Involvement of Gangliosides in Glycosylphosphatidylinositol-anchored Neuronal Cell Adhesion Molecule TAG-1 Signaling in Lipid Rafts*

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The association of ganglioside GD3 with TAG-1, a glycosylphosphatidylinositol-anchored neuronal cell adhesion molecule, was examined by coimmunoprecipitation experiments. Previously, we have shown that the anti-ganglioside GD3 antibody (R24) immunoprecipitated the Src family kinase Lyn from the rat cerebellum, and R24 treatment of primary cerebellar cultures induced Lyn activation and rapid tyrosine phosphorylation of an 80-kDa protein (p80). We now report that R24 coimmunoprecipitates a 135-kDa protein (p135) from primary cerebellar cultures. Treatment with phosphatidylinositol-specific phospholipase C revealed that p135 was glycosylphosphatidylinositol-anchored to the membrane. It was identified as TAG-1 by sequential immunoprecipitation with an anti-TAG-1 antibody. Antibody-mediated cross-linking of TAG-1 induced Lyn activation and rapid tyrosine phosphorylation of p80. Selective inhibitor for Src family kinases reduced the tyrosine phosphorylation of p80. Sucrose density gradient analysis revealed that the TAG-1 and tyrosine-phosphorylated p80 in cerebellar cultures were present in the lipid raft fraction. These data show that TAG-1 transduces signals via Lyn to p80 in the lipid rafts of the cerebellum. Furthermore, degradation of cell-surface glycosphingolipids by endoglycosidase H induced an alteration of TAG-1 distribution in lipid rafts. In addition, the TAG-1-mediated Lyn activation and tyrosine phosphorylation of p80. These observations suggest that glycosphingolipids are involved in TAG-1-mediated signaling in lipid rafts.

Gangliosides, sialic acid-containing glycosphingolipids (GSLs),¹ 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 (1–4). In the nervous system, where gangliosides are particularly abundant, the species and amounts of gangliosides undergo profound changes during development, suggesting that they may play fundamental roles in this process (5). The accumulation of gangliosides within the neurons in ganglioside storage diseases results in extensive neurite outgrowth (6). Exogenously administered gangliosides accelerate the regeneration of neurons in the central nervous system in vivo after lesioning (7). The addition of exogenous gangliosides to primary cultures of neurons and neuroblastoma cells in vitro stimulates cellular differentiation with concomitant neurite sprouting and extension (8–10). Glucosylceramide synthesis, the first glycosylation step of GSL synthesis, is required for axonal growth in hippocampal neurons (11) and for embryonic development (12). Transfection of the ganglioside GD3 synthase cDNA into neuroblastoma cells induces their cholinergic differentiation and neurite sprouting (13). Finally, mice lacking complex gangliosides exhibit axonal degeneration (14). These data show that gangliosides are involved in neural cell differentiation and brain development. However, the molecular mechanisms underlying the ganglioside-dependent neural functions remain obscure.

GSLs are known to exist in clusters and form microdomains containing cholesterol at the surface of the plasma membrane. These microdomains are variously referred to as lipid rafts, detergent-resistant membranes, detergent-insoluble glycosphingolipid-enriched domains, or caveolae membranes (15–20). The GSL microdomains have been implicated in signal transduction because a variety of signaling molecules, such as the Src family kinases, are associated with them. However, the precise functions of GSLs in the microdomains remain to be explored.

We investigated the association of gangliosides with specific proteins in the central nervous system by coimmunoprecipitation experiments using an anti-ganglioside antibody. We previously demonstrated that anti-ganglioside GD3 antibody

lipase C; NMDA, N-methyl-D-aspartate; FITC, fluorescein isothiocyanate; EGCase, endoglycosidase; MT8, 3-[4-(5-diethylthiazol-2-yl)-2,5-diphenylytetracilazolium bromide]; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; MES, 4-morpholinethanesulfonic acid; PBS, phosphate-buffered saline; RSA, bovine serum albumin; CHO, Chinese hamster ovary, PCR, polymerase chain reaction.

