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RGS8 Protein Is Distributed in Dendrites and Cell Body of Cerebellar Purkinje Cell

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RGS8 was originally identified as an RGS protein specifically expressed in neuronally differentiated P19 cells. We generated a polyclonal antibody specific to rat RGS8 using a synthetic peptide. When nonneural cells (DDT1MF2, CHO, and NIH3T3) transfected with rat RGS8 cDNA were immuno-stained with this antibody, the RGS8 protein was mainly detected in the nuclei. Since RGS8 mRNA was exclusively expressed in Purkinje cells of the cerebellum in the rat brain, we further examined the cellular distribution of the RGS8 protein in Purkinje cells using cultured cerebellar cells and tissue sections of the cerebellum. The RGS8 protein was excluded from the nuclei and distributed in the cell body and dendrites, but not in the axons of Purkinje cells. These results demonstrate the presence of a mechanism controlling the distribution of RGS8 protein in cerebellar Purkinje cells. © 2001 Academic Press

Key Words: **G protein; RGS; desensitization; receptor; Purkinje cells; nuclear localization.**

RGS (regulators of G protein signaling) proteins comprise a large family of more than 20 members, which modulate heterotrimeric G protein signaling (1, 2). This protein family was originally identified as a pheromone desensitization factor in yeast (3). Subsequent studies have identified many RGS proteins by virtue of a common stretch of 120 amino acids termed the RGS domain in organisms ranging from yeast to humans (1, 2, 4, 5). It has been reported that several RGS proteins (RGS1, RGS3, RGS4, GAIP) attenuate the G protein signaling in cultures (4, 6, 7). Biochemical studies have demonstrated that RGS members function as GTPase-activating proteins (GAP) for the α–subunits of heterotrimeric G proteins (8–10). Hence, RGS proteins are thought to down-regulate G protein signaling *in vivo* by enhancing the rate of Gα GTP hydrolysis. However, our group and another have demonstrated that the RGS proteins significantly accelerated the turning on and off of the G protein-coupled inwardly rectifying K^+ channels (11–13).

Since Gα subunits are usually associated with signaling events at the plasma membrane, the study of the subcellular distribution and its regulation of the RGS proteins will help to understand the nature of the G-protein signaling pathways that they regulate. Recently, several observations concerning the cellular distribution of RGS proteins have been reported. Druey *et al.* have reported that the majority of RGS4 is found as a soluble protein in the cytoplasm of mammalian cultured cells, and that the expression of a GTPase-deficient Gαi resulted in the translocation of RGS4 to the plasma membrane (14). In yeast, the short N-terminal domain conserved in RGS4 and RGS16 was reported to be required for membrane localization and the ability to inhibit pheromone response (15, 16). In contrast, RGS3 has a long unique N-terminus, and it was reported to be predominant in the cytoplasm and to be translocated to the plasma membrane upon agonist stimulation. The N-terminal domain of RGS3 was also reported to be important for this translocation (17). A truncated isoform of RGS3, termed RGS3T, lacking a large portion of the N-terminus of RGS3 but retaining a core RGS domain and a smaller N-terminal tail has been identified by PCR analysis (18). Recently, it was found that RGS3T is localized in the nucleus and induces apoptosis (19). Thus, multiple RGS proteins within a given cell might be differentially localized and their intracellular localization might be regulated to determine a physiological response to a G protein-linked stimulus.

Using a culture system of P19 cells, we have previously isolated the cDNA of RGS8 and identified it as an RGS protein induced in neuronally differentiated P19

Abbreviations used: G proteins, heterotrimeric guanine nucleotide-binding proteins; RGS, regulators of G protein signaling.

