A Mouse Monoclonal Antibody Against the $\gamma 2$ Subunit of GABA_A Receptors

Suchitra Joshi, Chengsan Sun, and Jaideep Kapur

A mouse monoclonal antibody directed against the N terminal extracellular epitope of rat γ amino butyric acid (GABA) type-A (GABA_AR) receptor $\gamma 2$ subunit was generated. This antibody identified a protein of approximately 42 kDa in Western blot assays using rat and mouse hippocampal proteins. The antibody also detected the expression of $\gamma 2$ subunit by immunohistochemistry and could immunoprecipitate the $\gamma 2$ subunit.

Introduction

GABA_A RECEPTORS (GABA_ARs) are ligand-gated neurotransmitter receptors that are widely distributed in the central nervous system and mediate the majority of fast synaptic inhibition in the brain. GABA_ARs are pentamers composed of subunits derived from different gene families— α , β , γ , δ , π , ϵ , τ , and ρ (see reviews^(1,2))—some of which contain multiple members, such as $\alpha 1$ -6, $\beta 1$ -3, and $\gamma 1$ -3. There is 40% sequence homology between gene families whereas members of same family share 70% homology. GABA_ARs are structurally similar to the nicotinic acetylcholine receptor, and each subunit has an N-terminal extracellular domain, four putative transmembrane domains (TM1-4), a large cytoplasmic loop between TM3 and TM4, and a short extracellular C-terminus.⁽²⁾ The N terminal extracellular domain and the cytoplasmic loop show greatest variation.

The $\gamma 2$ subunit is required for clustering of GABA_ARs at synapses,⁽³⁾ and changes in $\gamma 2$ subunit-containing GABA_ARs are observed under pathological conditions,⁽⁴⁻⁶⁾ which could be due to alterations in internalization or insertion of receptors at the cell membrane. Available anti- $\gamma 2$ antibodies directed against the N-terminal extracellular epitope or the cytoplasmic loop include those prepared in the laboratory of Dr. W. Sieghart^(7,8) (Medical University, Vienna, Austria), Millipore antibody Ab5954 (Billerica, MA), Novus antibody NB300-190 (Littleton, CO), and Alpha Diagnostics antibody GAG21-A (San Antonio, TX). However, all of these are polyclonal antibodies and identify multiple epitopes. The only mouse monoclonal anti- $\gamma 2$ antibody available is directed against the cytoplasmic loop (antibody ab52773; Abcam, Cambridge, MA) and cannot be used to detect surface expressed receptors for live imaging. To determine rate of internalization or insertion of GABA_ARs, antibodies identifying the extracellular domain can be used.^(5,9,10) Rate of insertion can be studied by antibody feeding technique.⁽¹¹⁻¹³⁾ In this

technique, surface expressed receptors are blocked by incubation with saturating concentration of unlabeled antibody and newly inserted receptors are determined with labeled antibody directed against the same epitope. However, polyclonal antibodies cannot be used for this technique because they identify multiple epitopes.

We have generated a mouse monoclonal antibody against the $\gamma 2$ subunit of GABA_ARs using a peptide from the N-terminal extracellular domain of the rat GABA_AR $\gamma 2$ subunit. This antibody detects a single protein of approximately 42 kDa in the rat brain lysates and can be used in Western blotting, immunoprecipitation, and immunohistochemical assays.

Materials and Methods

Animals were handled according to guidelines set by the University of Virginia Health Sciences Center Animal Care and Use Committee. Efforts were made to minimize animal stress and discomfort.

Generation of peptide

The peptide corresponding to amino acids 37-53 (c s q k s d d d y e d y a s n k t w) at the N-terminal region of rat $\gamma 2$ subunit was custom-synthesized at the University of Virginia's peptide synthesis facility. The peptide had an additional N-terminal cysteine cap, which was used to couple it with keyhole limpet hemacyanin (KLH) or bovine serum albumin (BSA). KLH-conjugated peptide was used to immunize the mice whereas BSA-conjugated peptide was used in ELISA assays.

Immunization of mice

Pre-immunization sera samples were obtained by tail bleeding. Immunization of mice, hybridoma production, and cloning were carried out at the University of Virginia

lymphocyte culture center as described previously.⁽¹⁴⁾ Briefly, A/J male mice were immunized by subcutaneous and intraperitoneal injection of 1:1 peptide (100 µg) and Freund's complete adjuvant (AdjuLite, Pacific Immunology, Ramona, CA). The injections were repeated three times with an interval of 4 weeks between subsequent injections. The last injection was intrasplenic 50 µg of peptide in PBS. The mice were bled 2 weeks after last injection and sera samples were subjected to ELISA analysis.

