Translocation of Activated Heterotrimeric G Protein \( \alpha_\text{G}\) to Ganglioside-enriched Detergent-resistant Membrane Rafts in Developing Cerebellum*

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The association of gangliosides with specific proteins in the central nervous system was examined by co-immunoprecipitation with an anti-ganglioside antibody. The monoclonal antibody to the ganglioside GD3 immunoprecipitated phosphoproteins of 40, 53, 56, and 80 kDa from the rat cerebellum. Of these proteins, the 40-kDa protein was identified as the \( \alpha \)-subunit of a heterotrimeric G protein, \( \alpha_\text{G} \) (\( \alpha_\text{G}\)). Using sucrose density gradient analysis of cerebellar membranes, \( \alpha_\text{G} \), but not \( \beta_\gamma \), was observed in detergent-resistant membrane (DRM) raft fractions in which GD3 was abundant after the addition of guanosine 5′-O-(thiotriphosphate) (GTP\( \gamma \)S), which stabilizes \( \alpha_\text{G} \) in its active form. On the other hand, both \( \alpha_\text{G} \) and \( \beta_\gamma \) were excluded from the DRM raft fractions in the presence of guanyl-5′-yl thiophosphate, which stabilizes \( \alpha_\text{G} \) in its inactive form. Only \( \alpha_\text{G} \) was observed in the DRM fractions from the cerebellum on postnatal day 7, but not from that in adult. After pertussis toxin treatment, \( \alpha_\text{G} \) was not observed in the DRM fractions, even from the cerebellum on postnatal day 7. These results indicate the activation-dependent translocation of \( \alpha_\text{G} \) into the DRM rafts. Furthermore, \( \alpha_\text{G} \) was concentrated in the neuronal growth cones. Treatment with stromal cell-derived factor-1\( \alpha \), a physiological ligand for the G protein-coupled receptor, stimulated \[^{35}\text{S}]\)GTP\( \gamma \)S binding to \( \alpha_\text{G} \) and caused \( \alpha_\text{G} \) translocation to the DRM fractions and RhoA translocation to the membrane fraction, leading to the growth cone collapse of cerebellar granule neurons. The collapse was partly prevented by pre-treatment with the cholesterol-sequestering and raft-disrupting agent methyl-\( \beta \)-cyclodextrin. These results demonstrate the involvement of signal-dependent \( \alpha_\text{G} \) translocation to the DRM in the growth cone behavior of cerebellar granule neurons.

Gangliosides, which are sialic acid-containing glycosphingolipids (GSLs),3 are found in the outer leaflet of the plasma membrane of all vertebrate cells and are thought to play functional roles in cellular interactions and the control of cell proliferation (1, 2). In the nervous system, where gangliosides are particularly abundant, the species and amounts of gangliosides undergo profound changes during development, suggesting that they play fundamental roles in this process.

Exogenously administered gangliosides accelerate the regeneration of neurons in the central nervous system in vivo after lesioning (3). The addition of exogenous gangliosides to primary cultures of neurons and neuroblastoma cells in vitro stimulates cellular differentiation with concomitant neurite sprouting and extension (4). Glucosylceramide synthesis, the first glycosylation step in GSL synthesis, is required for embryonic development (5). The transfection of the ganglioside GD3 synthase cDNA into neuroblastoma cells induces cholinergic differentiation and neurite sprouting (6). GD3 synthase gene knock-out mice exhibit impairment in the regeneration of lesioned hypoglossal nerves (7). Finally, ganglioside-deficient mice exhibit central nervous system degeneration (8). These findings show that gangliosides are involved in neural cell differentiation and brain development. However, the molecular mechanisms and signal transduction pathways underlying the ganglioside-dependent neural functions remain unclarified.

GSLs exist in clusters and form microdomains containing cholesterol at the surface of the plasma membrane called rafts (9). Rafts are insoluble in cold nonionic detergents such as Triton X-100 (Tx) and can be isolated from the nonraft domains of the cell membrane (10), and these rafts are called detergent-resistant membrane (DRM) rafts. GSL microdomains have been implicated in signal transduction because various signaling molecules, such as Src family kinases, are associated with them. However, the precise functions of GSL-enriched microdomains remain to be clarified.

