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Immunohistochemical Localization of an Isoform of TRK-Fused Gene-Like Protein in the Rat Retina

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The TRK-fused gene (*TFG*) was originally identified in chromosome translocation events, creating a pair of oncogenes in some cancers, and was recently demonstrated as the causal gene of hereditary motor and sensory neuropathy with proximal dominant involvement. Recently, we cloned an alternative splicing variant of *Tfg* from a cDNA library of the rat retina, tentatively naming it retinal *Tfg* (*rTfg*). Although the common form of *Tfg* is ubiquitously expressed in most rat tissues, *rTfg* expression is localized to the central nervous system. In this study, we produced an antibody against an rTFG-specific amino acid sequence and used it to examine the localization of rTFG-like protein in the rat retina by immunohistochemistry and Western blots. Western blot analysis showed that the antibody detected a single band of 24 kDa in the rat retina. When we examined rTFG recombinant protein, the antibody detected two bands of about 42 kDa and 24 kDa. The results suggest that the 24 kDa rTFG-like protein is a fragment of rTFG. In our immunohistochemical studies of the rat retina, rTFG-like immunoreactivity was observed in all calbindin D-28K-positive horizontal cells and in some syntaxin 1-positive amacrine cells (ACs). In addition, the rTFG-like immunopositive ACs were actually glycine transporter 1-positive glycinergic or glutamate decarboxylase-positive GABAergic ACs. Our findings indicate that this novel 24 kDa rTFG-like protein may play a specific role in retinal inhibitory interneurons.

Key words: amacrine cells, antibodies, horizontal cells, immunohistochemistry, retina

I. Introduction

The *TRK*-fused gene (*TFG*) has been previously identified in chromosome translocation events involving two protein kinases, creating pairs of oncogenes with *NTRK1*, *ALK* and *NOR1* in thyroid carcinoma, anaplastic large cell lymphoma and extraskeletal myxoid chondrosarcoma, respectively [3–7]. TFG protein has a PB1 domain, a coiled-coil domain, a proline/glutamate enriched domain, a Src homology 2 (SH2) binding motif and some SH3 binding motifs. In addition, the TFG protein contains putative phosphorylation sites for protein kinase C and casein kinase 2. *TFG* is reported to be ubiquitously expressed in adult tissues, and encodes a cytoplasmic protein [3, 9, 10, 14]. In *Caenorhabditis elegans*, the homolog of *TFG* is reported to suppress apoptosis and to be essential for normal cell size [2]. In human cell lines, TFG interacts with several proteins such as TANK and NEMO and is suggested to function in the nuclear factor-kappa B pathway [11]. Additionally, TFG is a novel SHP-1 auxiliary docking protein that participates in the downregulation of phosphatase activity [13]. Mass spectrometry analysis has identified TFG as a Src kinase substrate [1]. Recently, it has been reported that TFG forms hexamers to facilitate the co-assembly of SEC-16 with COPII subunits to endoplasmic reticulum exit sites [16]. More recently, *TFG* was reported to be the causal

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gene of hereditary motor and sensory neuropathy with proximal dominant involvement [8].

Rat *Tfg* spans 26.3 kb of genomic DNA on chromosome 11 and encodes an approximately 1.9 kb mRNA (NCBI Reference Sequence: NM_001012144.1). Recently, we cloned a variant of the *Tfg* gene from rat retinal tissue that contains a coding region of 849 bp (GenBank: AB218900.1) [9]. In the retinal *Tfg* (*rTfg*) an additional exon is present between exons 7 and 8 in the common *Tfg*. Common TFG encodes a 43.1 kDa protein consisting of 398 amino acids (NCBI Reference Sequence: NP_001012144.1), while rTFG encodes a predicted 31.0 kDa protein consisting of 282 amino acids (GenBank: BAE00105.1) [9].

While *cTfg* is ubiquitously expressed in adult tissues, *rTfg* is mainly detected in the central nervous system [9]. However, little information is available about the localization of the rTFG protein. In this study, therefore, we produced an antibody against an rTFG-specific amino acid sequence and used it to examine the localization of rTFG-like protein in the rat retina by immunohistochemistry and Western blot analysis.

II. Materials and Methods

Animals

Six Wistar rats weighing 200–300 g were used in this study. Rats were purchased from CLEA Japan (Tokyo, Japan). The animals were housed under a 12:12 hr light-dark schedule and had food and water available *ad libitum*. The study was performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1985) and the Animal Welfare Act (7 U.S.C. et seq.). The animal use protocol was approved by the Institutional Animal Care and Use Committee of Shiga University of Medical Science.

Production of antibody against rTFG-specific synthetic peptide

Antisera were raised using a synthetic peptide corresponding to an rTFG-specific region of the rTFG protein as an antigen (SGFQSMERFHC: amino acid number 271–281, GenBank accession number BAE00105.1). The peptide was conjugated to keyhole limpet hemocyanin. Two rabbits were immunized with the hapten antigen and antisera were collected after each booster immunization.