Screening of mice and generation of hybridomas

The fusions were carried out as described previously.^(15,16) Briefly, the spleen cells from immunized mice were washed once with Iscove's MDM and mixed with Sp2/0-Ag14 myeloma cells at a ratio of 5:1. The fusion is accomplished by the addition of 50% polyethylene glycol to pelleted cells over a period of 1 min. The cells were washed with Iscove's MDM containing 15% fetal bovine serum, hypoxanthine (H), and thymidine (T). The cells were suspended in HT medium and grown in 96-well tissue culture plates at a density of $2\text{--}4 \times 10^5$ cells/well. Twenty-four h later the cultures were fed with HT medium supplemented with aminopterin. The supernatant was screened for specific antibody production and positive hybridomas were cloned twice.

Preparation of cell lysates and immunoblotting

Hippocampal tissue lysates were prepared as described previously using a RIRA lysis buffer composed of 0.5 M Tris-HCl (pH 7.4), 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, and 10 mM EDTA (Millipore).⁽⁴⁾ Ten to 100 µg total protein was separated on 10% SDS-PAGE, transferred to hybond-P PVDF membrane using standard procedures, and Western blotted with 1:200–1:500 diluted sera samples or purified antibody (1–5 µg/mL) using standard procedures. As a control, 2 µg/mL of a previously characterized rabbit polyclonal antibody (Alpha Diagnostic International) was also used in parallel in the Western blotting assays. The signal was detected using Kodak Gel Logic imaging system (Kodak, New Haven, CT).

Immunoprecipitation

The $\gamma 2$ subunit from hippocampal lysates corresponding to 500 µg proteins was immunoprecipitated as described previously⁽⁴⁾ with a mammalian immunoprecipitation kit (Thermo Scientific, Rockford, IL), using 100 µg 10F10-C1-B8 antibody, a rabbit polyclonal antibody (Alpha Diagnostics), or normal mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Western blot analysis was performed using 10F10-C1-B8 antibody against the $\gamma 2$ subunit of GABA_ARs (2 µg/mL dilution).

Immunohistochemical analysis

Hippocampal neurons were cultured as described previously.⁽¹⁷⁾ Surface and total expression of the $\gamma 2$ subunit was studied in the neurons cultured *in vitro* for 10–14 days using 1 µg/mL purified antibody using standard immunochemical technique.⁽¹⁸⁾ Briefly neurons were fixed in 4% paraformaldehyde in PBS at room temperature (RT) for 10 min. Cells were washed with PBS three times for 5 min each; cells were permeabilized (0.01% Triton X-100 in PBS for 10 min at RT)

to detect total expression of $\gamma 2$ subunit. Permeabilized cells were washed with PBS to remove excess Triton X-100, and non-specific sites were blocked by incubation in a blocking solution (5% normal goat serum and 0.05% BSA in PBS) for 30 min at RT. Cells were incubated with primary antibody (1 µg/mL antibody diluted in PBS containing 0.05% normal goat serum) overnight at 4°C. Following extensive washing with PBS, the cells were incubated with secondary antibody (4 µg/mL, Molecular Probes, Eugene, OR) for 1 h at RT. As a control, some cultures were also incubated with primary antibody that was pre-adsorbed with five molar excess of immunizing peptide for 2 h in cold and centrifuged at 14,000 g for 1 h to separate precipitated antigen-antibody complexes.

Immunohistochemical detection of the $\gamma 2$ subunit in the brain slices was performed as described previously.⁽⁸⁾ Briefly, animals were anesthetized with an overdose of pentobarbital sodium and perfused with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Brains were removed and post-fixed in the same fixative for 2 h at 4°C. After overnight incubation in 25% sucrose in 0.1 M PB for cryoprotection, the brains were frozen by immersion in -70°C isopentane. Coronal sections from the anterior block were cut at 40 µm. The sections were then incubated with a blocking solution containing 5% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) and 0.1% Triton X-100 in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 1 h. The sections were subsequently incubated with primary antibodies, 10F10-C1-B8, or rabbit polyclonal antibody (5 µg/mL; a kind gift from Dr. W. Sieghart, Medical University, Vienna, Austria) at 4°C for 48 h on a shaker. The primary and secondary antibodies were diluted in PBS containing 2% normal goat serum and 0.2% bovine serum albumin. The sections were washed three times with PBS and incubated in fluorescent secondary antibodies (5 µg/mL, Molecular Probes) for 1 h in the dark. Sections were then mounted on slides with Gel/Mount (Foster City, CA).