We have been investigating the association of gangliosides with specific proteins in the central nervous system. We previously demonstrated that an anti-ganglioside GD3 antibody (R24) co-immunoprecipitates phosphorylated proteins of 40,
53, 56, and 80 kDa and proteins of 135 and 16 kDa from rat cerebellar neurons. Of these proteins, the 53- and 56-kDa phosphoproteins were identified as the Src family kinase Lyn (11). The 135-kDa protein was identified as the glycosylphosphatidylinositol-anchored neuronal cell adhesion molecule TAG-1 (12). We have demonstrated that TAG-1 transduces a signal via Lyn in the lipid rafts of primary cerebellar granule neurons and promotes neurite outgrowth (13).

In this study, we identified the 40-kDa phosphoprotein as the α-subunit of the heterotrimeric G protein \( G_\alpha \) (\( G_\alpha_o \)) and demonstrated the activation-dependent translocation of \( G_\alpha_o \) to lipid rafts, leading to the growth cone collapse of cerebellar granule neurons.

**EXPERIMENTAL PROCEDURES**

**Materials—** The anti-ganglioside GD3 monoclonal antibody (R24), anti-transferrin receptor monoclonal antibody, anti-Lyn monoclonal antibody (Lyn8), and anti-growth-associated protein (GAP)-43 monoclonal antibody were obtained from Signet Laboratories, Zymed Laboratories Inc., Wako Chemicals, and Chemicon International, respectively. The anti-\( G_\alpha_o \) polyclonal IgG (K-20), anti-\( G_\alpha_o \) monoclonal antibody (A2), and anti-\( G_\beta \) polyclonal IgG (T-20) were purchased from Santa Cruz Biotechnology, except for the anti-\( G_\alpha_o \) (GC/2) polyclonal antibody (PerkinElmer Life Sciences) used for the *in vitro* kinase assay. The anti-\( G_\alpha_o \)-1 and anti-\( G_\alpha_o \)-2 polyclonal IgGs were obtained from Calbiochem. The anti-Lyn polyclonal IgG and anti-p44/42 mitogen-activated protein kinase polyclonal IgG were purchased from Cell Signaling Technology, Inc.; the anti-neuronal filament IgG was purchased from Sigma. Alexa Fluor 488-conjugated anti-rabbit IgG and Alexa Fluor 568-conjugated phalloidin were obtained from Molecular Probes. An Enhanced Cy3-conjugated anti-rabbit IgG and Alexa Fluor 568-conjugated phalloidin were obtained from Molecular Probes. An Enhanced Chemiluminescence kit was purchased from Amersham Biosciences. Complete Mini, a protease inhibitor mixture containing (p-amidinophenyl)methanesulfonyl fluoride, aprotinin, bestatin, calpain inhibitor 1, calpain inhibitor II, chymostatin, E-64, hirudin, leupeptin, α2-macroglobulin, Pefabloc SC, pepstatin, phenylmethylsulfonyl fluoride, 1-chloro-3-tosylamido-7-amino-2-heptanone-HCl, 1-1-tosylamido-2-phenylethyl chloromethyl ketone, trypsin inhibitor, egg white, and soybean, was purchased from Roche Applied Science.

**Immunoprecipitation and in Vitro Kinase Assay—** Membrane preparation from adult Wistar rat cerebella, immunoprecipitation with anti-ganglioside GD3 antibody, and an *in vitro* kinase assay were performed as described previously (11). Briefly, the membrane fractions from the cerebella were solubilized in a lysis buffer (1% Tx, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM Na$_3$VO$_4$, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml pepstatin A) at 4 °C for 20 min. The supernatants were incubated with R24 and precipitated with protein G-Sepharose. Following immunoprecipitation, the *in vitro* kinase reaction was started by the addition of the 5 μCi of [γ-32P]ATP (3,000 Ci/mmol; PerkinElmer Life Sciences). Phosphorylation was stopped by the addition of Laemmli sample buffer, and the samples were subjected to SDS-PAGE followed by autoradiography. In a immunoprecipitation experiment, following the kinase reaction, the samples were boiled for 5 min in the lysis buffer with 1% SDS, diluted 10-fold with the lysis buffer, and then reimmunoprecipitated with anti-\( G_\alpha_o \) (GC/2).

**Expression of \( G_\alpha_o \) in Chinese Hamster Ovary (CHO) Cells—** CHO cells or C3H cells (CHO stable transfectants expressing ganglioside GD3 synthase) (14) were transfected transiently with the pcDNA-1 plasmid containing the rat \( G_\alpha_o \) gene (provided by Dr. K. Okamoto, RIKEN, Saitama, Japan) using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer’s instructions. Forty-eight hours after transfection, immunoprecipitation with R24 was performed as described previously (11). \( G_\alpha_o \) was not endogenously expressed in the CHO cells.