¹ The nomenclature for gangliosides follows the system of Svensson-Holm (79).
Gangliosides and TAG-1 Signaling in Lipid Rafts

(R24) coimmunoprecipitates the Src family kinase Lyn from the rat cerebellum (21). R24 treatment of primary cerebellar cultures induced Lyn activation and rapid tyrosine phosphorylation of an 80-kDa protein (p80). Furthermore, sucrose density gradient analysis showed that Lyn, in both cerebella and CHO cells transfected with Lyn cDNA, was detected in the lipid raft fraction. R24 immunoprecipitated caveolin, a caveolar marker protein, from CHO cells transfected with GD3 synthase cDNA (22). These observations suggest that GSL may regulate the functions of Lyn in lipid rafts.

In the present study, we attempted to identify the cell-surface molecules involved in Lyn signaling because Lyn is a nonreceptor-type kinase, and we found that R24 coimmunoprecipitates TAG-1, a glycosylphosphatidylinositol (GPI)-anchored neuronal cell adhesion molecule and that the antibody-mediated cross-linking of TAG-1 induced Lyn activation and rapid tyrosine phosphorylation of p80. Furthermore, we investigated the roles of GSLs in TAG-1 signaling.

EXPERIMENTAL PROCEDURES

Materials—Endoglycosidase II and activator II (EGCase II ACT) and the rat contacrin-specific phospholipase C (PI-PLC) were purchased from Takara Biomedicals (Osaka, Japan) and Funakoshi (Tokyo, Japan). Alternatively, OptiPrep was purchased from Nycomed Pharma (Oslo, Norway). Triton X-100 and EGTA were purchased from Sigma. PPI, PP2, and PP3 were purchased from Alexis Biochemicals (San Diego, CA) and Calbiochem, respectively. The 3.1C12 mouse monoclonal IgG for rat TAG-1 was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health, and maintained by the Department of Biological Sciences, University of Iowa. The mouse IgG3 anti-ganglioside GD3 monoclonal antibody, R24, anti-Lyn (Lyn8) monoclonal antibody, and anti-NCAM polyclonal antibody were obtained from Signet Laboratories (Dedham, MA), Wako Chemicals (Osaka, Japan), and Affinity Research Products (Mamhead, UK), respectively. The anti-caveolin polyclonal antibody, horseradish peroxidase-conjugated anti-phosphotyrosine antibody (PY20), and fluorescein isothiocyanate caveolin polyclonal antibody, horseradish peroxidase-conjugated anti-Affinity Research Products (Mamhead, UK), respectively. The anti-Lyn antibody and precipitated with protein G-Sepharose. The

Anti-contactin/F3 serum was generated against recombinant proteins expressed in E. coli using the pET vector system from rat contactin/F3 encoding immunoglobulin domains I–II (amino acids 22–255). Based on the sequence information of rat contactin/F3 cDNA, two degenerate oligonucleotides were synthesized. The sequences of the 5’- and 3’-primers were 5’-TTACATTACGAGGACCCACGACTCTTCTATTG3’ and 5’-TAGGAGCCAGCGTGACGACGACACACC-3’. For PCR amplification, first strand cDNA was synthesized from the total RNA of rat brain. Thirty cycles (94°C for 10 s, 55°C for 30 s, and 72°C for 45 s) were run. The PCR product, around 1 mg of the protein were emulsified with complete or incomplete Freund’s adjuvant and used to immunize rabbits.

Metabolic Labeling and Immunoprecipitation—Primary cerebellar cultures were labeled with the EXPRESSTM/S/3 Protein Labeling Mix (PerkinElmer Life Sciences) for 3 h in a methionine-/cysteine-depleted medium, at a final concentration of 0.1 mCi/ml. The cells were subcloned in lysis buffer (1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM Na2VO4, 1 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride) at 4°C for 20 min. The postnuclear supernatants were collected after centrifugation at 14,000 rpm for 3 min. Aliquots containing about 1 mg of the protein were emulsified with complete or incomplete Freund’s adjuvant and used to immunize rabbits.

