G  N  D  43
 181 ACAGTAAGAAATTTAAAGGAGAAGATAAGATGGACGGGGCGCCCTCTCGTGTGCTTCACATTCGAAAGTTACCTGGTGAAGTGACTGAGA
      S  K  K  F  K  G  E  D  K  M  D  G  A  P  S  R  V  L  H  I  R  K  L  P  G  E  V  T  E  T  73
 271 CCGAAGTAATTGCGTTAGGCTTACCTTTTGGTAAGGTGACTAACATCCTTATGCTGAAAGGAAAGAACCAGGCATTTTTGGAACTGGCAA
      E  V  I  A  L  G  L  P  F  G  K  V  T  N  I  L  M  L  K  G  K  N  Q  A  F  L  E  L  A  T 103
 361 CAGAGGAAGCAGCTATTACTATGGTTAATTACTATTCTGCTGTGACACCTCATCTTCGTAACCAACCAATTTACATTCAGTACTCCAATC
      E  E  A  A  I  T  M  V  N  Y  Y  S  A  V  T  P  H  L  R  N  Q  P  I  Y  I  Q  Y  S  N  H 133
 451 ACAAAGAACTAAAGACAGATAACACATTAAACCAACGTGCTCAAGTAGTTCTTCAAGCTGTGACAGCTGTCCAGACAGCAAATACACCTC
      K  E  L  K  T  D  N  T  L  N  Q  R  A  Q  V  V  L  Q  A  V  T  A  V  Q  T  A  N  T  P  L 163
 541 TCAGTGGCACCACAGTCAGTGAGAGTGCGGTGACTCCAGCTCAGAGTCCAGTGCTTAGAATAATTATTGACAATATGTACTACCCTGTAA
      S  G  T  T  V  S  E  S  A  V  T  P  A  Q  S  P  V  L  R  I  I  I  D  N  M  Y  Y  P  V  T 193
 631 CACTTGATGTCCTTCACCAAATATTTTCTAAGTTTGGTGCTGTATTGAAGATAATCACATTTACAAAAAACAACCAGTTTCAGGCTTTGC
      L  D  V  L  H  Q  I  F  S  K  F  G  A  V  L  K  I  I  T  F  T  K  N  N  Q  F  Q  A  L  L 223
 721 TCCAGTATGGTGATCCAGTAAACGCTCAACAGGCAAAGCTAGCCCTAGATGGTCAAAATATTTATAATGCTTGCTGTACCCTAAGGATTG
      Q  Y  G  D  P  V  N  A  Q  Q  A  K  L  A  L  D  G  Q  N  I  Y  N  A  C  C  T  L  R  I  D 253
 811 ATTTTTCCAAACTTGTGAATTTGAATGTAAAATACAACAATGATAAAAGTAGGGATTATACACGACCTGATCTTCCATCTGGAGACGGCC
      F  S  K  L  V  N  L  N  V  K  Y  N  N  D  K  S  R  D  Y  T  R  P  D  L  P  S  G  D  G  Q 283
 901 AGCCTGCATTAGACCCAGCTATTGCTGCAGCATTTGCCAAGGAGACGTCCCTTTTAGCTGTCCCAGGAGCTCTCAGTCCTTTGGCTATTC
      P  A  L  D  P  A  I  A  A  A  F  A  K  E  T  S  L  L  A  V  P  G  A  L  S  P  L  A  I  P 313
 991 CTAATGCTGCTGCGGCAGCTGCTGCCGCTGCCGCTGGCCGAGTGGGCATGCCTGGAGTCTCAGCTGGTGGCAACACAGTCCTGTTGGTTA
      N  A  A  A  A  A  A  A  A  A  A  G  R  V  G  M  P  G  V  S  A  G  G  N  T  V  L  L  V  S 343
1081 GCAATTTAAATGAAGAGATGGTTACGCCCCAAAGTCTGTTTACCCTCTTCGGTGTTTATGGCGATGTGCAGCGCGTGAAGATTTTGTACA
      N  L  N  E  E  M  V  T  P  Q  S  L  F  T  L  F  G  V  Y  G  D  V  Q  R  V  K  I  L  Y  N 373
1171 ATAAGAAAGACAGTGCTCTGATACAAATGGCCGATGGGAACCAGTCACAGCTTGCCATGAATCATCTTAATGGGCAGAAGATGTATGGAA
      K  K  D  S  A  L  I  Q  M  A  D  G  N  Q  S  Q  L  A  M  N  H  L  N  G  Q  K  M  Y  G  K 403
1261 AAATTATTCGTGTTACTCTCTCTAAACATCAGACTGTGCAACTACCTCGAGAGGGACTTGATGATCAAGGGCTAACAAAAGATTTTGGGA
      I  I  R  V  T  L  S  K  H  Q  T  V  Q  L  P  R  E  G  L  D  D  Q  G  L  T  K  D  F  G  N 433
1351 ATTCACCCCTGCACCGTTTTAAAAAACCGGGATCCAAAAACTTTCAGAACATTTTCCCTCCTTCTGCAACCCTTCACCTGTCCAACATCC
      S  P  L  H  R  F  K  K  P  G  S  K  N  F  Q  N  I  F  P  P  S  A  T  L  H  L  S  N  I  P 463