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cells. A sequencing analysis of the RGS8 cDNA revealed that the N-terminal sequence was significantly homologous to the N-terminal domains of RGS4 and RGS16, in addition to the RGS domain well conserved among all members of the RGS proteins (12). We further examined the distribution of RGS8 mRNA in the brain by *in situ* hybridization. The RGS8 mRNA was found to be densely expressed in Purkinje cells of the cerebellar cortex (13). Recently, to examine the subcellular distribution of the RGS8 protein, we expressed RGS8 as a fusion protein with RFP (red fluorescent protein) in nonneural DDT1MF2 cells. We found that RGS8-RFP was concentrated in the nuclei of the cells. Furthermore, we found that coexpression of constitutively active Gαo resulted in the translocation of RGS8-RFP to the plasma membrane. The N-terminal domain of RGS8 was then demonstrated to play important roles in subcellular localization and in physiological functions as well (20).

Here, we investigated how the RGS8 protein is distributed in Purkinje cells of the cerebellar cortex by using a newly generated specific antibody for RGS8.

MATERIALS AND METHODS

Cell cultures. A Syrian hamster leiomyosarcoma cell line, DDT1MF2, was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. A Chinese hamster ovarian cell line, CHO, was grown in Ham's F12 medium supplemented with 10% fetal bovine serum. A mouse embryo cell line, NIH3T3, was grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Rat RGS8 cDNA was cloned into the pCXN2 expression vector provided by Professor Miyazaki (21). The resultant plasmid DNA was transfected into cultured cells using Fugene 6 (Roche Molecular Biochemicals). A primary culture of the cerebellar neurons of Wister rats was prepared by the method of Hirano and Kasono (22). Briefly, cerebella were dissected out from 20-day-old fetuses. The cerebella were incubated in Ca^{2+} and Mg^{2+}-free Hank's balanced salt solution containing 0.1% trypsin and 0.05% DNase for 15 min at 37°C. The neurons were then dissociated by trituration and cultured on glass coverslips coated with poly-L-lysine in a defined medium.

Antibodies. A polyclonal antibody against RGS8 was prepared. A peptide (CSDFTAILPDKPNRAL) corresponding to residues 27–42 of rat RGS8 outside of the RGS domain was synthesized and conjugated with keyhole limpet hemocyanin. Rabbits were immunized with this hemocyanin-conjugated peptide. The specific immunoglobulin was purified by Sepharose 4B coupled to the synthetic peptide. Mouse anti-calbindin monoclonal antibody (Sigma) and rabbit anti-Gαo polyclonal antibody (Santa Cruz) were also used. Peroxidase (HRP)-labeled goat anti-rabbit IgG (GAR) and HRP-labeled goat anti-mouse IgG (GAM) were purchased from ICN. Cy3 conjugated goat anti-rabbit IgG and fluorescein (FITC)-conjugated donkey anti-mouse IgG were from Jackson Immuno Research.

Western blotting. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using 13.5% polyacrylamide gel in a discontinuous Tris-glycine buffer system. The proteins were electrophoretically transferred from the SDS–polyacrylamide gel to nitrocellulose paper. The transferred nitrocellulose paper was treated with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TTBS) and incubated with primary antibody for 90 min, followed by treatment with HRP-labeled GAM or GAR for 1 h. After immunoreaction, the paper was washed with TTBS. The HRP-labeled antibodies bound to the paper were detected with an ECL system (Amersham Pharmasia Biotech).

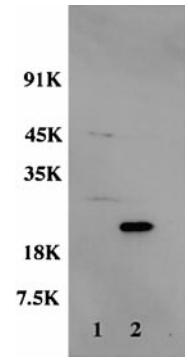

FIG. 1. Specificity of anti-RGS8 antibody. The DDT1MF2 cells were transfected with empty vector (1) or with RGS8 cDNA in the expression vector (2). After 48 h, each whole SDS extract was prepared, electrophoresed on an SDS–polyacrylamide gel, and transferred to nitrocellulose paper. The transferred paper was reacted with anti-RGS8 antibody.