Antibody-mediated Cross-linking of TAG-1 and Measurement of Lyn Activity in Primary Cerebellar Cultures—Cerebellar cultures of 3 days in vitro in a 35-mm dish were incubated with 20 μg/ml anti-TAG-1 monoclonal antibody (3.1C12) on ice for 30 min in BSA medium (culture medium without fetal bovine serum containing 1% BSA and 20 mM HEPES (pH 7.4). After washing with cold BSA medium, the cells were incubated with 20 μg/ml anti-rabbit IgG and anti-mouse IgG antibodies and then partially purified as inclusion bodies. After gel electrophoresis, the protein were emulsified with complete or incomplete Freund’s adjuvant and used to immunize rabbits.

For cross-linking of ganglioside GD3, cells were incubated with 20 μg/ml R24 on ice for 30 min in BSA medium. After washing with cold BSA medium, the cells were incubated with 20 μg/ml anti-rabbit IgG affinity-purified polyclonal antibody in BSA medium at 37°C for 3 min. For mock cross-linking of NMDA receptors, cells were incubated with 20 μg/ml anti-mouse IgG affinity-purified polyclonal antibody in BSA medium at 37°C for 3 min. For mock cross-linking of TAG-1 or ganglioside GD3, cells were incubated with 20 μg/ml normal mouse IgG on ice for 30 min in BSA medium. After washing with cold BSA medium, the cells were incubated with 50 μg/ml anti-rabbit IgG affinity-purified polyclonal antibody in BSA medium at 37°C for 3 min.
polyclonal antibody (secondary antibody) in BSA medium at 37 °C for 3 min.

Phosphacan-mediated Stimulation and Detection of Tyrosine Phosphorylation of Proteins in Primary Cerebellar Cultures—Two methods were used for the phosphacan-mediated stimulation. For method 1, the cerebellar cultures were incubated with 10 μg/ml phosphacan in BSA medium at 37 °C for 3 min. For method 2, the cerebellar cultures were incubated with 10 μg/ml phosphacan in BSA medium on ice for 30 min. After washing with cold BSA medium, the cells were incubated with a 10-fold concentrated culture supernatant of hybridoma 6B4 (anti-phosphacan) (25) at 37 °C for 5 min. As a negative control experiment, the cells were incubated with the concentrated 6B4 supernatant at 37 °C for 3 min without phosphacan pretreatment.

For the detection of tyrosine phosphorylation of proteins, cells were lysed with lysis buffer and subjected to SDS-PAGE and immunoblotting with the anti-phosphotyrosine antibody. Tyrosine phosphorylation of p80 was quantified by densitometry.

Protein Kinase Activity Coprecipitated with Anti-TAG-1 Antibody or R24—Membrane fractions were prepared from newborn rat cerebellum. The cerebella were homogenized in ice-cold buffer A (0.32 M sucrose, 1 mM Tris-HCl (pH 7.4) and 0.1 mM EDTA) using a Teflon motor-driven glass homogenizer. The homogenate was centrifuged at 900 × g for 1 min. The supernatant was centrifuged at 11,500 × g for 3 min. The resulting pellet of the rat brain or the centrifuged cultured cells was solubilized in this buffer (1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM Na3VO4, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin and 5 μg/ml peptatin A) at 4 °C for 20 min. The supernatants were collected after centrifugation at 14,000 rpm for 3 min. Aliquots (0.5 ml, 750 μg of protein) of the supernatants were precleared with protein G-Sepharose (7.5 μl), then incubated with anti-TAG-1 antibody or R24 (2.5 μg) for 1 h, and precipitated with protein G-Sepharose (7.5 μl). Following immunoprecipitation, the beads were washed three times with lysis buffer, once with kinase buffer (30 mM HEPES (pH 7.5), 10 mM MgCl2, and 2 mM MnCl2), and then resuspended in 20 μl of kinase buffer. The reaction was started by the addition of 5 μCi of [γ-32P]ATP (3,000 Ci/mmol, PerkinElmer Life Sciences), and the samples were incubated for 10 min at room temperature. Phosphorylation was stopped by the addition of the Laemmli sample buffer, and the samples were subjected to SDS-PAGE followed by autoradiography. We used normal mouse IgG, anti-NMDA receptor antibody, and anti-integrin β1, antibody as negative controls.