1441 CTCCTTCTGTAGCAGAAGAGGATCTGCGAACTCTGTTTGCCAACACCGGGGGCACTGTGAAAGCATTTAAGTTTTTTCAAAGAGATCACA
      P  S  V  A  E  E  D  L  R  T  L  F  A  N  T  G  G  T  V  K  A  F  K  F  F  Q  R  D  H  K 493
1531 AAATGGCTCTTCTTCAGATGGCAACAGTGGAGGAAGCTATTCAGGCTTTGATTGATCTTCATAATTATAACCTTGGTGAAAACCATCATC
      M  A  L  L  Q  M  A  T  V  E  E  A  I  Q  A  L  I  D  L  H  N  Y  N  L  G  E  N  H  H  L 523
1621 TGAGAGTGTCTTTCTCCAAATCAACAATTTAAGGACGGGAGATGAAGATGGCGGGCAGATCCCATTGTTTGGTGTCATCACCTATTGACT
      R  V  S  F  S  K  S  T  I  *                                                              532
1711 GTTCAGAAAAGTGGGGACCAGAGTTGATTTTTTCATGCTGTTCTCATTCCTTGGTTATAAATGAAATGGCGTATGTAAGGCAGAGTGCTA
1801 ACTGCTGTATTTCATCTGTTCAGTAGGGAAGCCATTTTGTCTGTTAAATTTTAGTTTAATTTTGCTTCTTTTTTTTTTTTTCAACTTAG
1891 TGACATACGTGCCTTACAAAGGAAAGCTAGTGTTGCTATTGTGCATTTACTAGGAAAAAGGAATTGGTTGTTTAGGGCACATTGTTATGT
1981 GGAAATTAAAATCTGTTTAGGCAGGGGTGTGTCAAAAGGTTAAGTTTTTGTTTCTCCTGCTTGGAACTTATTTTGAATTATTGGCTTATC
2071 ACATTTCTTTCTATTTAATCTAATAAGATACTTGATACTGAAAGTGTAAAACAGCATTTTTAGTTCCTACTATCTTAGGCTTTATTGCTT
2161 TGAAAACATTGGCCTTTTGTATCTCACAAATCTGGTCTAGATTCAGTTATGAATATAGGCATTAGTTAAAATTAACAAGATGCAGAGTAT
2251 TAATTTCTTAAGACAAGTGATTTCTGTAAGTTTGAGCCCTATGTGGAAAGCATTGTGGAACCTTAACCTTTTTGTACACACTCTTGTGGG
2341 ACGTATCATATAAATGTCAGCACTAAGTAATGTCTTGTTTGTGGCTGAATATTTTTCGTAGATGTTTTTGAAGTTGACATGACTTATGTG
2431 CATTTAAATATATATTGCCATCCTTAGTTTGTAATTAAGATTTGGAAAATGGTTGTGGATTTCTGAGCATGTGCAGACTGGTCTAGCTAG
2521 TTCAGGAACTGGTGCATGTATTTTCAAAGACAAAGAAAAGTGTACTGCGAAAACTTGCAGGAAGATTAATTTTGTGGCAGTTTTCTAAAA
2611 CTGACAACCAGGTGGGACCAAAGTTTATGTGCCTTTAGTCTTAATTTACCTTGCATTGTAATATTCAGTTTTATAAATCTTCAAAATATT
2701 TTGTATTTAGGAATAGATCTGACTTTAATAAAAACATGGCTCAGAATCTACAGGTCAAATTTATTTGAACAGTTCTTGTCAATCTGAATT
2791 GTTGATTCTGTTAAATGACAATACTTTTTGAAATTGATGTACTTAGTTTCAAGATTCATAGATTCTGTTATCTATGTAGACAGAATGGTC
2881 ATGTATATTTTCTATTAGTTGAGTTTTTACATCTTTAGAAATGTAAAATTCAGTATAGTTTGAAAGCGGCACAATTAAAAATTAATTTTC
2971 TAA[A]AAGTTGGGAGGTCTTGAATGGTTGTTTAATTTCATTTTGTGTGTACTCTGCTTACCTCTGTAGCATGCTCAATAAACACTTCTGT
3061 AGCTCTG[T]ATTCACCTTTTCTGTCTTTCTCTGCTGCCTTTTCTCTCTCTCCTTTGTTTTCACTCCACTGTGCTTCTGAATTCATGTTTAC
3151 TCTCTGCCAGGGTGGGAAAGGAGTAATAATACTACAATCCTATGGCTTTATACCATGAATAAATCCAGATGCTGTGAAAATG[A]AAAAAAA
3241 AAAAAAAAAAAAAAA
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Fig. 1. **Nucleotide sequence of PTBLP and the deduced amino acid sequence of the putative encoded protein.** The translation initiation codon AUG and the termination codon UAA are underlined. Sequences in open boxes and black boxes show variations with polyadenylation sites and the polyadenylation signals. This sequence has been deposited in the EMBL Nucleotide Sequence Database under accession number AJ010585.