Immunofluorescence staining of the cultured cells. Cell cultures on glass coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature. They were then permeabilized with 0.5% triton X-100 in PBS for 5 min and blocked with 5% skim milk in PBS for 90 min. The cultures were immuno-stained for RGS8 or doubly immuno-stained for calbindin and RGS8. Mouse anti-calbindin antibody was visualized with FITC-anti-mouse antibody and rabbit anti-RGS8 antibody was detected by Cy3-anti-rabbit antibody. After immunoreaction, cells were treated with 50 nM DAPI (4′,6-diamidino-2-phenylindole,dihydrochloride, Molecular Probes) for 10 min and mounted with Fluoro Guard antifade reagent (Bio Rad).

Immunofluorescence staining of the tissue sections. Under anesthesia, adult Wister rats were perfused through the ascending aorta with freshly prepared 4% paraformaldehyde in PBS. The whole brains were removed, postfixed in 4% paraformaldehyde in PBS overnight, and immersed in 30% sucrose in PBS for 48 h at 4°C. The cerebella were dissected, embedded in OCT compound (Tissue Tek II, Miles), and rapidly frozen. Sagittal frozen sections mounted on silanized slides were used for the immunofluorescence staining. The sections were fixed with 4% paraformaldehyde in PBS for 30 min, treated with 0.5% triton X-100 in PBS for 30 min, and then blocked with 5% skim milk in PBS for 2 h. The blocked sections were immuno-stained for calbindin and further for RGS8 as cultured cells. After antibody staining, sections were incubated with 50 nM DAPI for 10 min and mounted with Fluoro Guard.

RESULTS

Generation of anti-RGS8 antibody. A polyclonal antibody against RGS8 was prepared by immunizing rabbits with a synthetic peptide (SDFTAILPDKPNRAL) corresponding to part of the N-terminal region of RGS8 outside of the RGS domain. The obtained antiserum was purified using an affinity column coupled with this peptide. The specificity of the purified antibody was first tested by a Western blot of the whole SDS extract of the transfected DDT1MF2 cells expressing RGS8. As shown in Fig. 1, an approximately 21 kDa band was observed in the cells transfected with RGS8 cDNA. No significant protein band was detected in the lysates from vector-transfected cells.