The antisera were purified by affinity chromatography using cyanogen bromide-activated Sepharose gel bound with the antigenic peptide (SGFQSMERFHC). One ml of the antiserum was applied to the affinity chromatograph. After washing the unbound protein with 10 mM phosphate buffered saline (PBS; pH 7.4, unbound fraction), bound IgG was eluted by 0.1 M glycine-HCl (pH 2.3). The purified IgG was neutralized with 1 M Tris-HCl (pH 8.0). The eluted fraction was dialyzed with 10 mM PBS, and then stored in 50% glycerol/PBS. The immunoactivity was confirmed by enzyme-linked immunosorbent assay. The production and purification of the antibody were done by Medical & Biological Laboratories, Co. Ltd. (Nagoya, Japan).

Tissue preparation

For Western blots, under pentobarbital anesthesia (80 mg/kg), three male Wistar rats were perfused via the ascending aorta with 10 mM PBS (pH 7.4) to remove blood. The eyes were removed from each rat. Under a stereoscopic microscope, the retinas were isolated and frozen at –80°C until use. For immunohistochemistry, another three male Wistar rats were transcardially perfused with 10 mM PBS followed by an ice-cold fixative of 0.1 M phosphate buffer (PB; pH 7.4) containing 4% paraformaldehyde. The eyes were removed from each rat and post-fixed for 48 hr in the same fixative at 4°C. The tissues were then immersed for at least 48 hr in 0.1 M PB containing 15% sucrose and 0.1% sodium azide for cryoprotection. The tissues were cut into 20-μm thick sections using a cryostat. The sections were used in a free-floating state.

Immunohistochemistry

To facilitate penetration of the rTFG antibody into the fixed tissues, the sections were kept at 4°C for at least 1 month in 0.1 M PBS containing 0.3% Triton X-100 (PBST; pH 7.4) before staining. Sections were incubated for 20 min with PBST containing 0.3% hydrogen peroxide and 0.1% sodium azide at room temperature to eliminate endogenous peroxidase. After three 10-min washes with PBST, the sections were incubated with the purified rTFG antibody (0.156 μg/ml) at 4°C overnight. After washing, the sections were incubated for 1 hr with biotinylated anti-rabbit IgG (1:1,000; BA-1000, Vector Laboratories, Burlingame, CA) at room temperature. The sections were then washed and incubated for 1 hr at room temperature with avidin-biotin-peroxidase complex (1:3,000; PK-6100, Vector Laboratories). Sections were again washed and a purple color was developed with 0.02% 3,3'-diaminobenzidine, 0.3% nickel ammonium sulfate and 0.0045% hydrogen peroxide in 25 mM Tris-HCl buffer (pH 8.0). The free-floating sections were mounted on triethoxyaminopropylsilane-coated glass slides (Dako, Glostrup, Denmark) and air-dried.

For immunohistological controls, an absorption test was employed. In order to adjust the antibody:peptide molar ratio to 1:20, 0.156 μg of rTFG antibody and 0.028 μg of peptide were added to 1 ml of PBST. This mixture was incubated while rotating overnight at 4°C. The treated solution was then used for immunohistochemical staining of control sections.

Double immunofluorescence histochemistry

To identify what kinds of cells demonstrated rTFG-like immunoreactivity, we performed double immuno-

Table 1. *List of primary antibodies used for immunohistochemistry (IHC), immunofluorescence (IF), and Western blotting (WB)*

Antigen	Host	Concentration/dilution	Detectors	Source
rTFG	Rabbit	IHC, IF; 0.156 μg/ml, WB; 0.156–0.312 μg/ml	Unknown	Home made
cTFG	Rabbit	WB; 0.13 μg/ml	Unkown	Home made
Calbindin D-28K	Mouse	IF; 1:2,500, WB; 1:3,000	Horizontal cell	Sigma-Aldrich
Syntaxin 1	Mouse	IF; 1:2,000, WB; 1:2,000	Amacrine cells	Sigma-Aldrich
Brn3a	Mouse	IF; 1:100	Retinal ganglion cells	Merck Millipore
GlyT1	Goat	IF; 1:5,000	Glycinergic amacrine cells	Merck Millipore
GAD65	Mouse	IF; 1:1,000	GABAergic amacrine cells	Merck Millipore
GAD67	Mouse	IF; 1:1,000	GABAergic amacrine cells	Merck Millipore
TH	Mouse	IF; 1:5,000	Dopaminergic amacrine cells	Merck Millipore
ChAT	Goat	IF; 1:1,000	Cholinergic amacrine cells	Merck Millipore

fluorescence histochemistry using the rTFG antibody and retinal cell type specific antibodies or neurotransmitter marker antibodies. We used the following antibodies: mouse monoclonal calbindin D-28K antibody (1:2,500; C9848, Sigma-Aldrich), mouse monoclonal syntaxin 1 antibody (1:2,000; S0664, Sigma-Aldrich), mouse monoclonal Brn3a antibody (1:100; MAB1585, Merck Millipore), goat polyclonal glycine transporter 1 (GlyT1) antibody (1:5,000; AB1770, Merck Millipore), mouse monoclonal glutamate decarboxylase 65 (GAD65) antibody (1:1,000; MAB351, Merck Millipore), mouse monoclonal GAD67 antibody (1:1,000; MAB5406, Merck Millipore), mouse monoclonal tyrosine hydroxylase (TH) antibody (1:5,000; MAB318, Merck Millipore), and goat polyclonal choline acetyl transferase (ChAT) antibody (1:1,000; AB144p, Merck Millipore). The antibodies used in this study are listed in Table 1.