KLH conjugate was injected into New Zealand White rabbits in complete Freund's adjuvant (BRL Gibco). Three weeks later, the animals were injected with the peptide/KLH conjugate in incomplete Freund's adjuvant. Three additional injections of the peptide/KLH conjugate in incomplete Freund's adjuvant were given at three-week intervals. Two weeks after the final injection, the animals were anesthetized with pentobarbital and the serum was collected from each animal.

The anti-PTBLP antibody was purified from the antisera by use of a peptide affinity column. The peptide column was prepared from the peptide and FMP-activated cellulofine (Seikagaku Kogyo) according to the manufacturer's protocol.

Immunoblot Analysis—SDS-soluble proteins were prepared from homogenates of rat retina and other tissues. The proteins were separated on 10% polyacrylamide gel containing 1% of SDS and transferred to a nitrocellulose membrane. After blocking with 5% dry skim milk in TBST, the membrane was incubated with an affinity-purified anti–PTBLP IgG overnight at 4°C. After washing with TBST three times, the membrane was incubated with a secondary antibody to rabbit IgG conjugated to alkaline phosphatase. The membrane was incubated with BCIP and NBT to visualize immune complexes.

Immunohistchemistry—Tissues from rats were used for immunocytochemical study of PTBLP protein. Animals were euthanized with pentobarbital. Immediately after the animals stopped breathing, their eyes were enucleated and fixed in 0.1 M sodium phosphate, pH 7.2 with 4% paraformaldehyde at 4°C for 6 h, after which the cornea, lens, and vitreous were removed. The eyecups were cryoprotected by overnight incubation with 30% sucrose in PBS. The eyecups were embedded in OCT compound (Miles), frozen, and sectioned at a thickness of 15 μm. The sections were preincubated in blocking solution containing 10% normal goat serum at room temperature for 1 h and incubated with the affinity-purified anti-PTBLP antibody in PBS containing 5% normal goat serum, 0.05% sodium azide, and 0.05% Tween 20 at 4°C for 18 h. After washing with PBS, the sections were incubated with the secondary antibody, FITC-conjugated goat affinity purified IgG to rabbit IgG diluted 1:100, for 1 h at room temperature. The sections were stained in 0.5 mg/ml of propidium iodide to visualize cell nuclei. Immunofluorescence images were observed with a Zeiss LSM 410 inverted scanning laser confocal microscope.

To prepare retinal whole mounts for immunocytochemistry, rat retinas were separated from the eyecups that had been pre-fixed in phosphate-buffered paraformaldehyde at 4°C for 1 h, and further fixed in the fixative for 3 h at 4°C. The retinas were washed three times in PBS containing 2 mM $MgCl_2$, then permeabilized in PBS containing 0.1% Triton-X100 for 30 min. The retinas were processed for immunostaining as described above. After staining for cell nuclei with the propidium iodide, the retinas were washed and flatmounted in the Slow Fade antifade solution (Molecular Probes), with the ganglion cell layer facing up. The retinas were examined under a confocal scanning laser microscope.

Controls were carried out with primary antibody that had been pre-incubated with the PTBLPN1 peptide used as immunogen in the antibody dilution buffer at 4°C overnight. A second set of control sections was incubated with the secondary antibody omitting the primary antibody.

RESULTS

Cloning and Characterization of the PTBLP—Eighteen cDNA clones were isolated from the cDNA library of rat neonatal retina. Among the 18 clones, 2 were identified as recoverin by nucleotide sequence analysis. The remaining 16 clones were partial cDNA clones that encompassed the 3′ portion from the same unknown gene. The cDNA was comprised of 3,240 bp that potentially encoded a 532-amino-acid protein (Fig. 1). Six of the clones contained a full-length coding sequence and 2 of them had a 53-bp of the longest 5′-flanking sequence. The 5′-end of the shortest clone was the sequence position 1,042. The 3′-ends of the clones were dispersed to three polyadenylation sites just before the poly-A tail indicated by open boxes, and their polyadenylation signals are also shown in black boxes (Fig. 1). Four of the clones had a variation in sequence beginning at nucleotide 958, and the cDNA sequence of those clones contained an additional 15 nucleotides (GGCTTCCTGTTGCAG) at this position. The trinucleotide CAG was found at the 3′ boundary of the additional sequence and was part of the consensus 3′-splice site sequence. This sequence variation may be generated by alternative splicing. The additional 15 nucleotides extend the open reading frame of PTBLP by 5 amino acids (LPVAA) and convert the alanine of the 302nd amino acid to glycine.

The predicted amino acid sequence of the cDNA showed a strong similarity to PTB (73.5% identity) (*38*) and to a regulator of differentiation 1 (ROD1) (68.8% identity) (Fig. 2A) (*39*). Functional domains in the PTB (*40–43*), such as nuclear localization signal (NLS) and RNA recognition motifs (RRMs), were highly conserved in the PTBLP (Fig. 2A). Bipartite NLSs, shown as open boxes in Fig. 2A, were found at the N-terminal region of PTBLP and PTB, while ROD1 has only second NLS. The consensus sequence of bipartite NLS was derived from nucleoplasmin (*44*). In addition, a putative casein kinase II phosphorylation site (the consensus sequence: S/TxxD/E) (*45*), located 10–30 amino acids from the NLS and appearing to be important for the efficiency of nuclear protein transportation (*46*), was also found. In PTBLP, the casein kinase II site (TETE) was located at 23 amino acids from the NLS of PTBLP (asterisks, Fig. 2A).

RRM is an 80–90 amino acid domain commonly found in a number of RNA-binding proteins (*47*, *48*). PTB contains four RRMs shown as a solid line with arrows in Fig. 2A. Figure 2B shows sequence alignments of RRMs from PTBLP, PTB, and ROD1. Three highly conserved regions were found in the RRMs. Two of the regions are the well-known RNP1 and RNP2 that are commonly observed in RRM, and the three regions are predicted to form a β-sheet structure (*49*).

Epitope Mapping of PTBLP Fusion Protein—To determine whether the patient's serum recognizes a specific sequence within PTBLP, four truncated fragments of the PTBLP clone were constructed (Fig. 3A) and tested for immunoreactivity to the serum by Western blot analysis (Fig. 3B). Construct 4 containing the fourth RRM domain and C-terminus of PTBLP was recognized by the serum. Construct 3 including a 20 aa sequence of the N-terminus of

construct 4 did not react to the serum. Other constructs also had no immunoreactivity to the serum. These results suggest that the epitopes of PTBLP recognized by serum of the CAR patient are present within the last 89 aa sequence of the C-terminus containing the fourth RRM domain.