Transfection of HEK293 cells and detection of $\gamma 2$ subunit

HEK293 cells grown on poly L-lysine (100 µg/mL) coated coverslips were transfected with 1 µg of rat $\gamma 2$ L subunit cDNA (a kind gift from Dr. R. Macdonald, Vanderbilt University Medical Center, Nashville, TN) using lipofectamine 2000 (Invitrogen, Carlsbad, CA) reagent according to the manufacturer's instructions, and used for immunochemical detection of $\gamma 2$ subunit 36 h post-transfection. Briefly, cells were fixed with 4% paraformaldehyde in PBS for 10 min at RT, washed three times with PBS for 5 min each at RT, and permeabilized in 0.1% Triton X-100 in PBS for 10 min at RT. After washing with PBS three times for 5 min each at RT, non-specific sites were blocked by incubation in PBS containing 5% normal goat serum and 5% fetal bovine serum albumin for 30 min at RT. The cells were incubated with 10F10-C1-B8 antibody (1 µg/mL) at 4°C overnight. Following three washes with PBS for 5 min each at RT to remove unbound primary antibody, the cells were incubated with fluorescent secondary antibody (2 µg/mL) for 1 h at RT in the dark. HEK293 cells cultured in 60 mm Petri dishes were transfected with 3 µg of $\gamma 2$ subunit cDNA as described above using lipofectamine reagent and lysed in RIPA lysis buffer 48 h post-transfection. Expression of $\gamma 2$ subunit was detected using 75 µg of total

proteins by Western blot analysis. As a negative control, expression of γ 2 subunit in equal protein from untransfected cells was also studied.

As a control, we also determined expression of γ 2 subunit in hippocampal neurons or HEK293 cells transfected with γ 2 subunit siRNA, which has been characterized previously.⁽¹⁹⁾ Briefly, 6-day-old hippocampal neurons were transfected with 5 μ g siRNA/35 mm culture dish for 48 h using HiPerfect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. HEK293 cells were transfected with a plasmid containing γ 2L cDNA (1 μ g/35 mm dish) along with γ 2 siRNA (5 μ g/35 mm dish) using lipofectamine reagent. Expression of γ 2 subunit was detected as above 36 h post-transfection.

Results and Discussion

Screening of mice

Four mice were immunized with the peptide, and sera were screened for the presence of antibody using the BSA-conjugated peptide in ELISA assays. Sera from all four mice reacted positive in the ELISA assay, whereas pre-immunization sera, used as negative control, did not react (data not shown). The mouse that showed the highest titer in the serum

obtained from tail bleed following the third injection was used for fusions.

Screening of fusions

Thirty-seven fusions obtained from the selected mouse tested positive in ELISA assay. Only four fusions reacted with a protein of approximately 40–50 kDa size in Western blotting using hippocampal tissue lysates (data not shown). Fusion 10F10 gave a strong signal in Western blot assays.

Single clones were isolated from fusion 10F10 by limited dilution, tested in ELISA, and purified by two rounds of selection. Supernatant from a clone 10F10-C1-B8 showed highest titer in the ELISA assays. Isotyping of the clone revealed that it was IgG2b kappa. These antibodies were thoroughly characterized in subsequent experiments.

Characterization of antibodies

Antibodies generated by clone 10F10-C1-B8 were tested in a Western blot assay using hippocampal tissue lysates. We used different concentrations of antibody (1–5 μ g/mL) in the Western blot assays. A protein of ~40–50 kDa reacted with the antibody 10F10-C1-B8. The Western blot signal intensity of antibody concentration at 1 μ g/mL was comparatively

FIG. 1. Detection of γ 2 subunit in a Western blot assay using 10F10-C1-B8 antibody. (A) 10, 25, 50, and 100 μ g rat or mouse hippocampal proteins were Western blotted using 2 μ g/mL of 10F10-C1-B8 antibody. Arrowhead indicates γ 2 signal. Position of molecular weight marker 30, 40, and 50 kDa is also shown for reference (lane M). (B) Expression of γ 2 subunit in 10–100 μ g rat or mouse hippocampal proteins in a Western blotting assay using 2 μ g/mL rabbit polyclonal antibody. Arrowhead indicates γ 2 signal. Lane M shows position of marker proteins of 30, 40, and 50 kDa sizes. (C) Expression of rat γ 2L subunit in HEK293 cells (lanes T) transfected with a cDNA expressing rat γ 2L subunit. 75 μ g total proteins were Western blotted using 10F10-C1-B8 antibody (2 μ g/mL, panel Ms) or rabbit polyclonal antibody (2 μ g/mL, panel Rb). Proteins from untransfected cells were used as control (lanes NT). Position of molecular weight proteins 40 and 50 kDa is also shown (lane M). (D) γ 2 subunit was immunoprecipitated from 500 μ g hippocampal proteins using 100 μ g of 10F10-C1-B8 antibody (lane Ms), a rabbit polyclonal antibody against γ 2 subunit (lane Rb), or normal mouse IgG (lane MslgG). Arrowhead indicates γ 2 signal. In lane M, molecular weight marker, 40 and 50 kDa is marked.