**Treatment with GTPγS or GDPβS—** The membrane fraction of the rat cerebellum was prepared as described previously (11). Membrane aliquots (5 mg of protein) were suspended in 100 μl of the reaction buffer (20 mM Tris-HCl, 1 mM dithiothreitol, 250 mM (NH$_4$)$_2$SO$_4$, and Complete Mini) containing 5 mM GTPγS and 5 mM MgCl$_2$, or 5 mM GDPβS. After brief sonication and incubation for 30 min at 30 °C, the suspension was centrifuged at 11,500 × g for 10 min at 4 °C. The pellets as membrane fractions treated with GTPγS or GDPβS were solubilized in lysis buffer B (2% octyl glucoside, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM Na$_3$VO$_4$, 1 mM EGTA, and Complete Mini) at 4 °C. Immunoprecipitation manipulations were performed as described above using protein G-Sepharose and anti-\( G_\alpha_o \) antibody (A2), followed by the detection of \( G_\alpha_o \)-binding \( G_\beta \) or GAP-43 by immunoblot analysis.

**Sucrose Density Gradient Analysis—** For treatment with mastoparan (Wako Chemicals) or mouse recombinant chemokine stromal cell-derived factor (SDF)-1α (DAKO), rat cerebellar membranes (5 mg of protein) were incubated with 10 μM mastoparan or 100 μg/ml SDF-1α in 100 μl of reaction buffer B containing 100 μM GDP, 0.1 μM GTPγS, and 10 mM MgCl$_2$ at 30 °C for 30 min. For comparison between a developing cerebellum (postnatal day 7) and an adult cerebellum, the membrane fractions of each cerebellum were prepared. In *vitro* pertussis toxin (PTX)-mediated ADP-ribosylation of \( G_\alpha \) proteins in the membrane fractions (5 mg of protein) isolated from a developing cerebellum was performed with nonradioactive NAD using a standard technique (15). Then sucrose gradient analysis with Tx was performed as described previously (11). Briefly, the pellets as membrane fractions were homogenized using a Teflon glass homogenizer in 2 ml of TNE/Tx buffer (0.5% Tx, 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 mM EGTA). The sucrose content of the homogenate was then adjusted to 40% by adding 80% sucrose. A linear sucrose gradient (5–30%) in 6 ml of TNE without Tx was layered over the lysate. The gradients were centrifuged for 17 h at 200,000 × g at 4 °C using a Hitachi RPS40T rotor. Ten fractions were collected from the top of the gradient, followed by immunoblot analysis using various antibodies. The distribution of GD3 in the gradient fractions was observed by dot blotting with R24.

\[ ^{35}S \text{GTP} \gamma \text{S Binding Assay—} \] Rat cerebellar membranes (postnatal day 7, 100 μg of protein) were resuspended in 50 μl of 50 mM Tris-HCl, pH 7.6, 2 mM EDTA, 100 mM NaCl, 5 mM MgCl$_2$, 1 μM GDP, Complete Mini, and 50 nm \([^{35}S] \text{GTP} \gamma \text{S} \) (2000 Ci/mmol) and incubated in the presence or absence of the indicated concentrations of mastoparan or SDF-1α at 30 °C.
**Gαo Translocation to Rafts in Cerebellar Neurons**

After 30 min, the reaction was terminated by adding 500 μl of immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM MgCl₂, 0.5% Nonidet P-40, and Complete Mini). The extracts were incubated with 10 μl of protein G-Sepharose and centrifuged after 30 min to remove nonspecifically bound proteins. The extracts were incubated with anti-Gαo antibody (A2) for 1 h at 4 °C. The immune complex was then incubated with 10 μl of protein G-Sepharose, and the complexes were collected and washed three times in immunoprecipitation buffer. [35S]GTPγS binding in the immunoprecipitates was quantified by scintillation counting.

**Growth Cone Preparation**—The neuronal growth cones of the rat cerebellum (postnatal day 7) were prepared according to the method of Pfenniger et al. (16) with minor modifications. Briefly, rat cerebella were homogenized at 4 °C with six passes in a glass Teflon homogenizer in five volumes of 0.32M sucrose. After centrifugation at 100,000 g for 10 min, the supernatant was layered over a step gradient of sucrose at 0.75 and 1.0M. The gradient was centrifuged at 250,000 g for 1 h at 4 °C. The immune complex was then incubated with 10 μl of protein G-Sepharose, and the complexes were collected and washed three times in immunoprecipitation buffer. [35S]GTPγS binding in the immunoprecipitates was quantified by scintillation counting.