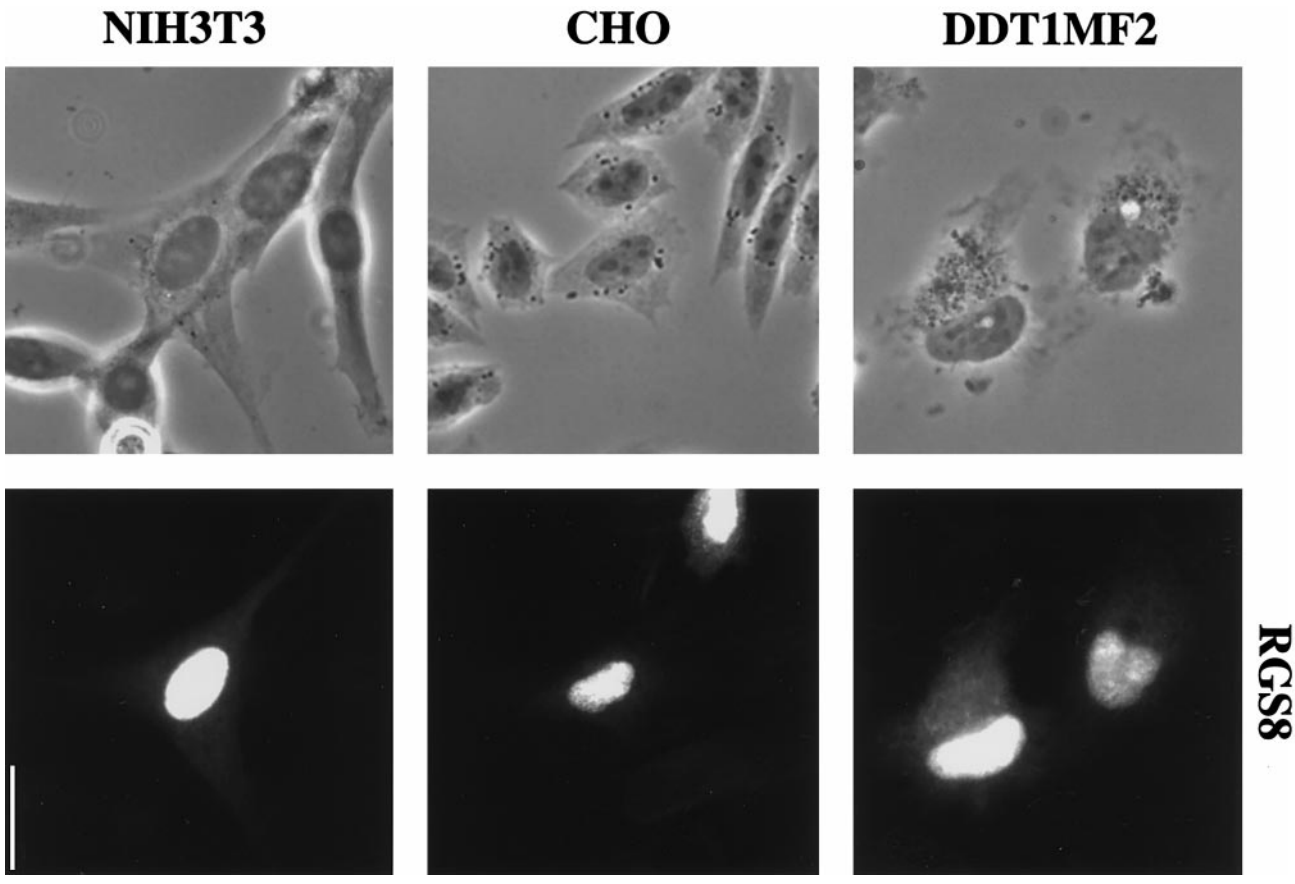

FIG. 2. Cellular distribution of the RGS8 protein in nonneural cells. DDT1MF2, CHO, NIH3T3 cells were transfected with RGS8 cDNA in the expression vector. After 48 h, cells were immuno-stained with anti-RGS8 antibody. Phase-contrast and corresponding immunofluorescence micrographs are shown. Bar indicates 25 μm.

Cellular distribution of RGS8 in nonneural cells. To further examine the specificity of anti-RGS8 antibody and to investigate the cellular distribution of RGS8, we carried out immunofluorescent staining of the cultured nonneural cells transfected with RGS8 cDNA. DDT1MF2, CHO, and NIH3T3 cells were transfected. In any line, the RGS8 protein was observed mainly in the nuclei of most of the transfected cells (Fig. 2). When RGS8 was expressed as a chimeric protein with a fluorescent protein, a similar distribution was observed as described (20). No staining was observed in the vector-transfected control cells (data not shown). Thus, this anti-RGS8 antibody was considered to be suitable for the study of the cellular distribution of the RGS8 protein, and the RGS8 protein was found to be accumulated at nuclei in the examined nonneural cells.

Cellular distribution of RGS8 in cultured Purkinje cells. We have previously reported that RGS8 mRNA was exclusively expressed in Purkinje cells of the cerebellum by *in situ* hybridization (13). Therefore, we examined the cellular distribution of RGS8 protein in cultured Purkinje cells by using an antibody specific for RGS8. The cerebella were isolated from 20-day-old rat fetuses and the cerebellar cells were cultured. The cultured cerebellar cells were analyzed by double immuno-staining using antibodies to RGS8 and calbindin, a calcium-binding protein specifically expressed in Purkinje cells (23). Purkinje cells were easily identified by both their distinctive morphology and immunoreactivity to calbindin antibody (Fig. 3). Purkinje cells cultured for 22 days had large cell bodies, thick dendrites, and typical axons. When the distribution of RGS8 was examined in the same cells, RGS8 was found to exist only in the cell bodies and the thick dendrites of Purkinje cells. The axons were not significantly stained by the anti-RGS8 antibody.