Northern Blot Analysis and RT-PCR—The mRNA of PTBLP in adult rat retina was detected by Northern blot analysis (Fig. 4A). The sequence of probe DNA contained a 3′-untranslated region of PTBLP that had no homology with PTB. One prominent band of approximately 3.7 kb was detected in lane 1. The cDNA clones therefore contained not only the entire coding sequence of PTBLP but also most, if not all, of the untranslated mRNA sequence. An identical blot was reprobed for the mRNA of GAPDH. The sequence of the probe DNA contained 983 bp of GAPDH coding sequence subcloned a product of RT-PCR

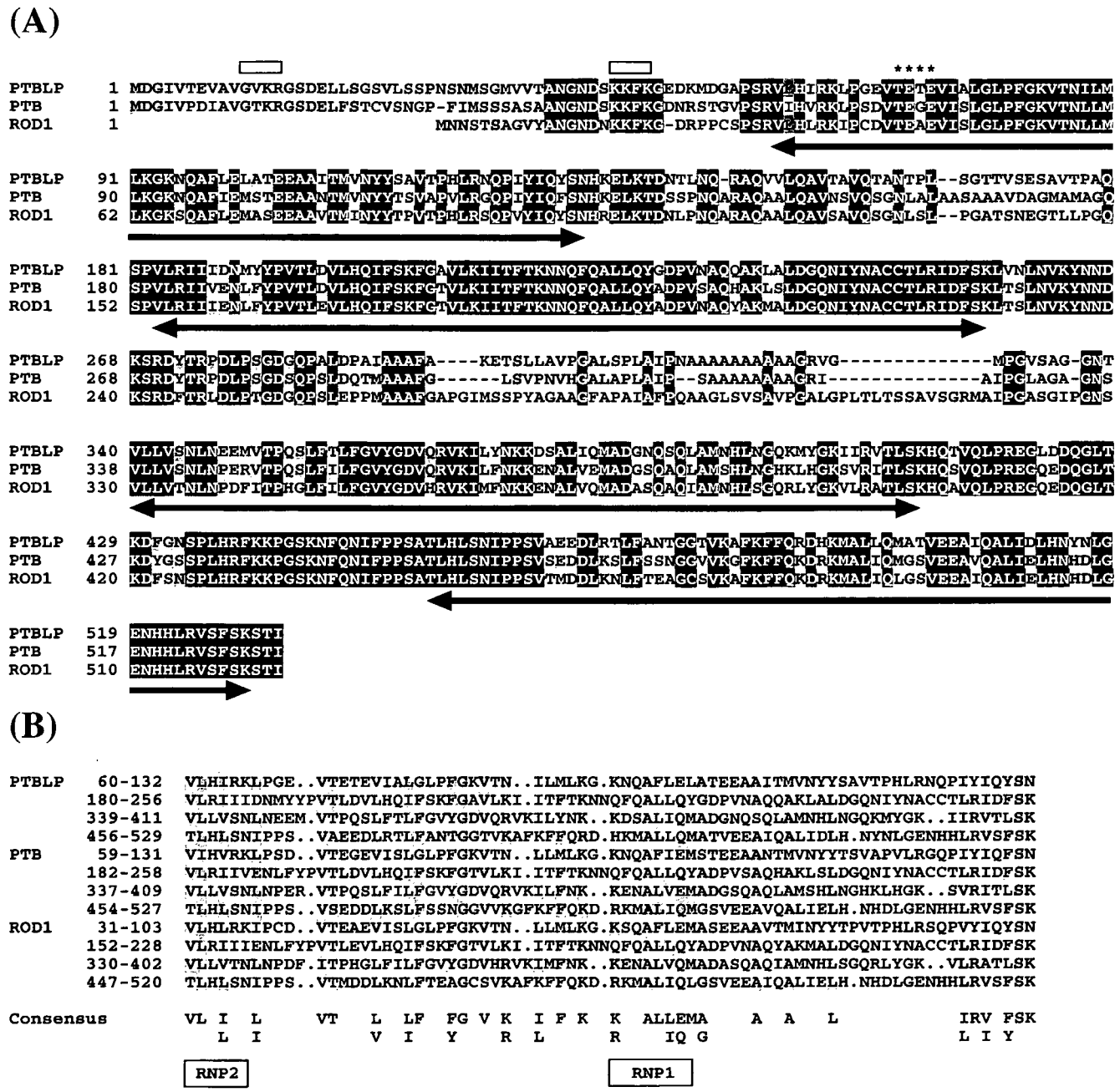


Fig. 2. **(A) Amino acid homology between PTBLP, rat PTB, and rat ROD1 sequences.** Amino acid sequences of PTB and ROD1 were obtained from GenBank data base under accession numbers X60789 and AB023966, respectively (*38*, *39*). Amino acid residues identical among these proteins are highlighted and two of three identical residues are shaded gray. Functional domains in the PTB protein were well-characterized (*43*), such as a nuclear localization signal (NLS) and four RNA recognition motifs (RRMs). Bipartite NLSs are indicated as open boxes. Asterisks show a casein kinase II phosphorylation site. Four RRMs are shown as solid line with arrows. **(B) Alignment of RRM sequences in PTBLP, PTB, and ROD1.** Amino acid residues which are identical or conserved (conservation is defined by: I=L=V, F=Y, S=T, K=R, and E=Q) in at least seven of the twelve repeats are indicated by gray shading. The two highly conserved regions of the RRM (RNP1 and RNP2) are boxed.