Density Gradient Analysis—TAG-1 distribution on a density gradient was investigated by three different methods. Sucrose gradient analysis with Triton X-100 was performed according to a method described previously (26). Rat primary cerebellar cultures in a 35-mm dish were homogenized using a Teflon glass homogenizer and sonicated in 2 ml of 50 mM sucrose containing 20 mM Tricine (pH 7.8)). The sucrose content in the homogenate was then adjusted to 45% by the addition of 90% sucrose in MBS (25 mM MES (pH 6.5), 0.15 M NaCl). A linear sucrose gradient (5–35%) in 6 ml of MBS containing 250 mM sodium carbonate was layered over the lysate. The gradients were centrifuged for 16–20 h at 39,000 rpm at 4 °C in a Hitachi RPS40T rotor. 10 × 1 ml fractions were collected from the top of the gradient.

Sucrose gradient analysis with Triton X-100 was performed according to a method described previously (27). The cells were homogenized using a Teflon glass homogenizer and sonicated in 2 ml of 500 mM sodium carbonate at pH 11.0. The sucrose content in the homogenate was then adjusted to 45% by the addition of 90% sucrose in MBS (25 mM MES (pH 6.5), 0.15 M NaCl). A linear sucrose gradient (5–35%) in 6 ml of MBS containing 250 mM sodium carbonate was layered over the lysate. The gradients were centrifuged for 16–20 h at 39,000 rpm at 4 °C in a Hitachi RPS40T rotor. 10 × 1 ml fractions were collected from the top of the gradient.

OptiPrep gradient analysis was performed according to a previously described method with some modifications (28). The cells were homogenized using a Teflon glass homogenizer and a sonicator in 2 ml of buffer A (0.25 M sucrose, 1 mM EDTA, and 20 mM Tricine (pH 7.8)). The OptiPrep content in the homogenate was then adjusted to 25% by the addition of 50% OptiPrep in buffer A. A linear OptiPrep gradient (10–40%) in 6 ml of buffer A was layered over the lysate. The gradients were centrifuged for 16–20 h at 39,000 rpm at 4 °C in a Hitachi RPS40T rotor. 10 × 1 ml fractions were collected from the top of the gradient.

After trichloroacetic acid precipitation of each fraction, the immunoprecipitates were subjected to SDS-PAGE and autoradiography. R24 coimmunoprecipitated two proteins of 135 and 16 kDa, as revealed by SDS-PAGE (Fig. 1). This observation suggests that ganglioside GD3 associates with these two proteins.

Anti-ganglioside GD3 Antibody Coprecipitates p135—The immunoprecipitate obtained with the anti-GD3 antibody (R24) from the Triton X-100 extract of rat primary cerebellar cultures metabolically labeled with [35S]methionine was analyzed by autoradiography. R24 immunoprecipitated two proteins of 135 and 16 kDa, as revealed by SDS-PAGE (Fig. 1). This observation suggests that ganglioside GD3 associates with these two proteins.

Identification of p135 as the GPI-anchored Neuronal Cell Adhesion Molecule, TAG-1—[35S]surface labeling and immunoprecipitation with R24 were performed to investigate whether or not the 135-kDa protein (p135) was a surface protein. p135, but not the 16-kDa protein, was also coprecipitated with R24 from the surface-labeled cells (Fig. 2A). The appearance of another smaller band was possibly due to the degradation of p135 by trypsin during cell culture, since the cell surface was labeled 12 h after trypsinization.

p135 was not coprecipitated with R24 after treatment of the surface-labeled cells with PI-PLC (Fig. 2B, lane 1). PI-PLC treatment released p135 from the cell surface (Fig. 2B, lane 2) into the culture medium (Fig. 2B, lane 3), indicating that p135 is a GPI-anchored protein.