Cellular distribution of RGS8 in the cerebellar cortex. To further analyze the cellular distribution of the RGS8 protein, frozen sections of rat cerebella were prepared and reacted with anti-RGS8 and anti-calbindin antibodies (Fig. 4). The cell bodies and dendrites in the molecular layer of Purkinje cells were stained by the RGS8 antibody, but no significant staining was detected in the granule cell layer. Comparison with DAPI staining revealed that the staining with

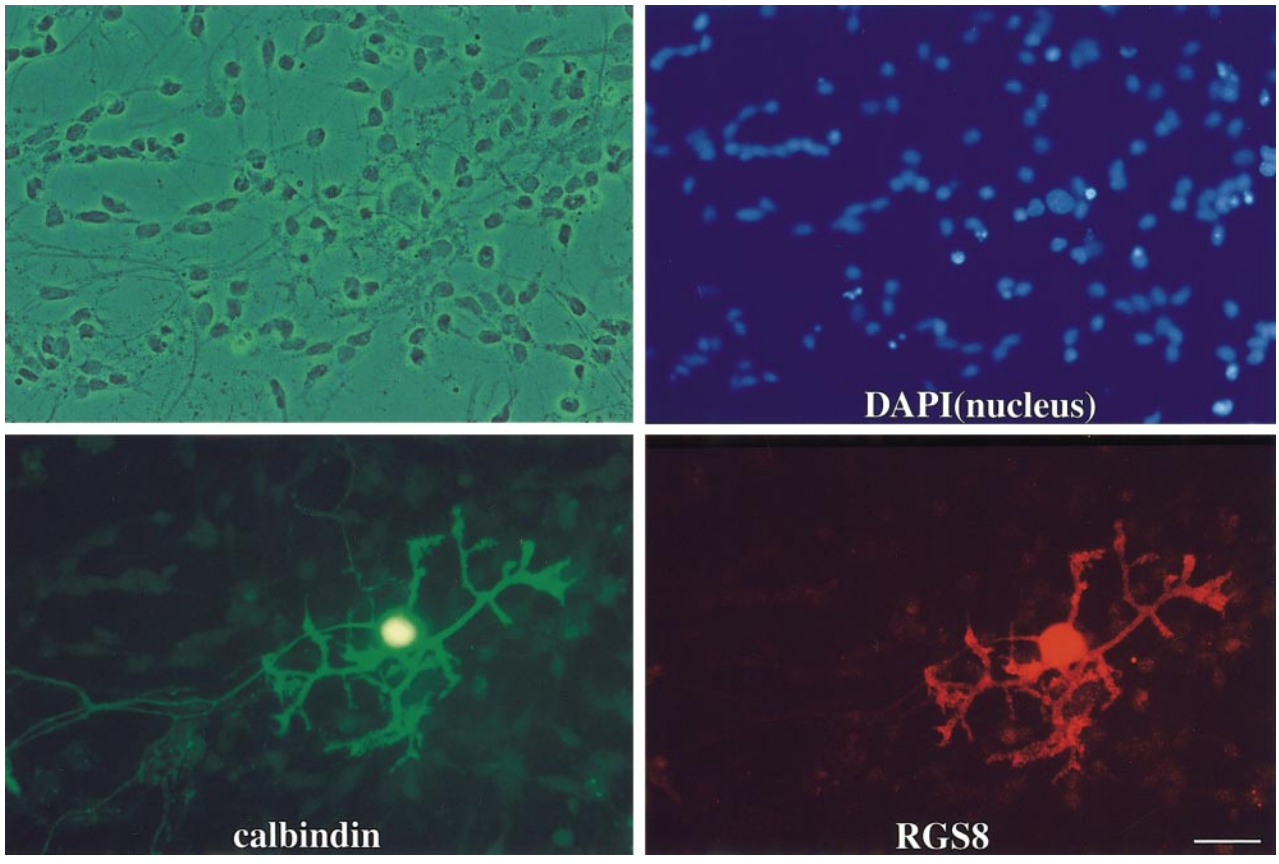

FIG. 3. Cellular distribution of RGS8 in cultured cerebellar Purkinje cells. The cerebella were dissected from 20-day-old rat fetuses. The cerebellar cells were isolated, cultured for 22 days, doubly immuno-stained with anti-calbindin and anti-RGS8 antibodies, and stained with DAPI. Phase-contrast and corresponding immunofluorescence micrographs are shown. Bar indicates 37 μm.

