Tubulin Anchoring to Glycolipid-enriched, Detergent-resistant Domains of the Neuronal Plasma Membrane*

(Received for publication, October 29, 1999, and in revised form, January 20, 2000)

Paola Palestini, Marina Pitto, Gabriella Tedeschi, Anita Ferraretto, Marco Parenti, Joseph Brunner, and Massimo Masserini

From the Department of Experimental, Environmental Medicine and Biotechnologies, Medical School, University of Milano-Bicocca, Hospital S. Gerardo, 20052 Monza, Italy, the Department of Medical Chemistry and Biochemistry and the Institute of Veterinary Physiology and CH-8092 Biochemistry, University of Milano, 20133 Milano, Italy, and the Eidgenössische Technische Hochschule, Zurich, Switzerland

After incubation of intact living cultured rat cerebellar granule cells at 37 °C with a new GM1 ganglioside analog, carrying a diazirine group and labeled with 125I in the ceramide moiety, followed by photoactivation, a relatively small number of radiolabeled proteins were detected in a membrane-enriched fraction. A protein of about 55 kDa with a pI of about 5 carried a large portion of the radioactivity even if incubation and cross-linking were performed at 4 °C and in the presence of inhibitors of endocytosis, suggesting that it is cross-linked at the plasma membrane. Immunoprecipitation and Western blotting experiments showed the positivity of this protein for tubulin. Trypsin treatment of intact cells ruled out the involvement of a plasma membrane surface tubulin. Release of radioactive from cross-linked tubulin after KOH treatment (but not hydroxylamine treatment) suggested that the photoactivated ganglioside reacts with an -ester-linked fatty acid anchor of tubulin. Low buoyancy, detergent-resistant membrane fractions, isolated from cells after incubation with the GM1 analogue and photoactivation, proved their enrichment in endogenous and radioactive GM1 ganglioside, sphingomyelin, cholesterol, signal transduction proteins, and tubulin. It is noteworthy that radioactive tubulin was also detected in this fraction, indicating the presence of tubulin molecules carrying a fatty acid anchor in detergent-resistant, ganglioside-enriched domains of the plasma membrane. Parallel experiments carried out with a phosphatidylycholine analogue, also carrying a diazirine group and labeled with 125I in the fatty acid moiety, showed the specificity of tubulin interaction with GM1. Taken together, these results indicate that some tubulin molecules are associated with a lipid anchor to detergent-resistant glycolipid-enriched domains of the plasma membrane. This novel feature of membrane domains can provide a key for a better understanding of their biological role.

Gangliosides are amphiphilic components of the plasma membrane of vertebrate cells and are known to participate in a number of cell surface-mediated events, such as cell-cell recognition processes, modulation of cell growth and differentiation, receptor function, and membrane-mediated transfer of information (1–5). This wide range of functions is likely attributable to their membrane topology. In fact, they are asymmetrically located in the outer leaflet of the plasma membrane bilayer, with the oligosaccharide moiety exposed toward the external medium and the ceramide portion embedded in the hydrophobic core of the bilayer. Therefore, they are available to interact either with membrane-associated molecules or with external ligands. As a matter of fact, the occurrence of ganglioside-protein interaction has been reported (6–9).

Another peculiar feature of glycolipids is their enrichment—along with sphingomyelin, cholesterol, and a series of functionally related proteins—within discrete plasma membrane domains, the involvement of which in the mechanisms of signal transduction, cell adhesion, and lipid/protein sorting has been postulated (10–12). In particular, among glycolipids, domains are typically enriched in GM1 ganglioside, which is often utilized as a lipid marker of these membrane structures (11, 13).

Owing to their peculiar membrane distribution, interaction of glycolipids with specific proteins of domains is expected. However, despite the reported co-segregation of glycolipids and specific proteins inside domains (14–16), proof of their direct interaction has not been provided, with the exception of caveolin in caveolae (17, 18).