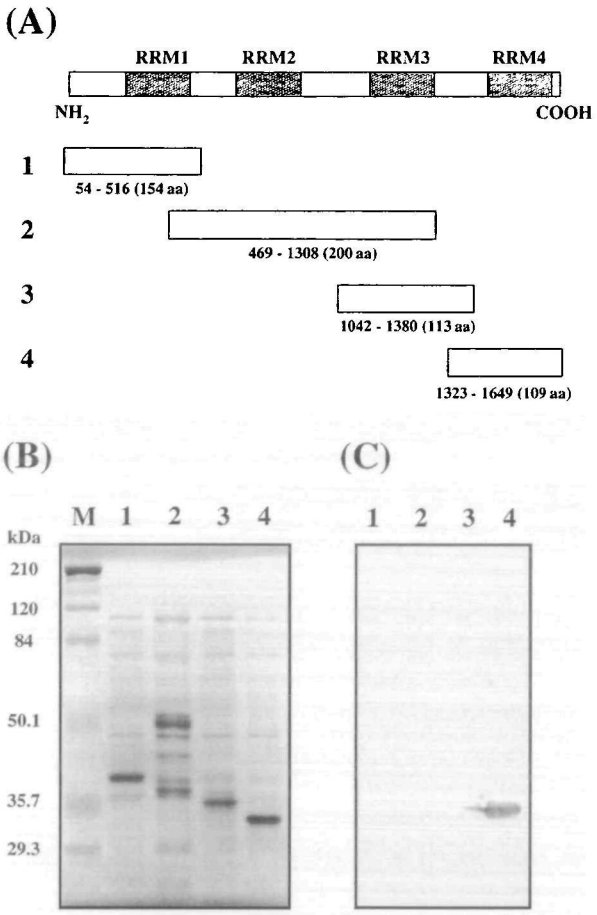


Fig. 3. **Schematic diagram of the recombinant proteins of PTBLP (A).** Four cDNA fragments were inserted into pET-32 expression vector. The amino termini of the recombinant proteins have an additional 159 aa sequence of His-Tag and S-Tag proteins derived from the pET-32 vector. The amino acid numbers corresponding to each clone are listed on the right. Closed boxes indicate four RRMs. **Expression and immunoreactivity of the recombinant protein of PTBLP (B, C).** The lysates of the *E.coli* cells carrying the constructed DNA were separated by SDS-PAGE. The proteins were stained with 0.25% Coomassie Brilliant Blue R250 (B) or transferred to nitrocellulose membrane and reacted with the patient's serum diluted 1:2,000 (C). Lane 1, construct 1; lane 2, construct 2; lane 3, construct 3; lane 4, construct 4; lane M, prestained SDS-PAGE standards, broad range (Bio-Rad).

described below. One band of approximately 1.7 kb was detected in lane 2 by only 20-h exposure to a phosphoimaging plate, although the mRNA of PTBLP was exposed for 10 days. The expression level of PTBLP was at least 30 times lower than that of GAPDH, compared with the blot signal and exposure time.

RT-PCR analyses showed specific amplification of PTBLP cDNA (600 bp) in the samples only from the retina and brain; no amplicons were obtained from the mRNA prepared from the liver, heart, lung or spleen. On the other hand, specific amplification of the target cDNA from retina, brain, liver, heart, lung, and spleen was possible by using specific primers for PTB (668 bp) and GAPDH (983 bp) (Fig. 4 B). The expression level of PTBLP was similar to that of PTB but much lower than that of GAPDH because the same amount of first strand cDNA synthesized by reverse transcriptase was used as a template DNA for PTBLP and PTB. In GAPDH, a 10-times lower amount of the cDNA was used for RT-PCR.

Generation of Anti-PTBLP Antibody—Sera specific to PTBLP were generated by immunization of rabbits with a synthetic peptide containing 19 amino acid residues of the N-terminal region between the bipartite NLSs. The amino acid sequence of the peptide had weak homology to PTB. Proteins from total homogenates of rat retina, brain, liver, kidney, and spleen were separated by SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was probed with the affinity-purified antibody to the PTBLP. The antibody dominantly labeled a 58-kDa band of the retinal proteins (Fig. 5). A faint band at 58 kDa was seen in the brain preparation of 1-week-old rat. No band was found in the brain extracts of adult rat, and the liver, kidney, and spleen preparations of 1-week-old rat.

Distribution of PTBLP Protein—The distribution of PTBLP was also examined by immunohistochemistry. Positive immunoreactivities to PTBLP were detected in rat retinal sections (Fig. 6, A, B, E, and F). Strong immunoreactivity was observed in the nuclei in the ganglion cell layer and some cells in the inner nuclear layer. These immunoreactivities were abolished when the antibody used was pre-adsorbed *in vitro* with a 10 mM concentration of the peptide used as antigen at 4°C overnight before application (Fig. 6, C and D). Some of the nuclei in the ganglion

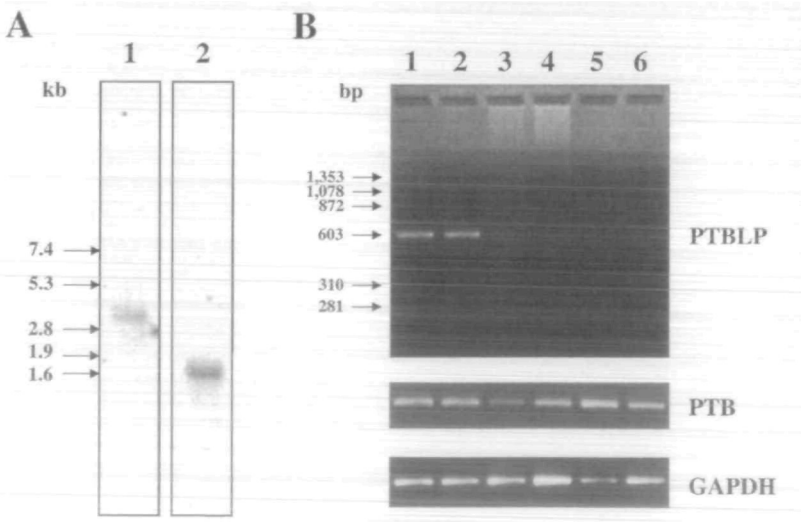


Fig. 4. **Expression of the RNA encoding the PTBLP-like protein in rat tissues.** (A) Northern blot containing 5 μg of poly(A)$^+$ RNA isolated from adult rat retina. The blot was probed with an 860-bp cDNA fragment containing the 3′-noncoding sequence of PTBLP and exposed to a phosphoimaging plate for 10 days. The size of the PTBLP mRNA detected in the lane 1 is 3.7 kb based on DIG-labeled RNA markers (Boehringer) and their molecular sizes in kilobases are indicated. The same blot was reprobed with a 983-bp cDNA fragment containing rat GAPDH coding sequence and was exposed to phosphoimaging plate for 20 h. A 1.7-kb strong band was detected in the lane 2. (B) RT-PCR analysis. Top, PTBLP; middle, PTB; bottom, GAPDH. Lanes 1, retina; 2, brain; 3, liver; 4, kidney; 5, spleen; and 6, lung. PTBLP is expressed only in retina and brain, whereas PTB is expressed ubiquitously. The concentration of the template cDNA used for the amplification of GAPDH was about 1/10th of those for PTBLP and PTB.