anti-RGS8 antibody was excluded from the nuclei of Purkinje cells. When the same section was reacted with anti-calbindin antibody, apparent staining of the axons of Purkinje cells in the granule cell layer was observed in addition to cell bodies and dendrites.

RGS8 is a membrane-bound protein. To examine whether the RGS8 protein is present as a cytoplasmic or membrane-bound protein in Purkinje cells, rat cerebellum was fractionated as previously described (20). The cytoplasmic and particulate fractions were subjected to Western blot analysis using the RGS8-specific antibody (Fig. 5B). RGS8 was present in the particulate fraction, as was Gα. However, when the same blots were reacted with anti-calbindin antibody, the presence of calbindin was easily observed in both fractions. A rather higher amount was detected in the cytoplasmic fraction.

DISCUSSION

We generated a specific antibody for RGS8 using a synthetic peptide and carried out immunostaining of the cultured cerebellar cells and cerebellar sections with this anti-RGS8 antibody. We found that the RGS8 protein is expressed specifically in Purkinje cells, and furthermore that RGS8 is distributed through the cell bodies and dendrites, except axons, in Purkinje cells. We have previously reported that RGS8 functions as a GAP for Gαo and Gαi3 (12). From these observations, it is suggested that RGS8 regulates the Gi/o-mediated signalings triggered by metabotropic receptors localized at the postsynaptic region of Purkinje cells.

Based on the observations of the immunostained sections of the cerebella, the RGS8 protein was found to be excluded from the nuclei of Purkinje cells. The cell fractionation analysis indicated that RGS8 is present mainly as a membrane-bound protein. However, when RGS8 was expressed in nonneural cells, the RGS8 protein was found to be concentrated in the nuclei. Moreover, we observed that RGS8 is translocated to the plasma membranes from the nuclei by the expression of constitutively active Gαo in DDT1MF2 cells, and that this translocation requires the short N-terminal region of RGS8 (20). Therefore, in unstimulated Purkinje cells, it seems that the RGS8 protein might be exported from the nuclei, and furthermore that the

exported RGS8 might be excluded from the axons by an unknown certain mechanism. PCP2 (Purkinje cell protein-2) might be involved in determining this subcellular distribution of RGS8 in cerebellar Purkinje cells. PCP2, which is specifically expressed in Purkinje cells, has been reported to function as a novel nucleotide exchange factor for Gαo (24). Expression of PCP2 might serve as introduction of active Gαo in Purkinje cells.

The present data demonstrate that the subcellular distribution of RGS8 is quite different in nonneural cells and neurons. Further investigations, however, are required to elucidate the molecular mechanism determining the distribution of the RGS8 protein and to understand how neural activity may modulate the distribution and function of RGS8 to regulate G protein signaling.