Among the tools utilized to investigate the interaction with membrane-associated proteins, photoactivatable radioactive analogs of lipids, gangliosides included, which are able to covalently cross-link and thus radiolabel neighboring molecules upon illumination, have been repeatedly used (17–23). In the present investigation, we used this approach in order to identify proteins interacting with gangliosides in neurons, in which these glycosphingolipids are particularly abundant (24), but in which the role of domains has been only partially investigated (13). For this purpose, cultured rat cerebellar granule cells were utilized, coupled with the use of a new GM1 ganglioside analog, TID-GM1, carrying a photoactivatable diazirine group and labeled with 125I in the ceramide moiety.
Chemicals—The reagents used (analytical grade) and high performance TLC plates (Kieselgel 60) were purchased from Merck GmbH (Darmstadt, Germany). Modified Eagle’s basal medium; fetal calf serum; trypsin; CAPS; MES; and antibodies against α-β, and acetyl-tubulin, against actin, and against GAP-43 were from Sigma. Antibodies against Fyn were from Transduction Laboratories (Lexington, KY); monoclonal anti-Tau was a kind gift of Dr. A. Matus (Friedrich Miescher Institute, Basel, Switzerland). Rabbit anti-GTP protein α-subunit (Gα) and polyclonal antiserum raised against a synthetic peptide corresponding to amino acids 2–17 of the Gα, was kindly provided by G. Milligan (University of Glasgow, Scotland, United Kingdom). All of the material for the electrophoresis was from Bio-Rad. 125I (IMS-300), 14C-labeled methylated protein standard for electrophoresis, protein A-Sepharose, and autoradiographic films were from Amersham Pharmacia Biotech. GM1 ganglioside was extracted and purified from calf brain (25). Preparation and purification of tritiated GM1, labeled at the 3-position of the long chain base, was accomplished as described (26, 27). Horseradish peroxidase-labeled cholera toxin B subunit was from List Biological (Vandell Way, CA).

Cell Cultures—Granule cells, obtained from the cerebella of 8-day-old Harlan Sprague-Dawley rats (Charles River, Milan, Italy), were prepared and cultured as described (28, 29). Morphological differentiation of granule cells in culture was followed by microscopic examination, and cell viability was monitored with fluorescein diacetate and propidium iodide (30). The experiments were performed with cells cultured for 8 days in vitro. The protein content was determined by the method of Lowry et al. (31).

Chemical Synthesis of Photoactivable, 125I-Labeled GM1 Ganglioside (TID-GM1) and of Photoactivable, 125I-Labeled Phosphatidylcholine (TID-PC)—For the preparation of TID-PC, the procedure previously described (32) has been followed. The final specific radioactivity was 2000 Ci/mmol, and the radiochemical purity, assessed by TLC using different solvent systems followed by autoradiography, was >98%. Samples were photolyzed using a UV lamp (300 W, Jelosyl, Milan, Italy). In order to define the time needed for photolysis of the TID reagents, a 10^-5 M solution of the stannylated, unlabeled photoactivable ganglioside (see above) was irradiated for different periods of time. Photolysis of the diazirine group was monitored spectrophotoscopically, by measuring the disappearance of the characteristic diazirine band at 350 nm (molar extinction coefficient at 350 nm = 300).

Treatment of Granule Cells with TID-GM1 and Cross-linking Experiments—Cells plated in 10-cm dishes were washed with Locke’s solution and incubated for 2 h at 37 °C, or for 4 h at 4 °C, with 2.5 ml of the same solution containing 10^-6 M TID-GM1. After incubation, the cells were washed three times with Locke’s solution at 4 °C, irradiated 5 min on ice with UV light (20), and then incubated with Eagle’s basal medium containing 10% fetal calf serum for 30 min at 37 °C. In some experiments, cell incubation with TID-GM1 at 4 °C was carried out in the presence of 20 μM nocodazole or 100 μM colchicine, added 1 h before the photoactivable ganglioside (34, 35).

In other experiments, after cell incubation with TID-GM1 at 4 °C and irradiation, cells were treated for 5 min at 37 °C with trypsin (0.1% in 2 ml of phosphate-buffered saline solution) (36).