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Gangliosides and TAG-1 Signaling in Lipid Rafts

The possible increase in tyrosine phosphorylation of cellular proteins as a result of antibody-mediated cross-linking of TAG-1 was investigated. After SDS-PAGE, phosphotyrosines were detected by anti-phosphotyrosine immunoblotting. Treatment with the secondary antibody induced tyrosine phospho-
ylation of several proteins, including the prominent phospho-
ylation of p80 (Fig. 5A). The phosphorylation peaked at 2 min and returned to the control level by 20 min. Treatment with the anti-TAG-1 monoclonal antibody only at 37 °C, even at 100 µg/ml, resulted in no change in tyrosine phosphorylation of p80 (data not shown). p80 was also phosphorylated by antibody-
mediated cross-linking of ganglioside GD3 (Fig. 5B). Mock cross-linking with normal mouse IgG and antibody-mediated cross-linking of NMDA receptor did not induce increase in Lyn activity (Fig. 4, lanes 6 and 7) and protein tyrosine phosphorylation (Fig. 5D). Phosphacan, a neural tissue-specific chondroitin sulfate proteoglycan, is known to be one of the high affinity ligands of TAG-1 (29). Sequential treatment of cerebellar cultures with phosphacan and anti-phosphacan antibody also induced tyrosine phosphorylation of p80 (Fig. 5C). Furthermore, PP1 and PP2, selective inhibitor for Src family kinases, significantly reduced tyrosine phosphorylation of p80 by antibody-mediated cross-linking of TAG-1 (Fig. 6, lanes 3–6). However, the inactive compound PP3 did not reduce (Fig. 6, lanes 7 and 8). These observations suggest that TAG-1 transduces signals via Lyn to p80 in the rat cerebellum.

Previously, we found that R24 treatment of primary cerebellar cultures induced Lyn activation and tyrosine phosphorylation of p80 and that Lyn was present in the lipid raft fraction of sucrose density gradient analysis (21). Therefore, we investigated whether tyrosine phosphorylation of p80 was detected in the lipid raft fraction. Antibody-mediated cross-linking of TAG-1 or GD3 induced tyrosine phosphorylation of p80 in low density lipid raft fractions 3–5 on sucrose density gradient (Fig. 7, B and C). TAG-1 was also detected in the lipid raft fraction by immunoblotting (Fig. 7D). In contrast, tyrosine phosphorylation of about 110-kDa protein, but not p80, and most cellular proteins were detected in high density sample fractions 7–10 (Fig. 7, A–C and E). These observations suggest that TAG-1 transduces signal via Lyn in lipid rafts of rat cerebellum and that antibody-mediated cross-linking of ganglioside GD3 can mimic the TAG-1 signaling.

Association of Protein Kinase Activity with TAG-1 and Gangliosides GD3—Previously we found that R24 coprecipitated protein kinase activity from the rat cerebellum (21). Therefore, we investigated whether the protein kinase activity was precipitated or not by the anti-TAG-1 monoclonal antibody (Fig. 8A). Proteins of 80, 56, 53, and 40 kDa were phosphorylated as...
revealed by an immune complex kinase assay using R24 from postnatal days 5–21. In addition, a 46-kDa protein was phosphorylated as revealed by an immune complex kinase assay using R24 from postnatal days 5–14. Proteins of 56 and 53 kDa were identified as two isoforms of the Src family kinase Lyn by reimmunoprecipitation with the anti-Lyn antibody (Fig. 8B, lane 3). The 46-kDa protein was also detected by the immune complex kinase assay using the anti-TAG-1 antibody from postnatal days 5–14 at the same level as that of R24. However, the phosphorylation of Lyn and the 80- and 40-kDa proteins was weak in contrast with that by R24. This indicates that association with TAG-1 of Lyn and the 40-kDa and 80-kDa proteins is weak or unstable in comparison to their association with ganglioside GD3. The anti-NMDA receptor antibody and anti-rabbit IgG secondary antibody for 3 min (lanes 3) are shown. Arrow indicates p80.

Density Gradient Analysis of TAG-1—Although GPI-anchored proteins associate with lipid rafts during signal transduction, there is some debate as to whether GPI-anchored proteins associate with lipid rafts during signal transduction, the Triton X-100 extracts were subjected to sucrose density gradient analysis. For the detection of tyrosine phosphorylation of proteins, each fraction was subjected to SDS-PAGE and immunoblotting with anti-phosphotyrosine antibody. A, mock cross-linking; B, TAG-1 cross-linking; C, ganglioside GD3 cross-linking. TAG-1 was detected by immunoblotting (D). The cellular proteins of D were stained with Ponceau S (E). Arrow indicates p80.