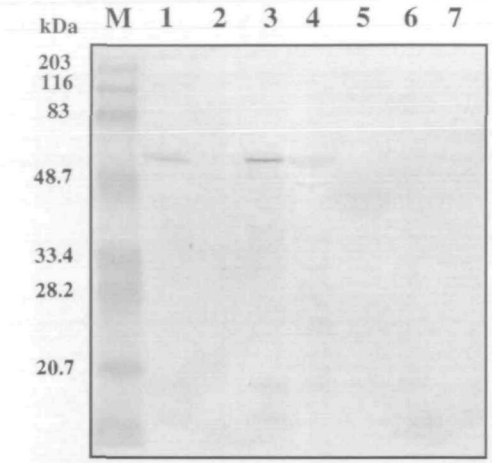


Fig. 5.
from homogenates of retina and brain in 12-week-old rat, and retina, brain, liver, kidney and spleen in 1-week-old rat were subjected to SDS-PAGE and immunoblot analysis. Each sample containing 20 μg of proteins was applied onto a 10% polyacrylamide gel containing 0.1% SDS. The antibody specific to PTBLP was prepared from immune sera generated with the PTBLP peptide. The antibody dominantly reacted to a 58-kDa band in the retinal preparations (lane 1, adult rat; lane 3, 1-week-old rat). Two weak bands are observed at 58 and 48 kDa in the brain of 1-week-old rat (lane 4). No band is seen in the brain of adult rat (lane 2), the liver (lane 5), kidney (lane 6), and spleen (lane 7). Prestained SDS-PAGE standards from Bio-Rad were used as molecular markers. The numbers to the left of the figure indicate the positions of molecular mass markers in kilodaltons.

cell layer, indicated by an arrow in Fig. 6F, did not react with the antibody.

To evaluate the number and type of cells in the ganglion cell layer have immunoreactivity to PTBLP, whole mounts of adult rat retina were used for immunostaining of PTBLP. Confocal images of the ganglion cell layer are shown in Fig. 7. Three different types of nuclei were found in the ganglion cell layer. Rounded nuclei were those of neuronal cells, *e.g.*, ganglion cells and displaced amacrine cells which make up about 90% of the cells in the ganglion cell layer. These nuclei were immunoreactive to PTBLP (Fig. 7A). Small and condensed nuclei may be those of the glial cells (arrows, Fig. 7, B and C). These nuclei were not immunoreactive to PTBLP (Fig. 7C). Thirdly, elongated and crescent-shaped nuclei are those of capillary endothelial cells (arrowheads, Fig. 7, B and C). These nuclei were also not immunoreactive to PTBLP (Fig. 7C). The immunostaining patterns described here were observed both in the central and the peripheral retina.

DISCUSSION

We previously reported that the serum from the same patient used in this study had immunoreactivities to three retinal proteins of different size (27, 58, and 78 kDa), and two of those proteins were identified as recoverin (23 kDa) and TULP1 (78 kDa) (*33*). In this study, we isolated a new retinal protein which has a predicted molecular weight of 58 kDa based on the amino acid sequence. This protein has a strong homology to PTB and ROD1, and thus it was named PTB-like protein (PTBLP). PTB is a well-known RNA-binding protein and a ubiquitous factor involved in RNA splicing and in the regulation of translation (*38*, *50*, *51*).