**Primary Culture**—Rat cerebellar granule neurons were cultured according to the method of Levi et al. (17) with some modifications. Briefly, cerebella were dissected from 7-day-old rats, and cerebellar neurons were prepared using a dissociation solution (Sumitomo Bakelite Co., Ltd.). The dissociated cells were plated in a poly-d-lysine-coated chamber slide (8-well; Becton Dickinson Labware) at a density of 3.0 × 10⁵ cells/well containing 0.5 ml of neurobasal medium (Invitrogen) supplemented with 25 mM KCl, 2 mM glutamine, and B27 supplement (Invitrogen). After centrifugation at 100,000 g for 30 min, the pellets were used as growth cone samples.

**Growth Cone Collapse Assay**—Cells cultured for 24 h were exposed to 10 μM mastoparan or 100 ng/ml SDF-1α and observed for their growth cone morphology by microscopy. For immunofluorescence analysis, after the treatment with mastoparan and SDF-1α for 5 min, the cells were fixed in 2% paraformaldehyde with 0.05% Tx for 20 min and incubated with an anti-neurofilament antibody for 1 h. The cells were incubated with the Alexa Fluor 488-labeled secondary antibody and Alexa Fluor 568-labeled phalloidin. Parts of the growth cones were stained red because of the high amount of actin filaments (18). The images were captured at 400× using a Zeiss laser-scanning confocal imaging system (LSM510). The areas observed for their growth cone morphology by microscopy. For immunofluorescence analysis, after the treatment with mastoparan and SDF-1α for 5 min, the cells were fixed in 2% paraformaldehyde with 0.05% Tx for 20 min and incubated with an anti-neurofilament antibody for 1 h. The cells were incubated with the Alexa Fluor 488-labeled secondary antibody and Alexa Fluor 568-labeled phalloidin. Parts of the growth cones were stained red because of the high amount of actin filaments (18). The images were captured at 400× using a Zeiss laser-scanning confocal imaging system (LSM510). The areas stained red were extracted with the image processing software Photoshop® (Adobe), and quantification was performed using the NIH Image program V1.62. The growth cone collapse percentage was calculated between the control and treated cells from three fields (0.2 mm²) selected randomly in three independent experiments.

**RESULTS**

**Antiganglioside GD3 Antibody (R24) Precipitates α-Subunit of Trimeric G Protein, Gαo (Gαo)—**The immunoprecipitates obtained using R24 from the Tx extract of rat cerebellar membranes were analyzed for the presence of protein kinase activity. An in vitro kinase reaction resulted in the phosphorylation of several proteins of 40, 53, 56, and 80 kDa, as determined by SDS-PAGE (Fig. 1, lane 1). We previously identified p53/56 as two isoforms of the Src family kinase Lyn by sequential immunoprecipitation with R24 and the anti-Lyn antibody (11). In this study, the same method was used in p40 identification. Briefly, the in vitro kinase assay was conducted, after which the immune complexes were disrupted by boiling in SDS-containing buffer and subjected to a second immunoprecipitation with the anti-Gαo antibody (Fig. 1, lane 3). As a result, the anti-Gαo antibody specifically precipitated p40 in the reimmunoprecipitation experiments. This result suggests that there is a specific association of Gαo with GD3 in the rat brain cell membrane.

**Gαo is Associated with GD3 in cDNA Expression System—**The association of Gαo with GD3 was confirmed using a cDNA expression system in CHO cells. GM3 is the only ganglioside synthesized in CHO cells and is an enzymatic substrate of GD3 synthase. We previously established a CHO cell line, namely, CST, which constitutively expresses GD3 synthase and demonstrated that R24 co-immunoprecipitated Lyn from CST cells expressing Lyn (11). Both CHO and CST cells were transiently transfected with an expression plasmid carrying the Gαo gene. Gαo was co-immunoprecipitated by R24 (Fig. 2, lanes 2 and 5) from the CST cells but not from the CHO cells. This finding confirms the interaction of GD3 with Gαo. In addition, the specific binding of R24 to GD3 was verified.