weak, whereas at 2 and 5 $\mu\text{g}/\text{mL}$ dilutions of the antibody a strong signal was observed in the Western blots (data not shown). For all subsequent experiments we used 2 $\mu\text{g}/\text{mL}$ dilution of the antibody. The reactivity of antibody was also determined by using different protein concentrations (10–100 μg). A protein of ~ 40 –50 kDa size was identified by 10F10-C1-B8 antibody (Fig. 1A), similar to that reported previously by our and other laboratories.^(5,7,8,10,20) Similar expression of $\gamma 2$ subunit was also observed in the Western blot assay performed using a rabbit polyclonal antibody from Alpha Diagnostics (Fig. 1B). To determine cross-reactivity of

the antibody to $\gamma 2$ subunits from other species, we also determined expression of $\gamma 2$ subunit from mouse hippocampal proteins using 10F10-C1-B8 antibody (Fig. 1A and B, mouse panel). The antibody 10F10-C1-B8 detected mouse and rat $\gamma 2$ subunit expressed in the hippocampus.

The specificity of IgG was further confirmed using lysates from HEK293 cells expressing rat GABA_AR $\gamma 2$ subunit. A major protein of ~ 50 kDa reacted with the IgG (Fig. 1D). In addition, a protein of smaller size also reacted with IgG, which could represent degradation of expressed $\gamma 2$ subunit. A faint signal was also observed in the lysates from non-transfected cells, which

FIG. 2. Immunohistochemical detection of $\gamma 2$ subunit using 10F10-C1-B8 antibody. (A) Expression of $\gamma 2$ subunit (red) in HEK293 cells transfected with rat $\gamma 2$ L subunit cDNA. (B) Immunoreactivity of 10F10-C1-B8 antibody in untransfected HEK293 cells. (C) Immunoreactivity of pre-adsorbed 10F10-C1-B8 antibody (1 $\mu\text{g}/\text{mL}$) in transfected HEK293 cells. (D) Immunoreactivity (red) of 10F10-C1-B8 in HEK293 cells transfected with 1 μg $\gamma 2$ L cDNA and 5 μg anti- $\gamma 2$ subunit siRNA (green). (E, F) Expression of $\gamma 2$ subunit (red) in cultured hippocampal neurons grown *in vitro* for 12 days using 10F10-C1-B8 antibody before and after pre-adsorption, respectively. Expression of MAP2 (blue) is also shown. (G) Expression of $\gamma 2$ subunit (red) in cultured hippocampal neurons grown *in vitro* for 6 days, and transfected with 5 μg of anti- $\gamma 2$ subunit siRNA (green) detected using 10F10-C1-B8 antibody. Expression of MAP2 (blue) is also shown. (H) Immunohistochemical detection of $\gamma 2$ subunit in a control neuron not expressing siRNA and grown *in vitro* for 8 days. (I) Immunoreactivity of the purified $\gamma 2$ antibody 10F10-C1-B8 in the dentate gyrus. The IgG demonstrated dense immunoreactivity in the dentate granule cell layer. Some inter neurons were also labeled. (J) Immunolabeling using a well characterized polyclonal antibody (gift from Dr. W. Sieghart). Scale bar in left panel, 25 μm .

were used as a control. This suggested a low-affinity non-specific reactivity of the IgG. As a control, equal amounts of proteins from transfected or non-transfected cells were also blotted with rabbit polyclonal antibody, which gave similar results. This further indicated activity of IgG against the γ 2 subunit of GABA_ARs.

We also studied whether this antibody was able to immunoprecipitate γ 2 subunit from hippocampal lysates. Immunoprecipitation was performed as described previously⁽⁴⁾ using 100 μ g 10F10-C1-B8 antibody or rabbit polyclonal antibody. A single band of $\sim$ 40–50 kDa was observed in the proteins immunoprecipitated using 10F10-C1-B8 or rabbit antibodies (Fig. 1C). A weak signal was also observed in the normal mouse IgG pull-down samples. Although the polyclonal nature of the normal mouse IgG used in these assays may explain presence of weak non-specific signal, the possibility that antibody 10F10-C1-B8 may interact non-specifically with other protein of comparable size cannot be ruled out. These results suggest that the antibody can be used in Western blot assays. It can also immunoprecipitate the γ 2 subunit from hippocampal lysates; however the efficiency appeared to be lower, most likely because of the monoclonal nature. Whether it also reacts with other γ subunits is currently not known. However, only a few amino acid residues in this domain are conserved between different subunits,⁽²¹⁾ and chances of this antibody recognizing other subunits are low.