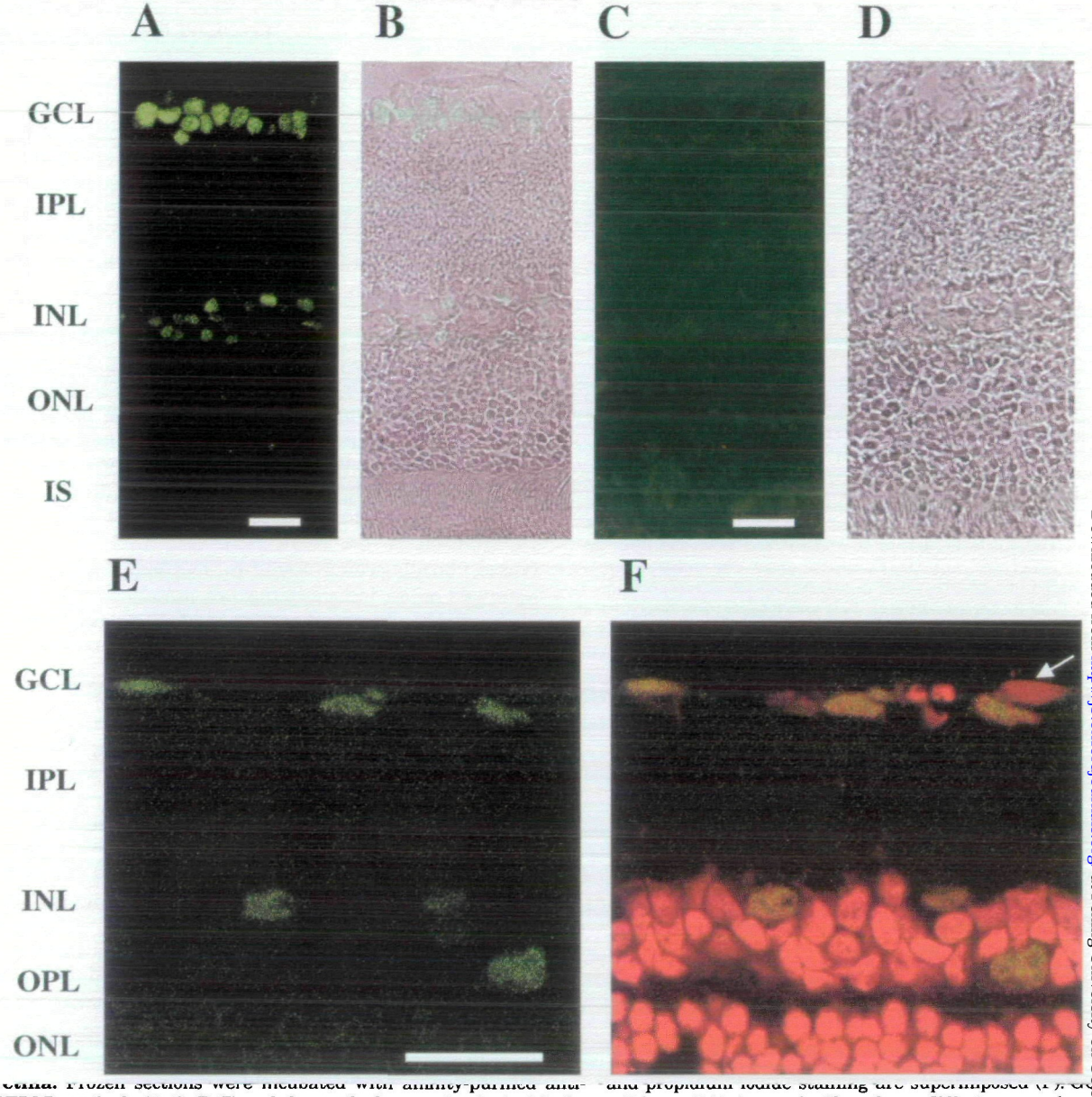
Functional domains of PTB, such as nuclear localization signal (NLS) and RNA recognition motifs (RRMs), were highly conserved in PTBLP. *ROD1* is currently isolated as a functional homologue of yeast nrd1$^+$ gene, and is known as an RNA-binding protein and a negative regulator of differentiation in the fission yeast, *Schizosaccharomyces pombe* (*39*, *52*). This protein also has NLS and RRMs. Although the function of the ROD1 protein is still unknown, the protein is expressed in hematopoietic cells throughout development.

Unlike PTB, the tissue distribution of PTBLP showed a characteristic pattern. PTBLP was predominantly expressed in the neurons in the ganglion cell layer of adult rat retina, and was also detected in neonatal rat brain. The expression was apparently more pronounced in neonatal than in adult rat brain. We conclude that PTBLP is a new member of PTB gene family and its neuron-specific analogue.

Recently, GAP-43 mRNA-binding protein was identified in neonatal bovine brain (*53*). GAP-43 is a membrane-bound phosphoprotein found in neuronal cells and is characterized as an inducible marker protein for neural differentiation (*54*). In undifferentiated PC12 pheochromocytoma cells, GAP-43 mRNA degrades rapidly but becomes stable when cells are treated with nerve growth factor (*55–57*). The stability of GAP-43 mRNA is controlled within a 26-base pyrimidine-rich sequence in the 3′-untranslated region of GAP-43 mRNA. GAP-43 mRNA binding protein was purified as a transfactor bound to the element of GAP-43 mRNA by biochemical methods (*53*). Two partial amino acid sequences derived from the triptic peptides of this protein completely matched the amino acid sequence of PTBLP at the amino acid positions 123–134 and 297–326, although two additional alanine residues in the alanine stretch sequence were found in the second peptide of GAP-43 mRNA-binding protein. In contrast, 2 of 12 or 8 of 28 amino acid residues in the peptides were different from the amino acid sequence of rat PTB. Even though the full-length amino acid sequence of the GAP-43 mRNA-binding protein has not been reported yet, this protein seems to be identical to a bovine analogue of PTBLP.

PTBLP protein is predominantly localized in the nucleus. However, PTB has multiple functions in the cytoplasm as well as the nucleus (*58*). It is possible that PTBLP also has multiple functions in the cytoplasm and nucleus during neurogenesis.

In a previous study, sera from CAR patients were demonstrated to react to photoreceptor and ganglion cells in the retina (*23*). A number of proteins, such as neurofilament (*22*), recoverin (*24*, *25*), neuron specific enolase (*29*), heat shock protein 70 (*59*), and TULP1 (*33*), have been identified as CAR antigens. However, most of these autoantigens, other than recoverin and TULP1, are ubiquitously distributed in many types of cells. Only recoverin and TULP1, photoreceptor proteins, are expressed in a cell-type-specific manner in the retina. PTBLP is the first ganglion and inner nuclear cell–specific protein that is identified as an autoantigen of CAR. In addition, some retinal degenerative diseases that give rise to clinical symptoms that are very similar to CAR are currently reported. In melanoma-associated retinopathy, which is a paraneoplastic syndrome of the



retina. Frozen sections were incubated with affinity-purified anti-PTBLP antibody (A, B, E, F) and the antibody pre-adsorbed with the peptide (C, D). After washing, the sections were reacted with FITC-conjugated antibody to rabbit IgG. The images (B, D) represent digital and propidium iodide staining are superimposed (F). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; IS, inner segment. Scale bars, 25 μm.

visual system, sera of patients react to retinal bipolar cells in the inner nuclear layer (*34*, *35*). In autoimmune retinopathy, which is similar to CAR without a tumor, serum of the patients reacts to Müller cells in the inner nuclear layer (*60*), and sera of the two patients labeled the inner plexiform layer of the retina by immunocytochemistry (*61*). However, the cell-type-specific proteins recognized by those sera have not yet been identified.