**Gαo is Abundant in DRMs in Developing Cerebellum but Not in Adult Cerebellum—**GSLs form microdomains called “lipid rafts” or “rafts” in cellular membranes. Lyn is associated with lipid rafts in several types of cell including cerebellar neurons (11, 19, 20). To investigate whether Gαo exists in lipid rafts, we isolated lipid rafts by treating cerebellar membranes with cold Tx and separating DRMs by sucrose gradient centrifugation. Go...
is a heterotrimeric GTP-binding protein composed of α, β, and γ subunits. The activation of G proteins was fundamentally initiated by the exchange of GDP by GTP bound to the α-subunits and the dissociation of the heterotrimer to an α-monomer and a βγ dimer. In this study, we examined the distribution of both monomeric and trimeric forms of Goα, on a sucrose gradient and compared the distribution between the membranes of a developing cerebellum (postnatal day 7) and an adult cerebellum. As shown in Fig. 3A, anti-Goα antibody precipitated Gβ from the adult cerebellum (lane 6, the band is indicated by the upper arrow with the solid line) but not from the developing cerebellum (lane 4). This indicates that the level of the monomeric forms of Goα was higher in the developing cerebellum than in the adult cerebellum. Sucrose density gradient analysis (Fig. 4B) showed that Goα predominantly existed in the DRM fractions (lanes 3–5) in the postnatal developing cerebellum (62% of total in DRM fractions). In contrast, few Goα molecules were present in the DRM fraction in the adult cerebellum (2% of total in DRM fractions). Most Gβ molecules existed in the non-raft fraction (lanes 7–10) in both the developing and adult cerebella (80 and 89% of total, in non-DRM fraction, respectively). The presence of Lyn in the DRM fractions and the exclusion of transferrin receptor, a nonraft marker protein, from the DRM fractions, confirmed the quality of the fractionation. The levels of Goα and Gβ proteins as determined by immunoblotting were comparable between the developing and adult cerebellar membranes (data not shown). PTX ADP ribosylates several G proteins including Go and stabilizes the G proteins in their heterotrimeric forms (15). In the presence of PTX, most Goα molecules were excluded from the DRM fractions in the developing cerebellum (92% of total in non-DRM fractions) (Fig. 3C). These observations suggest that Goα undergoes translocation to the lipid rafts in the early stage of cerebellar development in an activation-dependent manner. The pretreatment of the cholesterol-depleting agent, methyl-β-cyclodextrin (MβCD) significantly reduced Goα in the DRM fraction, suggesting that cholesterol depletion by MβCD disrupts the lipid rafts of the cerebellum (Fig. 3C).

**Goα Translocation to Rafts in Cerebellar Neurons**

**A.** Immunoprecipitation using R24 (membranes, respectively. The lum. As shown in Fig. 3

**B.** Confirmation of association between GD3 and Goα using CHO transfectants. Immunoprecipitation was investigated with anti-GD3 antibody (R24) using CHO transfectants expressing GD3 synthase and Goα. Immunoprecipitates using R24 (lanes 2, 3, and 5) or control mouse IgG (lane 4) from CHO (expressing ganglioside GM3) transfectants with Goα cDNA (lane 2), CST (expressing GD3) with Goα cDNA (lanes 4 and 5) and CST transfectants with vector only (lane 3). Lane 1 shows the lysate of CST transfectants with the Goα gene. IB, immunoblot for the molecules indicated.

**C.** Localization of Goα in sucrose density gradient in developing and adult cerebella. A, immunoprecipitation of developing (postnatal day 7) and adult cerebellar membranes with anti-Goα antibody. Immunoprecipitates were subjected to SDS-PAGE and immunoblotted using an anti-Gβ antibody. Precipitates were obtained using anti-Goα antibody (lanes 4 and 6) or control mouse IgG (lanes 3 and 5) from developing (lanes 3 and 4) and adult (lanes 5 and 6) cerebella. Lanes 1 and 2 show the lysate of the developing and adult cerebellar membranes, respectively. The top arrow with the solid line indicates the bands of Gβ proteins, and the bottom arrow with the dotted line indicates the non-specific binding protein to IgG or protein G-Sepharose during immunoprecipitation. B, the membrane fractions from a developing cerebellum (postnatal day 7) and an adult cerebellum were solubilized with TX, subjected to sucrose gradient centrifugation, and fractionated from the top to the bottom (fractions 1–5). Goα, Gβ, Lyn, and transferrin receptor (Tfr) in each fraction were detected by immunoblotting. Lanes 2–5 and 7–10 correspond to the DRM and non-DRM fractions, respectively. C, postnatal day 7 cerebellar membranes were treated with PTX or MβCD as described under “Experimental Procedures,” followed by solubilization and separation of the DRM and non-DRM fractions. Goα in each fraction was detected by immunoblotting (IB).
SDF-1 and Gαo,2 did not show apparent changes in their localization patterns between these treatments. These results suggest the lateral translocation of Gαo, on the membrane surface according to the state of activity of Gαo. Monomeric Gαo, is solely localized to the lipid rafts, whereas trimeric Gaβγ, may exist in adjacent regions of the membrane.