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proteins associate with lipid rafts under the steady state (32, 33). Therefore, three different methods of density gradient analysis were performed to investigate whether TAG-1 associates with lipid rafts in cerebellar cultures. Distribution of caveolin in lipid raft fractions for each method is shown in Fig. 9 (upper panels). Most of the TAG-1 was present in the lipid raft fractions 3–5 in sucrose gradients with sodium carbonate (pH 11) and sucrose gradient 5–35%; B, 1% Triton X-100 and sucrose gradient 5–30%; C, Tricine (pH 7.8) and OptiPrep gradient 10–20%. Caveolin of CHO cells was visualized by immunoblotting (upper panel).

Effect of GSL Hydrolysis by EGCase on TAG-1 Signaling—In the present study, anti-GSL antibody coimmunoprecipitated TAG-1, and antibody-mediated cross-linking of GSL mimicked TAG-1 signaling in lipid rafts. What is the role of GSL in TAG-1 signaling? EGCase is an enzyme that specifically hydrolyzes the linkage between carbohydrates and ceramides in GSLs (36). With the aid of an activator protein, it is capable of releasing the carbohydrate moiety of GSLs on the cell surface without affecting other lipids and glycoproteins (37, 38).

Treatment of cerebellar cultures with EGCase and an activator protein greatly reduced the cell-surface ganglioside GD3 content (Fig. 10A) and R24-mediated tyrosine phosphorylation of p80 (Fig. 10C, lane 4). Interestingly, EGCase treatment reduced TAG-1-mediated Lyn activation (Fig. 10B, lanes 3 and 4) and tyrosine phosphorylation of p80 (Fig. 10C, lane 5) with a concomitant neurite retraction (Fig. 11D, panel b). The EGCase effect on TAG-1-mediated tyrosine phosphorylation of p80 was dose- and time-dependent (Fig. 11, A and B) and reversible (Fig. 11C). Hydrolysis of cell-surface GSL by EGCase induces an increase in GSL synthesis de novo for maintaining the cell-surface GSL contents (36). Consistent with this, GD3 expression was restored by removal of EGCase (Fig. 11E, panel c). TAG-1-mediated tyrosine phosphorylation of p80 and neurites are also restored following the removal of EGCase (Fig. 11C, panel c, and D, panel c). EGCase treatment did not decrease...
cell viability as determined by the MTT method under this condition. No change in TAG-1-mediated tyrosine phosphorylation of p80 was observed following treatment with heat-inactivated EGCase (data not shown). Therefore, the inhibition of TAG-1-mediated signaling is probably due to the degradation of cell-surface GSLs. Furthermore, EGCase treatment altered TAG-1 distribution on the OptiPrep gradient to a low density fraction. This suggests that degradation of cell-surface GSLs may affect the properties of lipid rafts. Taken together, these results indicate that GSLs are involved in TAG-1 signaling in lipid rafts.

**DISCUSSION**

**TAG-1 Signaling via Src Family Kinase Lyn in Rat Cerebellum**—In our study, we have shown the association of ganglioside GD3 with GPI-anchored neuronal cell adhesion molecule, TAG-1, and the Src family kinase Lyn by a coimmunoprecipitation technique.

Several GPI-anchored proteins have been implicated in transmembrane signaling via Src family tyrosine kinases (39, 40). In leukocytes, antibodies against a variety of GPI-anchored proteins coimmunoprecipitate Src family kinases. Antibody-mediated cross-linking of GPI-anchored proteins induces transient tyrosine phosphorylation of several substrates and, concomitantly, cell activation (40). Some groups have reported coprecipitation of Src family kinases with GPI-anchored proteins in the nervous system. Src family kinase Fyn is coprecipitated with Thy-1 in the chick brain (41), with contactin/F3 in the mouse cerebellum (42) and with NCAM120 in oligodendrocytes (43). Antibody-mediated cross-linking of F11(chicken contactin/F3) on embryonic chick neuronal cells induces Fyn activation (44). Antibody-mediated cross-linking of contactin/F3 on oligodendrocytes induces Fyn activation (43). In the present study, we found that Lyn can interact with TAG-1 in the rat cerebellum and that antibody-mediated cross-linking of TAG-1 induced Lyn activation in rat primary cerebellar cultures. These observations show that TAG-1 transduces signals via Lyn in the rat cerebellum.