Rat retinal sections were incubated with a mixture of the rabbit polyclonal rTFG antibody (0.156 μg/ml) and mouse monoclonal or goat polyclonal antibodies to retinal cell type-specific antibodies or neurotransmitter marker antibodies for 2–3 days at 4°C. After three 10-min washes with PBST, the sections were incubated for 1 hr with a mixture of Alexa 488-conjugated anti-rabbit IgG (1:500; A-21441, Life Technologies, Carlsbad, CA) and Alexa 647-conjugated anti-mouse IgG (1:500; A-21463, Life Technologies) or Alexa 647-conjugated anti-goat IgG (1:500; A-21469, Life Technologies) at room temperature. The free-floating sections were washed and mounted on triethoxyaminopropylsilane-coated glass slides and the labeling was observed under a confocal microscope (Nikon C1si, TE2000-E; Nikon, Tokyo, Japan).

Western blot analysis

The rat retinas were homogenized in 5 volumes of ice cold 50 mM Tris-HCl (pH 7.4) containing 1% Triton-X100, 1 mM EDTA, 1 μg/ml pepstatin and Complete Mini protease inhibitor cocktail (1 tablet/7 ml; Roche Diagnostics, Basel, Switzerland). The homogenates were centrifuged at 20,000 *g* for 30 min at 4°C. The supernatants were collected as a crude protein fraction. Approximately 50 μg of the crude protein, prestained Precision Protein Standards (Bio-Rad Laboratories, Hercules, CA) and Protein Ladder One Triple-color (Nacalai Tesque, Kyoto, Japan) were electrophoresed on a 5%–20% sodium dodecyl sulfate-polyacrylamide gel (Wako Pure Chemical Industries, Osaka, Japan) under reducing conditions and then transferred to a polyvinylidene difluoride membrane (Immobilon-P, Merck Millipore, Billerica, MA). The membrane was incubated with 5% skim milk in 25 mM Tris-HCl buffer containing 150 mM NaCl and 0.1% Tween 20 (TBST; pH 7.4) for 30 min at room temperature. After washing with TBST, a further incubation with rabbit polyclonal rTFG antibody (0.312 μg/ml), mouse monoclonal syntaxin 1 antibody (1:2,000; S0664, Sigma-Aldrich, St. Louis, MO) or mouse monoclonal calbindin D-28K antibody (1:3,000; C9848, Sigma-Aldrich) in TBST containing 0.5% skim milk was conducted for 1 hr at room temperature. After three 10-min washes with TBST, the membrane was reacted for 1 hr with a peroxidase-labeled anti-rabbit IgG (1:20,000; 111-035-003, Jackson ImmunoResearch Laboratories; West Grove, PA) or a peroxidase-labeled anti-mouse IgG (1:20,000; 115-035-003, Jackson ImmunoResearch Laboratories) at room temperature. After washing, chemiluminescence signals were obtained by using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA).

Expression of rat rTFG

To examine the recombinant rTFG, cDNAs encoding rat rTFG from the rat retina were prepared using a primer set of 5'-GAGATCTCGAGACTATGGACGGACAGTT GGACCTAAG-3' and 5'-GGTGGATCCTACTTGCAATG AAACCT-3' into pCR2.1 vector. The upper primer contained Kozak consensus sequence and the lower primer included a stop codon. Then, the cDNAs were inserted into the pEGFP-N1 vector (Clontech, Palo Alto, CA). Four micrograms of pEGFP-N1 vector DNA with and without rat TFG were transfected into HEK293 cells using PolyFect (QIAGEN K.K., Tokyo, Japan) according to the manufacturer's instruction. At 48 hr after the transfection, the HEK293 cells were washed twice with cold PBS, and collected in PBS with gentle pipetting. The cells were precipitated by centrifugation at 150×g for 5 min, and resuspended in a lysis buffer consisting of PBS containing 0.5% TritonX-100 and protease-inhibitor cocktail (Complete-mini, Roche Applied Science, Penzberg, Germany; 1 tablet per 7 ml). The cell suspension was disrupted

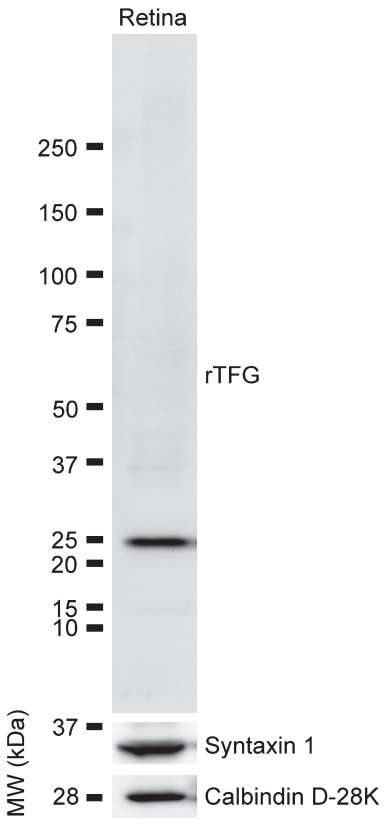

Fig. 1. Western blot analysis of rat retina homogenate probed with a purified antibody against retinal *TRK*-fused gene (rTFG)-like protein which recognized a protein of 24 kDa in the retina. Antibodies against syntaxin 1 and calbindin D-28K were used to confirm that the extract contained retinal tissue.