At the end of the treatments, cells were washed twice with Locke’s solution; scraped in 2.5 ml of a solution containing 250 mM sucrose, 0.1 mM EDTA, and 1 mM chymostatin, leupeptin, antiapain, and pepstatin protease inhibitor mixture (37) in 1 mM potassium phosphate buffer, pH 7.4; and finally centrifuged (8000 × g for 10 min). The pellet was homogenized and centrifuged (1000 × g for 10 min) three times in the same buffer, and the pooled supernatants were centrifuged at 100000 × g for 1 h. The pellet obtained, from now on called the “membrane-enriched fraction,” was used for further analysis (38).

Treatment of Granule Cells with TID-PC and Cross-linking Experiments—Cells plated in 10-cm dishes were washed with Locke’s solution and incubated for 2 h at 37 °C, or for 4 h at 4 °C, with 2.5 ml of the same solution containing 10^-6 M TID-PC. After incubation, the cells were washed three times with Locke’s solution at 4 °C, irradiated 5 min on ice with UV light (20), and then incubated with Eagle’s basal medium containing 10% fetal calf serum for 30 min at 37 °C. In some experiments, cell incubation with TID-GM1 at 4 °C was carried out in the presence of 20 μM nocodazole or 100 μM colchicine, added 1 h before the photoactivable ganglioside (34, 35).

2D Gel Electrophoresis of the Membrane-enriched Fractions—The membrane-enriched fractions were subjected to lipid extraction (25). The delipidized pellet was solubilized in...
a proper buffer (39), and 2D gel analysis was performed using the Bio-Rad Mini-protein II 2D system according to the manufacturer’s recommendations, except for the first dimension mixture, which was prepared as described (39). 10% gels were used for the second dimension. Radioactive spots were detected using Phosphorus Imager (Bio-Rad) and then subjected to autoradiography. The total protein pattern was assessed by silver staining. 30 μg (as protein) were used for each sample.

**Immunoprecipitation Experiments**—Aliquots of the membrane-enriched fraction were suspended at 37 °C for 20 min in 500 μl of lysis buffer (25 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% Triton X-100; 0.1% gelatin; and 0.1% chymostatin, leupeptin, antipapain, and pepstatin mixture). The lysate was successively incubated at 4 °C for 2 h with mouse anti-α-, anti-β-, or anti-acetyl-tubulin (1:200) antibodies. Protein A-Sepharose, preincubated with rabbit anti-mouse IgG for 2 h, was added, and the incubation prolonged for additional 2 h at 4 °C. The protein A-Sepharose beads, recovered by centrifugation (800 × g for 5 min, three times) were heated at 100 °C for 5 min in Laemmli buffer containing 1% 2-mercaptoethanol and centrifuged, and the supernatant was utilized for 10% SDS-polyacrylamide gel electrophoresis, followed by autoradiography.

**Preparation and Characterization of Detergent-resistant Membrane Fraction (DRF)**—Cells (60 × 10⁶), some after incubation with TID-GM1 or with TID-PC and illumination, were harvested in Locke’s solution and incubated in 2 ml of 1% Triton X-100 in 25 mM MES buffer, pH 6.5, containing 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 75 units/ml aprotinin, for 30 min on ice. The cell lysate was subjected to discontinuous sucrose density gradient centrifugation, for the separation of low density DRF, as described (40). 1.2-m fractions were collected from the gradient and subjected to radioactivity counting. Fraction 4 (DRF) and high density membrane fraction (HDF) (pool of fractions 8–10) were assayed for lipid and protein content, as follows.

**Lipids**—DRF and HDF were dialyzed against distilled water at 4 °C and then lyophilized. Lipids were extracted according to Ref. 41, with slight modifications, as described in Ref. 42. The lipids of the organic phases were separated by high performance TLC (solvent system chloroform/methanol/acetic acid/H₂O, 60:45:4:2, v/v/v/v) and revealed with I₂. For cholesterol visualization, the extracted lipid samples were separated by high performance TLC (solvent system hexane/diethylether/acetic acid, 20:35:1, v/v/v) and then sprayed with anisaldehyde reagent. Cholesterol was detected by heating the plate at 180 °C for 15 min. For detection and quantification of GM1, TLC separation, blotting with horseradish peroxidase-labeled cholera toxin B subunit, ECL detection, and quantification of GM1 were performed as described (9).