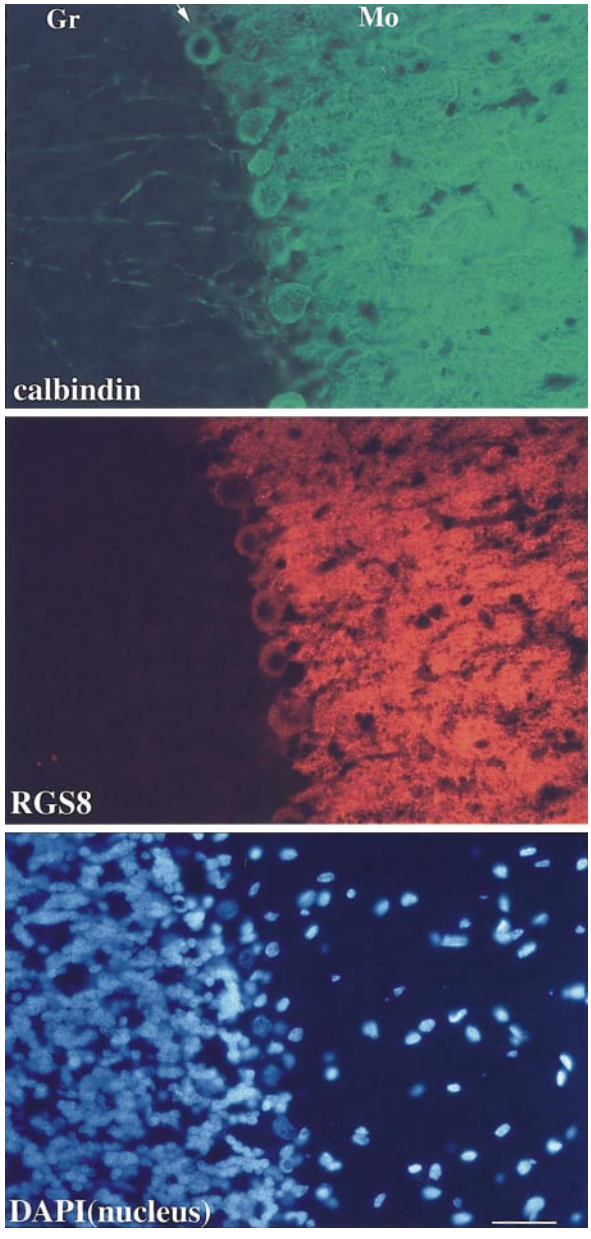

FIG. 4. Distribution of RGS8 in the cerebellum. The sagittal frozen section of the adult rat cerebella was stained with anti-calbindin antibody, anti-RGS8 antibody, and DAPI. Mo, molecular layer. Gr, granule cell layer. Arrows, Purkinje cell layer. Bar indicates 37 μm.

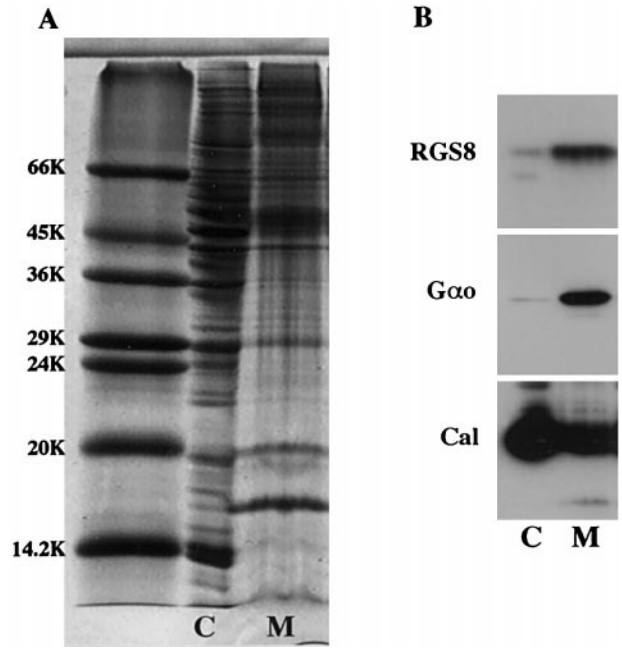

FIG. 5. RGS8 is a membrane-bound protein in the rat cerebellum. (A) Cytoplasmic (C) and particulate (M) fractions were obtained from the rat cerebella. They were electrophoresed on an SDS–polyacrylamide gel and stained with Coomassie brilliant blue. (B) After electrophoresis, both fractions were transferred to nitrocellulose paper. Western blotting using anti-RGS8 (RGS8), anti-Gαo (Gαo), or anti-calbindin (Cal) antibody was performed.

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