by passing repeatedly through a 23-gauge needle, and then the crude lysate was centrifuged at 12,000×g for 30 min at 4°C. The supernatant containing the soluble fraction of the lysate was collected and approximately 20 μg of the protein applied to Western blot analysis of rTFG as described above. In addition, we used cTFG antibody (0.13 μg/ml) as a positive control. This antibody is raised against a region common to cTFG and rTFG (SGPPSAPTEDRSGTP: amino acid number 194–208, GenBank Accession number BC078947) [9, 14].

III. Results

Western blot analysis

Western blot analysis showed that rTFG antibody detected a single band with a molecular weight of approximately 24 kDa in the rat retinal lysate (Fig. 1). In order to confirm that the lysate was isolated from the retina, we employed Western blots probed with syntaxin 1 antibody, a marker of amacrine cells (ACs), or calbindin D-28K antibody, a marker of horizontal cells (HCs). The syntaxin 1 and calbindin D-28K antibodies detected bands of 35 kDa and 28 kDa, respectively, indicating that the extract contained retinal tissue (Fig. 1).

Figures 2A and 2B show the results of Western blots

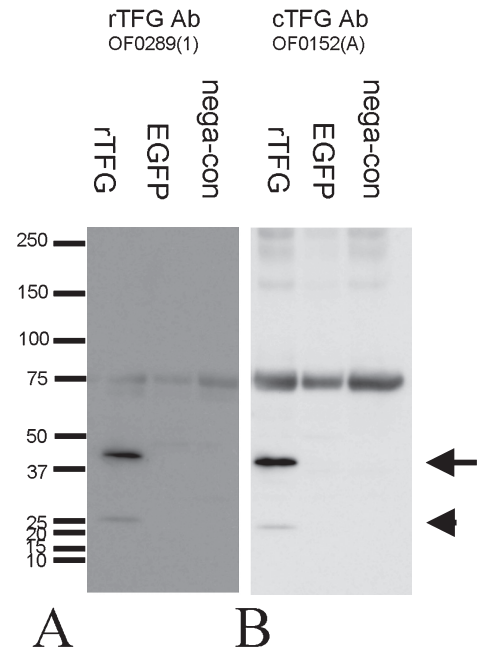

Fig. 2. Western blot analysis of homogenates of HEK293 cells expressing rTFG or pEGFP as well as no protein probed with a purified antibody specific to rTFG (**A**) and common to cTFG and rTFG (**B**). Both antibodies detected intense band of about 42 kDa (arrow) and weak band of 24 kDa (arrowhead) in HEK 293 cells transfected with pEGFP containing rat rTFG. These bands could not be recognized in HEK 293 cells transfected with pEGFP only.

using HEK293 cells expressing rat rTFG or EGFP probed with rTFG and cTFG antibodies, respectively (Fig. 2). Both antibodies detected intense band of about 42 kDa (arrow in Fig. 2) and weak band of 24 kDa (arrowhead in Fig. 2) in HEK 293 cells transfected with pEGFP containing rat rTFG. These bands could not be recognized in HEK 293 cells transfected with pEGFP only. These results indicate that both 42 kDa and 24 kDa proteins contain epitopes of the region specific to rTFG (SGFQSMERFHC) and common region to cTFG and rTFG (SGPPSAPTEDRSGTP).

Localization of rTFG-like immunoreactivity in the rat retina

Figure 3A shows hematoxylin and eosin staining of the layers of the rat retina: outer segment, inner segment (IS), outer nuclear layer, outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL) and ganglion cell layer (GCL). Figure 3B demonstrates a typical example of rTFG immunohistochemistry. In the rat retina, rTFG-like immunoreactivity was detected in some cells and fibers in the IS, OPL, INL, and GCL (Fig. 3B). No positive structures were seen when the rTFG antibody was not used (Fig. 3C) or when the rTFG antibody was pre-absorbed with the antigen peptide (SGFQSMERFHC; Fig. 3D).

Double immunofluorescence histochemistry for rTFG and cell-type markers

Figures 4A, 4B and 4C show the results of double immunofluorescence histochemistry using rTFG antibody and the antibody to calbindin D-28K, an HC marker, and