**Proteins**—DRF and HDF were subjected to trichloroacetic acid precipitation. The pellet washed with acetone was suspended in water for protein assay and then subjected to gel electrophoresis (EF). For detection of proteins by Western blotting, samples were suspended in Laemmli buffer containing 1% 2-mercaptoethanol, heated at 100 °C and resolved by SDS-polyacrylamide gel electrophoresis (10% acrylamide, 7 μg/lane) followed by Western blotting that was carried out as follows. Proteins were transferred to nitrocellulose membranes. Blots were incubated overnight at 4 °C in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% (w/v) dry skimmed milk. After washing with TBST, membranes were incubated at room temperature for 3 h with the primary antibody diluted in TBST/milk (anti-α-tubulin, 1:500; anti-β-tubulin, 1:500; anti-acetyl-tubulin, 1:2000; anti-actin, 1:500; anti-GAP-43, 1:500; anti-Fyn, 1:250; anti-Tau, 1:200; anti-G protein α-subunit, 1:1000) and then for 2 h with horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG (5000–10,000-fold diluted in TBST/milk, Pierce). Proteins were detected by chemiluminescence using the SuperSignal detection kit (Pierce).

In separate experiments, an aliquot (8 μg as protein) of the pellet obtained from fraction 4 (DRF) was subjected to 2D-EF, as described for the membrane-enriched fractions, and subjected to silver staining. Another aliquot, after 2D-EF, was transferred to polyvinylidene difluoride membrane and subjected to Western blotting and ECL detection, as described above, with the exception that a mixture of anti-α-tubulin (1:500), anti-β-tubulin (1:500), and anti-acetyl-tubulin (1:2000) antibodies was utilized.

**Hydroxylamine and KOH Treatment**—The immunoprecipitate obtained from the membrane-enriched fraction using a mixture of anti-α-, anti-β-, and anti-acetyl-tubulin (1:200) antibodies was divided into three identical aliquots and subjected to 10% SDS-polyacrylamide gel electrophoresis. The three lanes were cut from fresh gels and incubated for 2 h at room temperature in 1 mM Tris-HCl (pH 7.0), 1 mM hydroxylamine (pH 7.0), or 0.2 M KOH in methanol, respectively, as described (43) and then dried and subjected altogether to autoradiography.

**Assessment of the Specific Radioactivity of Tubulin in the Membrane-enriched and Detergent-resistant Fraction**—Two aliquots of the membrane-enriched and of the detergent-resistant fraction were subjected to immunoprecipitation using a mixture of anti-α-, anti-β-, and anti-acetyl-tubulin (1:200) antibodies. The immunoprecipitates were subjected to EF on the same gel, transferred to the same polyvinylidene difluoride membrane, and subjected to radioactivity quantification. Then, the polyvinylidene difluoride membrane was subjected to Western blotting using anti-tubulin antibody, followed by ECL detection and densitometric quantification. Finally, the specific radioactivity was calculated as the ratio of the radioactivity associated with the tubulin band (directly related to the amount of TID-GM1 bound to the protein) to the digitized value of the same band in the ECL film (related to the amount of tubulin as protein).

**Sequence Determination and Computer Analysis**—Automated sequence analysis was performed on a pulsed-liquid sequencer (model 477, Applied Biosystems, Foster City, CA) equipped with a 120A Applied Biosystems PTH analyzer. Homologies with the entries in the nonredundant GenBank™ were searched using the BLAST program (44).

**RESULTS**

After cell incubation with TID-GM1 for 2 h at 37 °C, 145 ± 2.5 pmol of probe/mg of protein were bound to granule cells. A parallel experiment performed with isotopically labeled [3H]GM1 (45) gave very similar figures (138 ± 1.9 pmol/mg of protein), indicating that the association of the two molecules was comparable. After illumination of cells with UV light, inducing cross-linking of TID-GM1 with neighboring molecules, a membrane-enriched fraction was prepared. Autoradiography of the 2D-EF of this fraction (Fig. 1A) showed the presence of a relatively small number of radioactive silver-stained spots, compared with those present in a 2D-EF after silver staining (Fig. 1C). The main radioactive spots corresponded to proteins with masses of about 55 and 30 kDa, with pl values of about 5.