The main site of TAG-1 signaling via Lyn in the cerebellum is probably the granule cells. Indeed, both TAG-1 and Lyn are predominantly expressed in the cerebellar granule cells, as has been shown by in situ hybridization and immunohistochemical studies (30, 31, 45–47). During development, cerebellar granule cells in the external granular layer undergo proliferation, axon outgrowth, migration to the internal granular layer, and formation of synaptic connections (48). Regulation of tyrosine phosphorylation through the Src family kinases is critical in the control of neural development (49). For example, Fyn-deficient mice exhibit impaired myelination (50). It has been shown that Src family kinases are involved in the intracellular signaling pathway for responses by the neural cell adhesion molecules of the immunoglobulin superfamily (51). TAG-1, a GPI-anchored neuronal cell adhesion molecule of the immunoglobulin superfamily, is involved in neurite outgrowth, pathfinding, and fasciculation (52). Therefore, TAG-1 signaling via Lyn might play a role in the development of cerebellar granule cells.

**Isolation of Lipid Rafts by Anti-GSL Antibody—GPI-anchored proteins are restricted to the outer leaflet of the lipid bilayer and are integrated into the membrane by GPI anchors. These lipid anchors have no direct contact with the cytoplasm. Src family kinases anchor onto the inner leaflet via N-terminal lipid modification, palmitoylation, and myristoylation. How do GPI-anchored proteins transduce signals via Src family kinases? This is probably possible via the lipid rafts that are rich in GSLs, cholesterol, GPI-anchored proteins, and signaling molecules such as Src family kinases (53, 54). The basic forces driving lipid raft formation are considered to be lipid interactions, which are weak and transient (16, 55). The saturated acyl chains and high acyl chain-melting temperature of GSLs mediate GSL clustering in combination with cholesterol, which has the properties of a “liquid-ordered phase” with restricted fluidity (16). In contrast, most phospholipids have unsaturated acyl chains, low melting temperature, and the properties of a “liquid phase” with high fluidity. Lipid rafts may exist as phase-separated domains in the membrane. Interactions between the carbohydrates of GSLs by hydrogen bonds is also assumed to contribute to raft formation (19). The linkage of GPI-anchored proteins and Src family kinases to saturated acyl chains is considered to facilitate targeting to lipid rafts. The lipid composition renders lipid rafts and their constituent proteins resistant to solubilization with non-ionic detergents. Thus, lipid rafts can be isolated as low density membrane fractions in sucrose density gradients with detergents. Lipid rafts can be also isolated in OptiPrep gradients without detergents or by immunoprecipitation with the anti-caveolin antibody (17). We found that the anti-GSL antibody coprecipitates TAG-1, Lyn, and caveolin, suggesting that immunoprecipitation with this antibody is an immunoabsorption method for isolating lipid rafts. Consistent with this idea, other anti-GSL antibodies such as anti-ganglioside GM3, anti-ganglioside αGalGd1b, and anti-globotriaosylceramide Glb3 can immunoprecipitate Src family kinases and GPI-anchored proteins (56–59).

**Role of GSL in GPI-anchored Protein Signaling in Lipid Rafts**—A number of studies have examined the influence of cholesterol on the physical properties and signaling of lipid rafts by (i) cholesterol depletion using cyclodextrin or by ox-
Gangliosides and TAG-1 Signaling in Lipid Rafts

dation to cholestenone, (ii) inhibition of cholesterol biosynthesis by compactin or lovastatin, and (iii) using cholesterol-binding agents, such as filipin or nystatin. Cholesterol depletion results in an increase in solubility of GPI-anchored proteins in non-ionic detergents and perturbation of raft-mediated signaling (16, 17, 60–64).