**Mastoparan and Chemokine SDF-1α Induce Recruitment of Gαo, to Lipid Rafts**—Mastoparan is a cationic amphiphilic tetradecapeptide isolated from wasp venom and has been reported to stimulate several G proteins including Gs in a manner similar to that of the G protein-coupled receptor (22). SDF-1α, which induces leukocyte chemotaxis, triggers the chemotraction of cerebellar granule cells (23). SDF-1α is the biological ligand for CXCR4, a G protein-coupled receptor. Both SDF-1α and CXCR4 are expressed in the developing cerebellum (24, 25). Mastoparan and SDF-1α significantly stimulated the Gαo-, specific binding of GTPγS in the cerebellar membranes (Fig. 5A). After the exposure of the cerebellar membranes to mastoparan or SDF-1α, DRMs were isolated as described under “Experimental Procedures.” As shown in Fig. 5B, mastoparan and SDF-1α induced the shift of Gαo, into the DRM fractions (44 and 55% of total in DRM fractions, respectively; 9% of total in the DRMs for control samples). Almost all of the β-subunits were localized in the non-DRM fractions, and there were no obvious differences between the control and mastoparan- and SDF-1α-treated cerebella (86, 86, and 90% of total in non-DRM fractions, respectively). CXCR4 was mainly localized in the non-raft fractions (data not shown). These results indicate that Gαo, translocates to the lipid rafts after activation by G protein-coupled receptor in the surrounding membrane region.

**Concentration of Gαo, in Cerebellar Growth Cones**—Growth cones are specialized neuronal compartments that are transiently generated at the tips of growing neurites. They play important roles in neurite outgrowth and the formation of neural circuits in a developing brain. Subcellular fractionation showed that the isolated growth cones (Fig. 6A, lane 1) contained higher levels of Gαo, and Lyn than the whole cerebellum (Fig. 6A, lane 2). GAP-43, a neuronal-specific protein, is particularly associated with growth cones (22). The abundance of GAP-43 in growth cones confirms the quality of the fractionation. The immunoprecipitates obtained with the anti-GAP-43 antibody from the Tx extract of the rat cerebellar membranes were analyzed. Gαo, was co-immunoprecipitated only after the treatment with GTPγS, not with GDPβS (Fig. 6B). However, Gαo-1 and Gαo-2 were not co-immunoprecipitated after the treatment with GTPγS. GAP-43 was localized in the DRM fractions in the cerebellum regardless of G protein activity, in both GTPγS- and GDPβS-treated membranes, consistent with previous reports (Fig. 6C) (26, 27). These observations suggest that the activation-dependent interaction of Gαo, with the lipid rafts occurs in the growth cones.

**Lipid Raft-dependent Translocation of RhoA to Membrane**

**Fraction by Treatments with SDF-1α and Mastoparan**—The small GTPase RhoA is one of the regulators of growth cone guidance in various neurons (28). For example, SDF-1α activates RhoA and represses axon formation of cerebellar granule neurons in a RhoA-dependent manner (23). It is known that the translocation of RhoA from cytosol to the plasma membrane occurs when RhoA is activated (29). Therefore, we investigated the role of the lipid rafts in RhoA translocation to the plasma membrane by treatment with SDF-1α. In Fig. 7, membrane-bound RhoA was detected by treatment with SDF-1α or mastoparan within 5 min. In the presence of PTX, the membrane-bound RhoA was not detected, suggesting that RhoA activation by SDF-1α is dependent on PTX-sensitive Gαo, heterotrimeric G proteins. Furthermore, the membrane-bound RhoA was not also detected in the presence of MJBCD, suggesting that RhoA activation by SDF-1α is dependent on the lipid rafts. In this study, we found that Gαo, undergoes translocation to the DRM raft fraction by treatment with SDF-1α or GTPγS and association with GAP-43, a lipid raft marker, by treatment with GTPγS. On the other hand, Gαo-1 and Gαo-2 do not undergo translocation to DRM by treatment with GTPγS and association with GAP-43 by treatment with GTPγS. These observations suggest that RhoA translocation to the plasma membrane by treatment with SDF-1α is probably mediated by activation-dependent Gαo, translocation to the lipid rafts in cerebellar granule neurons.