The IgG 10F10-C1-B8 was also tested in immunohistochemical assays. In the HEK293 cells expressing γ 2L subunit cDNA, incubation with 10F10-C1-B8 antibody resulted in intense cytoplasmic immunoreactivity (Fig. 2A), whereas in non-transfected cells only a minimal background fluorescence was observed (Fig. 2B). The cytoplasmic immunoreactivity completely disappeared when the transfected cells were incubated with pre-adsorbed antibody (Fig. 2C), suggesting specificity of the antibody. Furthermore, in HEK293 cells simultaneously transfected with rat γ 2L cDNA and anti- γ 2 subunit siRNA, immunoreactivity of 10F10-C1-B8 antibody was weak (Fig. 2D). Although expression of siRNA did not completely block immunoreactivity, the fluorescence intensity was reduced by $60 \pm 15\%$ ($n = 12$ cells).

Immunoreactivity of 10F10-C1-B8 antibody was also determined in cultured hippocampal neurons. In these neurons under permeabilizing conditions, intense γ 2 subunit reactivity was observed in the cell soma, whereas immunoreactivity in the dendrites appeared punctate (Fig. 2E). The pattern of γ 2 subunit immunoreactivity observed with 10F10-C1-B3 antibody was similar to that reported previously.⁽¹⁸⁾ In some assays, pre-adsorbed antibody was added and in these neurons, a minor immunoreactivity was observed (Fig. 2F), suggestive of specific immunoreactivity of peptide towards γ 2 subunit. To confirm that observed immunoreactivity was indeed due to activity of antibody against γ 2 subunit, neurons grown *in vitro* for 6 days were transfected with siRNA targeting γ 2L subunit, which has been characterized previously.⁽¹⁹⁾ Two concentrations (2, and 5 μ g) of siRNA were used to suppress expression of γ 2 subunit (data not shown). Immunoreactivity of 10F10-C1-B8 was studied 36 h after transfection. In the non-transfected neurons, γ 2 immunoreactivity was observed in the cell soma and dendrites. The punctate staining typical of γ 2 subunit expression observed at a later time was not observed in these neurons, most likely due to their younger age. In the neurons transfected with γ 2 subunit

siRNA (5 μ g/35 mm dish, Fig. 2G), weaker γ 2 immunoreactivity was observed, suggesting suppression of γ 2 expression. In the 10 neurons studied, siRNA reduced γ 2 subunit expression by $50 \pm 21\%$. We currently do not know the source of remaining immunoreactivity in siRNA expressing cells. Variation in transfection efficiency and stability of siRNA may have influenced observed variation in the suppression of γ 2 subunit expression in these cells. Together these results suggest specificity of 10F10-C1-B8 IgG towards γ 2 subunit.

In the dentate gyrus, γ 2 subunit immunoreactivity was observed in the soma of granule cell and some interneurons (Fig. 2I). Clusters of γ 2 subunit immunoreactivity were also observed in the molecular layer, which contains the dendrites of granule cells (Fig. 2J). Previous studies have reported similar distribution of the γ 2 subunit in hippocampal dentate gyrus by immunohistochemistry using rabbit polyclonal antibody (generated in Dr. W. Sieghart's laboratory) (Fig. 2J).⁽⁸⁾ The antibody showed specificity in the Western blot as well as immunochemical assays. The monoclonal antibody produced a pattern of staining that was identical to another antiserum (a polyclonal serum against a synthetic peptide) that was better characterized (and passed the adsorption test).⁽⁸⁾ Furthermore, the pattern of mRNA expression by using *in situ* hybridization histochemistry demonstrated similar results as the immunostaining for the protein.⁽²²⁾

In summary, we have generated a monoclonal antibody against the γ 2 subunit of GABA_ARs, which can be used in Western blotting and immunochemistry.

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Author Disclosure Statement

The authors have no financial interests to disclose.

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Address correspondence to:
 Jaideep Kapur, M.D., Ph.D.
 Department of Neurology
 Box 800394
 University of Virginia-HSC
 Charlottesville, VA 22908
 E-mail: jk8t@virginia.edu
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