In contrast to cholesterol, only a few studies have examined the influence of GSL, another core lipid of lipid rafts, on the physical properties and signaling in lipid rafts (65, 66). Inhibition of sphingolipid biosynthesis results in an increase in solubility of GPI-anchored proteins in non-ionic detergents (62). Single particle tracking of GPI-anchored proteins and GSL in native membranes showed their transient confinement in patches, which is assumed to represent lipid rafts in vivo (67). The size of the confining domain for the GPI-anchored protein is reduced by treatment with inhibitors of GSL biosynthesis (68). These observations suggest that GSLs can regulate the physical properties of lipid rafts.

What is the role of GSL in raft-mediated signaling? In the present study, we showed that GSL signaling and GPI-anchored protein signaling have many properties in common. (i) Both the anti-ganglioside GD3 antibody (R24) and anti-TAG-1 antibody (3.1C12) coprecipitate protein kinase activity. Proteins of 80 and 40 kDa, and 56/53 kDa:Lyn are phosphorylated as revealed by immune complex kinase assays. (ii) Cross-linking mediated by both R24 and 3.1C12 induces Lyn activation. (iii) Cross-linking mediated by both R24 and 3.1C12 induces rapid tyrosine phosphorylation of p80 in lipid rafts. It was reported that both the anti-ganglioside aGalGD1b antibody and anti-Thy-1 antibody coprecipitate Lyn, as well as phosphoproteins of 80 and 40 kDa. Cross-linking mediated by these antibodies also induces Lyn activation in lipid rafts, rapid tyrosine phosphorylation of proteins including an 80-kDa protein, and calcium flux in rat basophilic leukemia RBL-2H3 cells (58, 69–72). The 80-kDa phosphoprotein as revealed by the immune complex kinase assays in the presence of various GPI-anchored proteins and GSLs transduce signals into the cell using the same signaling pathway. Several groups also reported that antibodies against various GPI-anchored proteins and GSLs coprecipitate protein kinase activity. Proteins of 80 and 40 kDa and 60–53-kDa:Ssrc family kinases are phosphorylated at the tyrosine residue as indicated by the immune complex kinase assays (40, 41, 58, 73–75). GPI-anchored ephrin-A5 also induces tyrosine phosphorylation of an 80-kDa protein in the lipid raft fraction by binding its receptor, and the phosphorylation was abrogated in the presence of a selective inhibitor against Src family kinases and in Ssrc family kinase-deficient mice (76). These observations suggest that p80 may be a membrane protein in lipid rafts and a putative substrate for Src family kinases. Thus, Ssrc family kinases, p80 and 40-kDa protein are sets of signaling components in lipid rafts for both GPI-anchored proteins and GSLs.

We used the GSL-degrading enzyme, EGCase, to investigate the function of GSLs in lipid rafts. EGCase specifically hydrolyzes cell-surface GSLs of intact cells with the assistance of its activator protein (36–38). The application of EGCase to living cells provides a novel approach to determining the physiological roles of endogenous GSLs. In the present study, we showed that EGCase treatment reduced cell-surface GSL and impairs TAG-1-dependent signaling via Lyn. GD3 expression and TAG-1-mediated tyrosine phosphorylation of p80 was restored by removal of EGCase. The inhibition of TAG-1-mediated signaling by EGCase is not due to degradation of TAG-1 by protease contaminants in EGCase, because TAG-1 protein levels were not changed following EGCase treatment (Fig. 12). This is also not due to its cytotoxic effects, because EGCase treatment did not decrease cell viability and the EGCase effect was reversible. Therefore, the inhibition of TAG-1-mediated signaling is probably due to the degradation of GSLs and that cell-surface GSLs are probably necessary for TAG-1 signaling in lipid rafts. A recent report showed that exogenously administered ganglioside, which was incorporated in lipid raft fraction, displaced GPI-anchored proteins from lipid rafts and increased their detergent solubility (77, 78). This finding suggests that GSLs may affect distribution of GPI-anchored proteins in lipid rafts. Taken together, our results suggest that GSLs are involved in GPI-anchored protein signaling in lipid rafts. Further experiments are required to clarify the role of GSLs in signal transduction by GPI-anchored proteins.

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