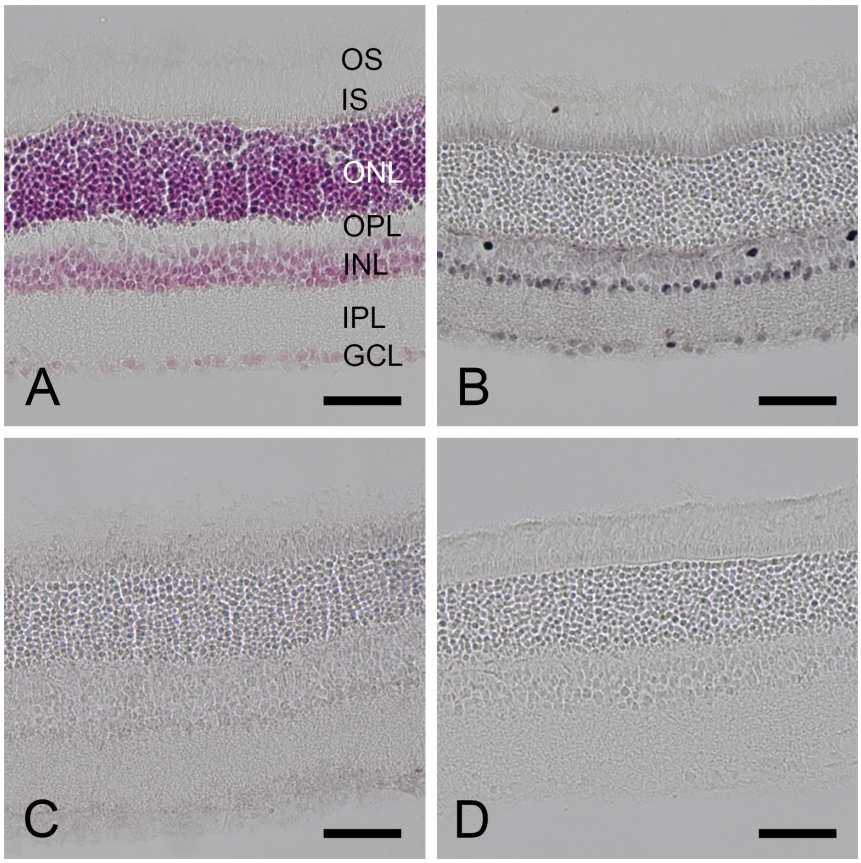

Fig. 3. Immunohistochemical staining using a purified antibody against the retinal TRK-fused gene (rTFG)-specific peptide. (**A**) Hematoxylin and eosin staining shows the layers of the rat retina: outer segment (OS), inner segment (IS), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL) and ganglion cell layer (GCL). (**B**) Immunohistochemistry shows intense rTFG-like immunoreactivity in the OPL, INL and GCL. (**C**) No staining is apparent in the experimental control without the rTFG antibody, (**D**) or following antibody pre-incubation with the rTFG peptide. Bars=50 μm.

their merged images, respectively. Calbindin D-28K immunoreactivity was detected in the cell bodies and processes in the OPL (Fig. 4A). All calbindin D-28K-positive HCs were also positive for rTFG (Fig. 4B and 4C).

Figures 4D, 4E and 4F show the results of double immunofluorescence histochemistry using antibodies to rTFG and the AC marker, syntaxin 1, and their merged images, respectively. Syntaxin 1 was localized in the membrane of the cell bodies in the INL and fibers in the OPL and processes in the IPL (Fig. 4D). In the INL, rTFG-positive cells were also positive for syntaxin 1 (arrows in Fig. 4D–F). Some syntaxin 1-positive cells were negative for rTFG (arrowheads in Fig. 4D–F). Thus, rTFG-positive cells in the INL were a subpopulation of syntaxin 1-positive-ACs.

Figures 4G, 4H and 4I show the results of double immunofluorescence histochemistry using antibodies to rTFG and the ganglion cell (GC) marker, Brn3a, and their merged images, respectively. Brn3a was localized in the nucleus of the GCs in the GCL (Fig. 4G). Immunoreactivity for rTFG was not seen in Brn3a-positive GCs (Fig. 4G–I).

Double immunofluorescence histochemistry for rTFG and neurotransmitter-related molecules

In order to identify what kinds of ACs express rTFG-immunoreactivity, we employed double immunofluorescence histochemistry for rTFG and neurotransmitter-related molecules. The glycinergic AC marker, GlyT1, was localized to cell membranes of ACs in the INL and processes in the IPL (Fig. 5A). In the INL, almost all GlyT1-positive ACs displayed rTFG-immunoreactivity (arrowheads in Fig. 5A–C), although some rTFG-positive cells were negative for GlyT1 (Fig. 5A–C).

Figures 5D and 5G show the results of immunostaining for the GABAergic markers, GAD65 and GAD67, respectively. Some GAD65-positive and GAD67-positive cells were also positive for rTFG (arrowheads in Fig. 5D–I). Some rTFG-positive cells were negative for GAD65 (Fig. 4D–F) and GAD67 (Fig. 5G–I).