**Modulation of Growth Cones by Mastoparan and SDF-1α**—To investigate the effects of mastoparan and SDF-1α on growth cones, time lapse microscopy was used to observe the responses of growth cones to treatment with mastoparan or SDF-1α.
Within 5 min of the addition of mastoparan or SDF-1α to the culture media at 10 μM or 100 ng/ml, respectively, most of the growth cones of the cerebellar primary cultures stopped extending, and their lamellipodia collapsed (Fig. 8A). The collapse of the growth cones is a response against repulsive factors expressed on the surface of cellular targets and along migratory pathways for the inhibitory regulation of growth cone motility (30, 31). To confirm growth cone collapse by Go activation, cerebellar cultures were treated with mastoparan or SDF-1α for 5 min, fixed, and stained with an anti-neurofilament antibody and phalloidin to detect F-actin. As shown in Fig. 8B (panel a), intense staining of F-actin in several growth cone regions was observed because F-actin is concentrated, and neurofilaments are absent in the growth cones (red areas indicated by arrows). The area of the red-stained growth cone regions was measured, and both the mastoparan and SDF-1α treatments for 5 min induced a significant decrease in the area of the growth cone regions (Fig. 8B, panels c and d) compared with that of the nontreated cells (Fig. 8B, panel b). Growth cone collapse was prevented by pretreatment with PTX. These results suggest that the activation of PTX-sensitive G proteins regulates the growth cone motility. Furthermore, the pretreatment of cerebellar cultures with the cholesterol-depleting and raft-disrupting agent, MβCD, significantly reduced the rate of growth cone collapse induced by mastoparan and SDF-1α treatments (Fig. 8C). These results imply that the disruption of the lipid rafts by treatment with MβCD interferes with the Go protein activation-induced growth cone collapse.
**DISCUSSION**

In this study, we demonstrated that a monoclonal antibody to ganglioside co-immunoprecipitated $G_{\alpha_o}$ from the rat cerebellum. We previously reported that the anti-ganglioside antibody also co-immunoprecipitated the glycosylphosphatidylinositol-anchored protein TAG-1, the Src family kinase Lyn, and caveolin. These proteins are raft-associated proteins, and gangliosides are a major lipid component of lipid rafts, suggesting that the anti-ganglioside antibody immunoprecipitates lipid rafts containing $G_{\alpha_o}$.

We observed the signal-dependent translocation of $G_{\alpha_o}$ to the lipid rafts of the cerebellar membranes in this study. What is the mechanism of the translocation of $G_{\alpha_o}$ to lipid rafts? One possible mechanism is a lipid-lipid interaction. The basic forces driving a lipid raft formation are considered to be lipid interactions. The saturated acyl chains and high acyl chain melting temperatures of GSLs mediate GSL clustering in combination with cholesterol, which has the properties of a “liquid-ordered phase.” In contrast, most phospholipids have unsaturated acyl chains, low melting temperatures, and the properties of a liquid phase. Lipid rafts are thought to exist as phase-separated domains in cerebellar membranes. The nature of phospholipids occupying the cytoplasmic side of lipid rafts is unknown; however, they probably also carry mainly saturated fatty acid chains to optimize packing. Phospholipids in the raft fraction of rat cerebellar granule cells are mainly dipalmitoylphosphatidylcholine (32). The linkage of $G_{\alpha_o}$ to the saturated acyl chains by palmitoylation and myristoylation is considered to facilitate $G_{\alpha_o}$ translocation to lipid rafts.