It is not yet clear whether autoantibodies to PTBLP are cytotoxic to retinal cells and are the cause of retinal degeneration. In our case, anti-PTBLP antibody was not the only autoantibody presented in the patient's serum. Indeed, among the 18 clones that the patient's serum recognized, 2 clones were identified as recoverin. However, it is noteworthy that autoantibodies against nuclear proteins have been described in paraneoplastic neurological disorders (*7*, *8*, *62*, *63*). In cases of paraneoplastic encephalomyelitis and sensory neuropathy, sera of the patients have reacted to a neuronal antigen, HuD. HuD protein contains three RRMs and is well known as an RNA-binding protein in neuronal cells. The major immunogenic sites of HuD that were recognized by autoantibodies of the patients were identified within two of the RRMs (*64*). In our case, serum of the CAR patient reacted dominantly to the last 89 aa sequence of the C-terminus of PTBLP, which contained the fourth RRM sequence. The third and fourth RRMs of PTB gene were necessary for RNA binding affinity and specificity (*42*, *43*). We suggest that autoantibodies against proteins that contain

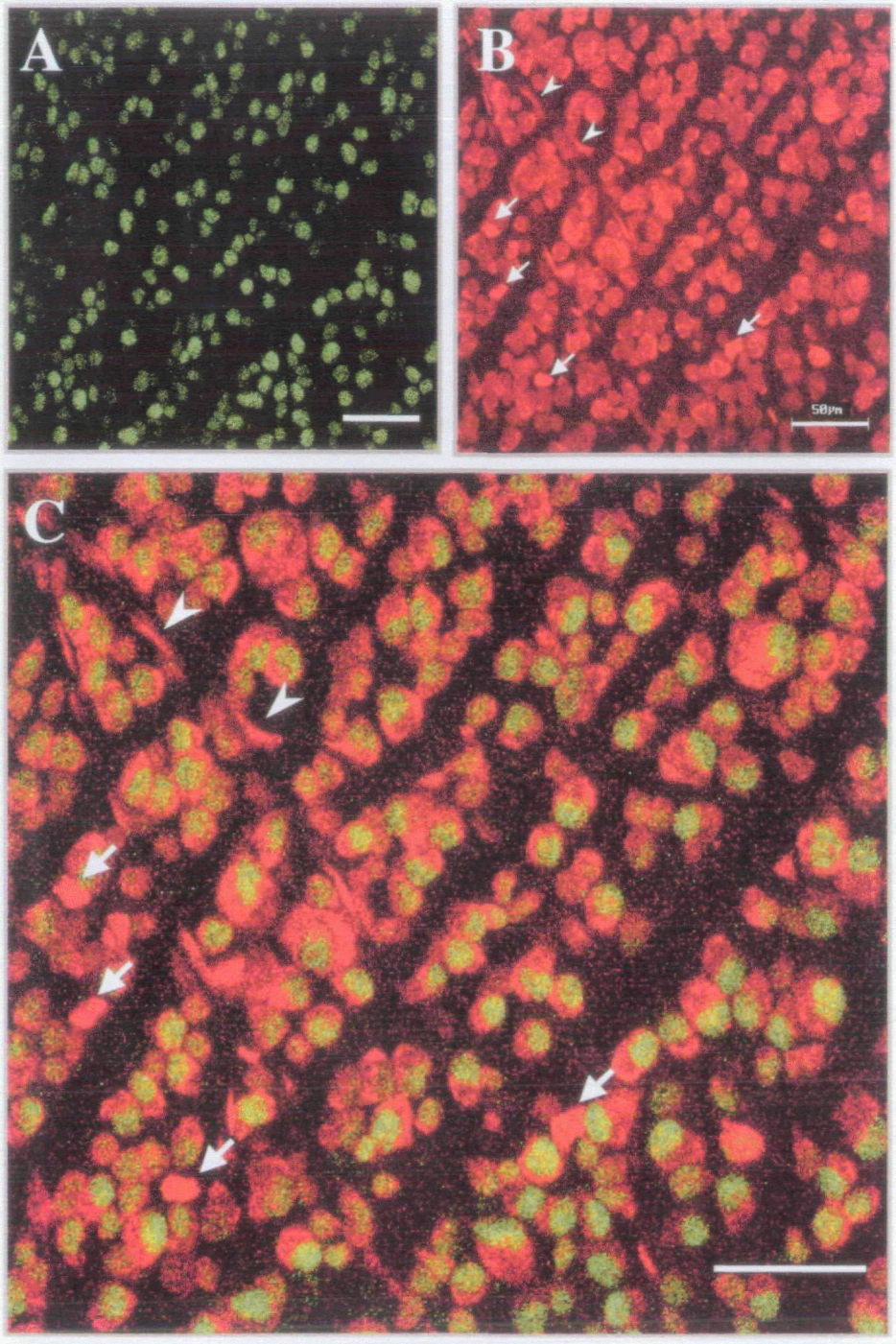


Fig. 7. **Confocal microscopy images of ganglion cell layer in whole mount of rat retina immunostained with anti-PTBLP antibody (A), couterstained with propidium iodide (B), and superimposed images (C).** Immunoreactivities of the antibody were found in most cells and restrictedly localized in the round-shaped nuclei. No signal was found in elongated and crescent-shaped nuclei belonging to the endothelial cells of capillaries (arrowheads) and in some nuclei strongly stained with propidium iodide (arrows). Scale bars, 50 μm.

the RRM may play an important role in the pathogenesis of paraneoplastic syndromes.

Recently, a mouse homologue of PTBLP was isolated as a protein that interacts with NOVA 2 (*65*). The NOVA 2 protein is known as an RNA-binding protein containing the KH domain, and one of the autoantigens reacted with sera of the patients of paraneoplastic neurologic disorder (*10*). The protein may play an important role in alternative splicing in the neuronal cells and in the development of these cells, although the exact functions of PTBLP are still unclear.

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