A marker of dopaminergic ACs, TH, was localized in cell bodies in the INL and processes in the IPL (Fig. 6A). However TH-positive cells did not contain rTFG-like protein (Fig. 6A–C). Moreover, ChAT, a marker of cholinergic ACs, was localized in the cell bodies of both type a ACs in

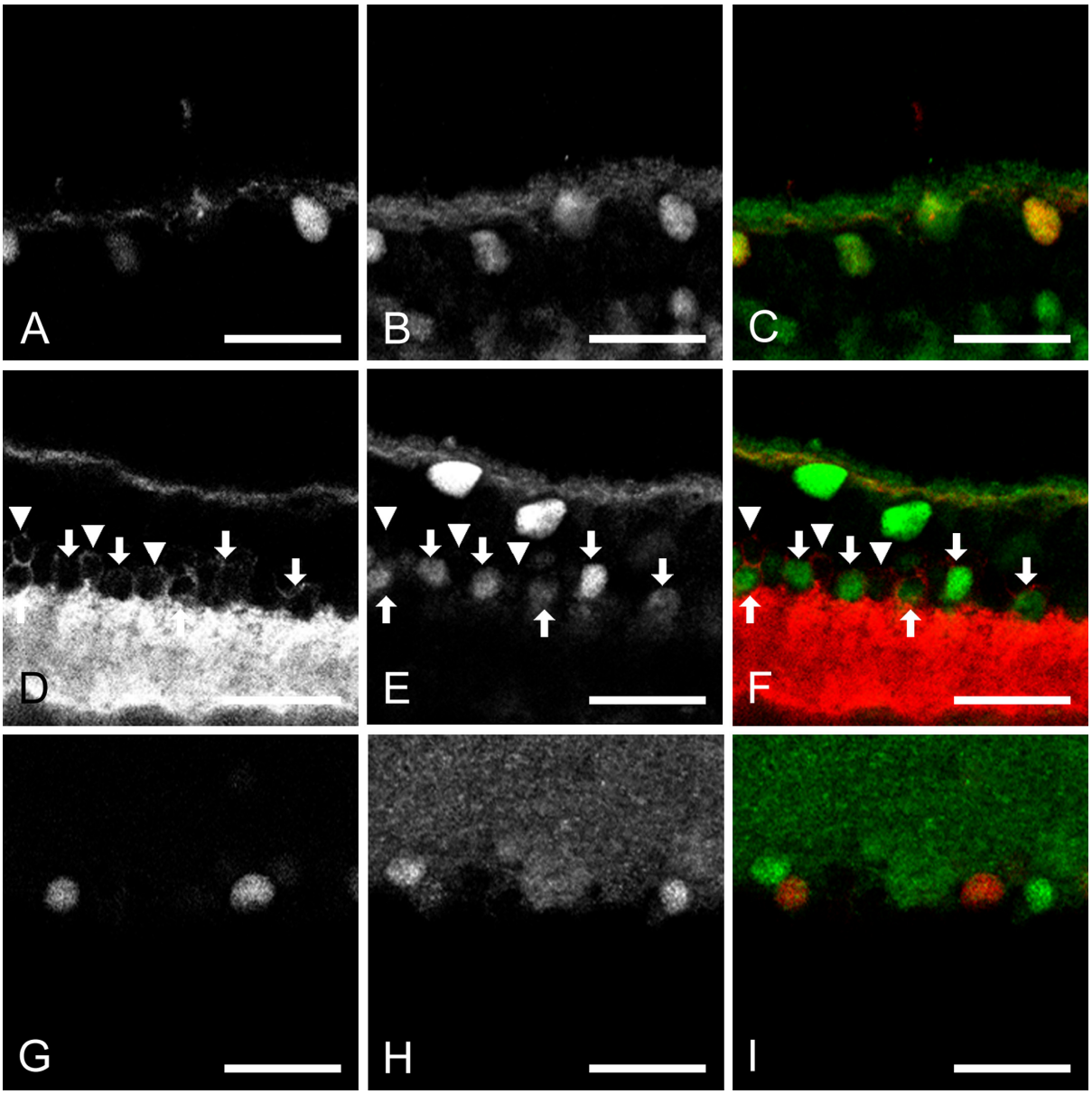

Fig. 4. Double-immunofluorescence histochemistry using the rTFG antibody and antibodies against calbindin D-28K (**A, B, C**), syntaxin 1 (**D, E, F**), or Brn3a (**G, H, I**). **A, B**, and **C** show rTFG, calbindin D-28K and their merged images, respectively. **D, E** and **F** show rTFG, syntaxin 1 and their merged images, respectively. Double-positive cells are shown by the arrows (**D–F**). Arrowheads show syntaxin 1-positive and rTFG-negative cells (**D–F**). **G, H** and **I** show rTFG, Brn3a and their merged images, respectively. Bars=100 μm.

the INL and type b ACs in the GCL (Fig. 6D). ChAT-positive fibers were also observed in the sublamina a and sublamina b of the IPL (Fig. 6D). ChAT-positive cells did not display rTFG-immunoreactivity (Fig. 6D–F).

IV. Discussion

Specificity of the rTFG antibody

In the present study, we produced antisera against an amino acid sequence specific to rTFG. Retinal *Tfg* contains an additional exon between exon 7 and 8 compared to *cTfg*. Since a frame shift occurs, the C-terminal region of rTFG differs from that of cTFG. Thus, we selected "SGFQSMERFHC" from the C-terminal region as the antigen. The antisera were purified by affinity chromatography, using a Sepharose column bound with the peptide. Immunohistochemistry using the purified antibody clearly demonstrated HCs, and some ACs and GCs in the rat retina. This staining was abolished by pre-incubation of the antibody with the antigenic peptide. Thus, the antibody recognized the protein with the epitope "SGFQSMERFHC".