The linkage of $G_{\gamma}$ to prenyl residues, which contain unsaturated bonds, is considered to facilitate exclusion from the lipid rafts (33). In this study, the $G_{\alpha_o}\beta\gamma$ heterotrimer was also excluded from the lipid rafts. This is probably due to the predominant effect of the $G_{\gamma}$ prenyl group over the fatty acids of $G_{\alpha_o}$ on the partitioning of the heterotrimer in the rat cerebellum. Therefore, the signal-dependent translocation of $G_{\alpha_o}$ to the lipid rafts may be a consequence of the dissociation of the heterotrimer. The following are also consistent with this idea: (i) $G_{\alpha_o}$, but not $G_{\beta\gamma}$, binds to the lipid rafts reconstituted in liposomes (34); (ii) myristoylation (G2A) and palmitoylation (C3S) mutants of $G_{\alpha_o}$ are poorly translocated to lipid rafts (35); (iii) many raft proteins are acylated, whereas few raft proteins are prenylated in Madin-Darby canine kidney cells (36); and (iv) test proteins containing double acyl chains (i.e. myristoyl and palmitoyl) are localized in lipid rafts. However, test proteins containing a prenyl tail are excluded from lipid rafts (37).

An alternative mechanism is a protein-protein interaction. GAP-43, a major growth cone protein that can interact with $G_{\alpha_o}$ (38), is modified by dual palmitoylation and localizes to lipid rafts (Fig. 6C) (26). In this study, GAP-43 could be co-immunoprecipitated with $G_{\alpha_o}$ only after GTP$\gamma$S treatment but not GDP$\beta$S treatment (Fig. 6B). Several RGS (regulators of $G$ protein signaling) proteins undergo palmitoylation and localize to lipid rafts (39). These proteins might contribute to the localization of $G_{\alpha_o}$ in the lipid rafts by direct interaction.

In contrast to our findings, $G_{\beta}$ has been detected in the raft fraction by several groups (39). $G_{\alpha_o}$, but not $G_{\beta}$, is abundant in chicken gizzard caveolae (40). Almost all $G_{\beta}$ is absent in the lipid rafts of Madin-Darby canine kidney cells (36). Therefore, the difference in the distribution of $G_{\beta}$ might depend on the cell type. We cannot rule out the possibility that $G_{\beta}\gamma$ binds to lipid rafts at a low affinity, and this interaction is susceptible to disruption by Tx.
In this study, we demonstrated that activation-dependent Goα translocation to DRM fraction is involved in SDF-1α-induced growth cone collapse. However, raft disruption by cholesterol depletion with MβCD partly prevented mastoparan- and SDF-1α-induced growth cone collapse (Fig. 8). Therefore, we can deny a possible presence of raft-independent signaling pathway of SDF-1α-induced growth cone collapse. We applied MβCD at 0.1%, which is lower than those used in other studies (41, 42), because the growth cones of cerebellar granule neurons are sensitive to cholesterol depletion. The disruption of lipid rafts by various approaches targeting cholesterol or GSLs selectively abolishes several signal-dependent growth cone attraction and repulsion processes. Raft disruption blocks semaphorin-3A-induced growth cone repulsion, inhibition, and collapse in Xenopus neurons (41). In differentiated PC12 cells, the recruitment of Pyk2/Cbl to lipid rafts contributes to the elongation of axons following growth factor stimulation (43). Growth cone migration mediated by L1 and N-cadherin is inhibited after raft disruption induced by microscale chromophore-assisted laser inactivation of ganglioside GM1 or by pharmacological treatments that deplete cholesterol or sphingolipids in dorsal root ganglion cells and cerebellar granule cells (44). These findings indicate that ganglioside-rich lipid rafts play an important role in the signaling cascade of modulating the growth cone movement.

The heterotrimeric G protein Goα is highly abundant in the mammalian nervous system (45). In growth cone membranes, Goα makes up 10% of the membrane proteins. Despite these interesting properties, the physiological roles of Goα in the nervous system have not been identified, and there is little knowledge of effector systems. The translocation of Goα to lipid rafts may promote Goα association with putative effector enzymes that are presumably abundant in growth cone rafts. A stimulus-dependent translocation of the heterotrimeric G protein has also been reported in the outer segments of the rod photoreceptor. The G protein transducin and its effector cGMP-phosphodiesterase undergo translocation to lipid rafts upon illumination (46, 47). The localization of signaling molecules in such a small area may markedly enhance the efficiency of signal transmission. The agonist stimulation of δ-opioid receptors increases Goα functional activity much more efficiently in lipid rafts than in the bulk of plasma membranes (48). Thus, gangliosides might function as platforms on neuronal membranes for the appropriate coupling of activated Goα to the effector system. Further studies are needed to elucidate the mechanism of Goα downstream signaling in lipid rafts.

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