When we examined HEK293 cells expressing rTFG, the rTFG recombinant protein showed about 42 kDa that is larger than the predict molecular weight of rTFG of 31

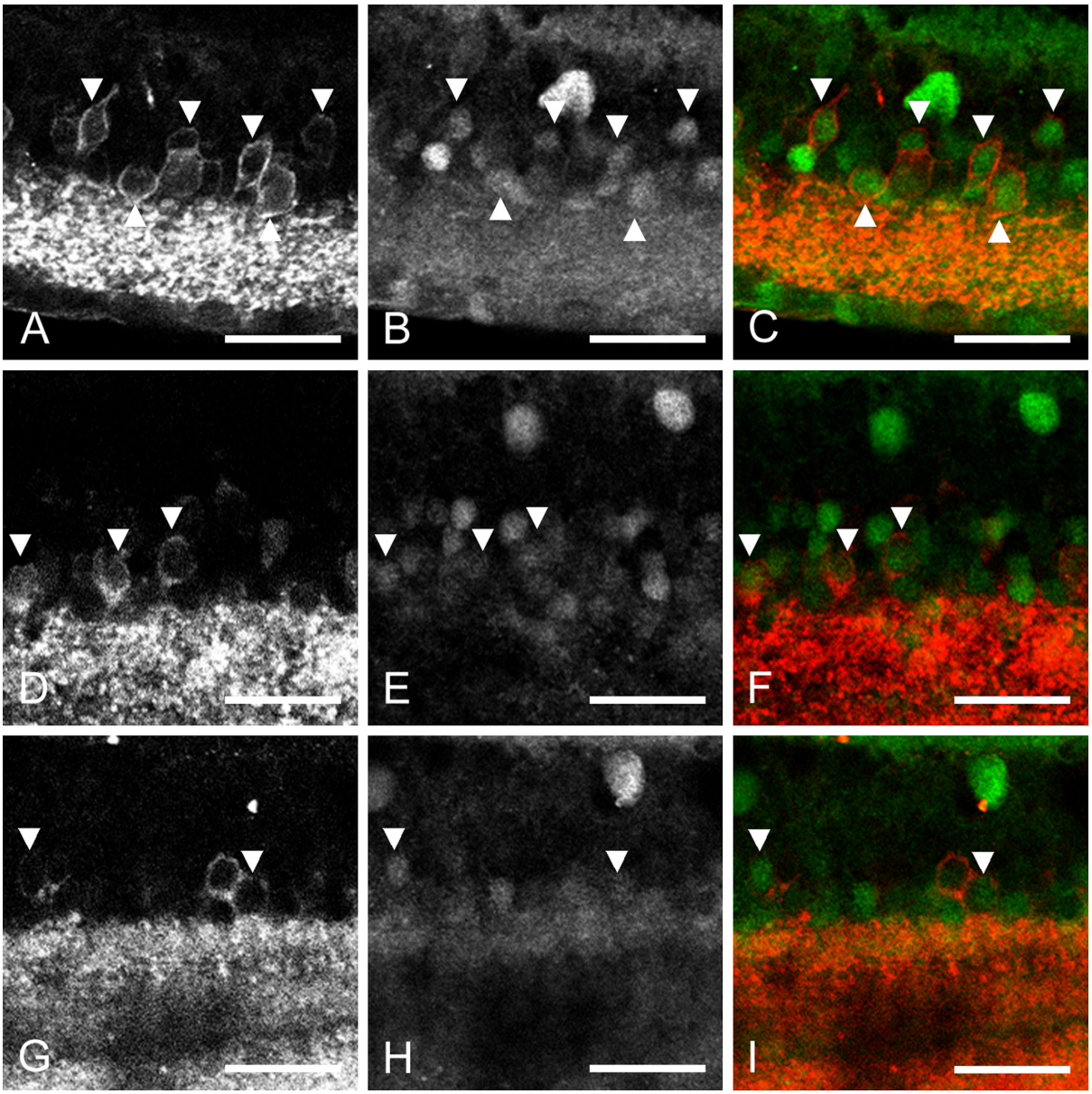

Fig. 5. Double-immunofluorescence histochemistry using the rTFG antibody (**A, D, G**) and antibodies against glycine transporter 1 (GlyT1) (**B**), glutamate decarboxylase 65 (GAD65) (**E**), or GAD67 (**H**). Merged images are shown in (**C, F, I**). Arrowheads show the double-positive cells (**D–I**). Bars=100 μm.

kDa. The similar phenomenon is reported in a case of cTFG. The predicted size of cTFG is 43.1 kDa while its actual molecular weight has been shown to be about 50 kDa [9, 16]. The results suggest that a mature protein of rTFG shows about 42 kDa. In addition to the 42 kDa band, a weak band of 24 kDa was also detected by both antibodies specific to rTFG (SGFQSMERFHC: amino acid number 271–281) and common to cTFG and rTFG (SGPPSAPTEDRSGTP: amino acid number 194–208). These results indicate that the 24 kDa protein contains both epitopes mentioned above. Thus, the 24 kDa rTFG-like protein in retinal homogenates may represent a cleavage product as suggested by a recent paper showing two potential sites of proteolysis for TFG [12].

The possibility that the 24 kDa TFG-like protein is not rTFG but another protein containing the same epitope of "SGFQSMERFHC" cannot be ruled out. However, using the Basic Local Alignment Search Tool (BLAST) analysis for protein of the National Center for Biotechnology Information (NCBI), we could not find a 24 kDa protein containing the epitope of "SGFQSMERFHC".

Localization of rTFG-like immunoreactivity in the rat retina

Retinal TFG-immunoreactivity was detected in the OPL, INL and GCL. Double immunofluorescence histochemistry demonstrated that rTFG-like protein was seen in

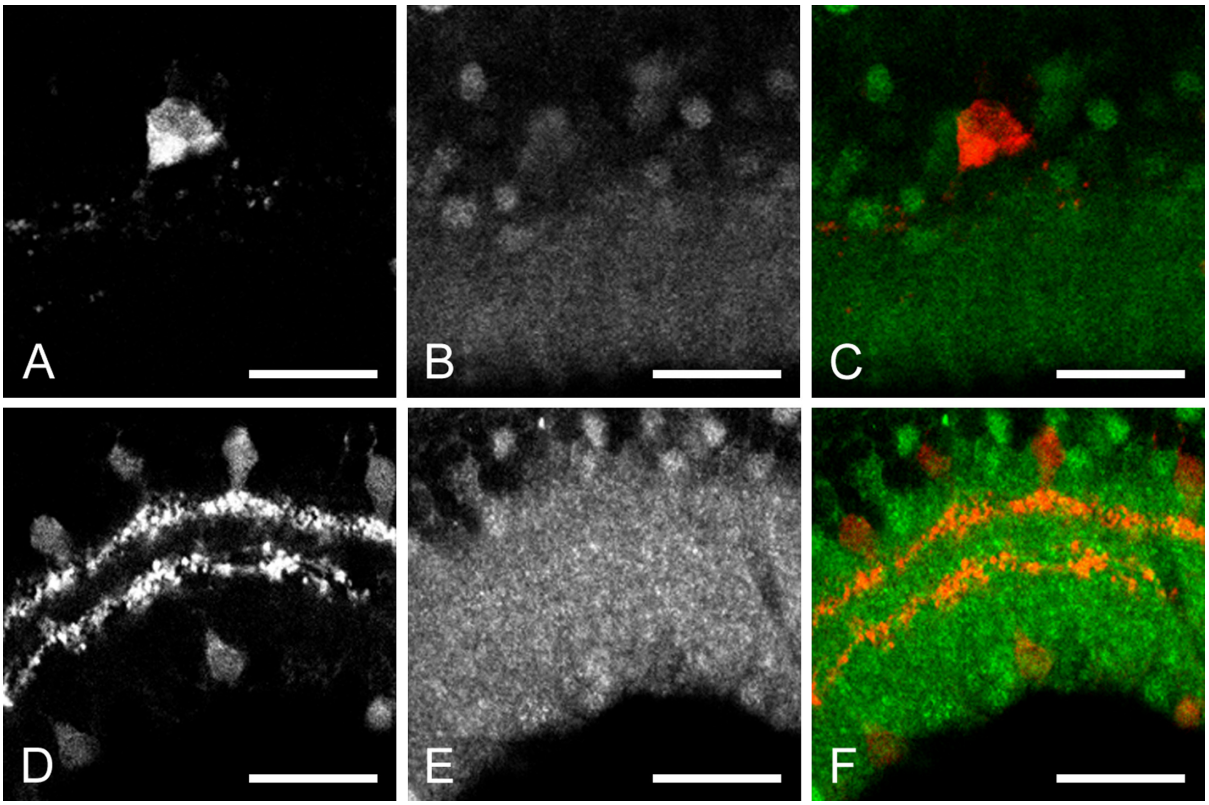

Fig. 6. Double-immunofluorescence histochemistry using the rTFG antibody (**B, E**) and antibodies against tyrosine hydroxylase (TH) (**A**), or choline acetyl transferase (ChAT) (**D**). Merged images are shown in (**C**) and (**F**). Bars=100 μm.

all calbindin D-28K-positive HCs and syntaxin 1-positive ACs. In the ACs, rTFG-like immunoreactivity was observed in most of the glycinergic cells. In addition, some GAD-positive GABAergic cells also demonstrated rTFG-immunoreactivity, while ChAT-positive and TH-positive ACs did not show any rTFG-immunoreactivity. Both HCs and ACs are known to be retinal inhibitory interneurons. Thus our findings indicate that rTFG-like protein may play a role in some populations of retinal inhibitory interneurons. It is well known that glycinergic amacrine cells synapse with the axons of OFF cone bipolar cells, which in turn synapse with OFF ganglion cells [15]. Our results suggest that rTFG may play an important role in the OFF light signal pathway in the retina.

In conclusion, we produced an antibody against an rTFG-specific amino acid sequence and used it to examine the localization of rTFG-like protein in the rat retina by immunohistochemistry. Western blots showed that the antibody detected a single band of 24 kDa in the rat retina. Immunohistochemically, rTFG-like immunoreactivity was observed in all HCs and in most glycinergic ACs and some GABAergic ACs. These results suggest that rTFG may play a role in the function of the inhibitory interneurons of the retina.

V. Acknowledgments

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