The experiment was repeated carrying out the incubation at 4 °C. After 4 h of incubation, 20 ± 0.18 pmol of TID-GM1/mg of protein were bound to granule cells. After photostimulation, the membrane-enriched fraction was prepared, and 2D-EF was
Membrane Tubulin-GM1 Interaction

The involvement of DRF in the interaction between GM1 and tubulin. Cells were treated with Triton X-100 at low temperature and spun on a sucrose gradient, and 1.2-ml fractions of the gradient were assayed for radioactivity, protein content, and lipid content. The low density fraction (fraction 4), located at the 5–30% sucrose interface and corresponding to the DRF (40), displayed the highest GM1 enrichment, almost mirroring the distribution of TID-GM1 associated to the cells (Fig. 6A). On the contrary, TID-PC was mostly localized outside the DRF, within the HDF.

A comparison between DRF and HDF showed a higher proportion of sphingomyelin in the detergent resistant-fraction (Fig. 6B). Moreover, DRF was also enriched in cholesterol (Fig. 6C). Western blotting experiments showed that the DRF, al-

FIG. 1. Detection of the proteins cross-linked by the photoactivable radioactive GM1 ganglioside analog TID-GM1. Cultured rat cerebellar granule cells were incubated with TID-GM1. After cross-linking induced by illumination with UV light, a membrane-enriched fraction was prepared, and 2D electrophoresis carried out. A, autoradiography of the electrophoresis obtained after incubation of TID-GM1 for 2 h at 37 °C; B, autoradiography of the electrophoresis obtained after incubation of TID-GM1 for 4 h at 4 °C; C, silver staining of the electrophoresis shown in B. The arrow shows the direction of the isoelectric focusing (IEF) or the direction of PAGE, pI, isoelectric point. pH range is 4–8.

carried out. A lesser number of radiolabeled proteins (Fig. 1B) were detected in comparison with the experiment carried out at 37 °C. The major radioactive spots were again corresponding to proteins of about 55- (from now on called P55) and 30-kDa mass. A nearly identical radiolabeling pattern was observed when cell incubation with TID-GM1 was carried out at 4 °C; for 2 h at 37 °C; B, autoradiography of the electrophoresis obtained after incubation of TID-GM1 for 4 h at 4 °C; C, silver staining of the electrophoresis shown in B. The arrow shows the direction of the isoelectric focusing (IEF) or the direction of PAGE, pI, isoelectric point. pH range is 4–8.

In the 2D-EF of the membrane-enriched fraction after silver staining, a major spot, apparently due to at least two proteins partially overlapping and having electrophoretic characteristics similar to P55, was present. This major spot, after transfer to PDVF membrane, elution, and amino acid sequencing, showed its identity with α- and β-tubulin, suggesting that P55 could correspond to the product of cross-linking between TID-GM1 and tubulin.

In order to verify this hypothesis, immunoprecipitation experiments were performed. For this purpose, the membrane-enriched fraction, prepared after cell incubation with TID-GM1 and photoactivation at 4 °C, was lysed at 37 °C and immunoprecipitation carried out using monoclonal antibodies against α-, β-, or acetyl-tubulin. As shown in Fig. 2, the radioactive protein P55 was present in the immunoprecipitates obtained with any of the three antibodies. Using preimmune serum instead of anti-tubulin antibody and submitting the cells to the immunoprecipitation procedure, P55 was undetectable in the immunoprecipitate under the same conditions.

In order to test the specificity of interaction between TID-GM1 and P55, parallel experiments were performed, in which TID-PC was used instead of the photoactivatable ganglioside. After incubation at 4 °C for 2 h, 1 ± 0.2 pmol of phospholipid/mg of protein were bound to cells. The pattern of radiolabeled proteins was different from that obtained with TID-GM1 (the autoradiography of the 2D-EF of the membrane-enriched fraction is reported in Fig. 3).

In further experiments, intact living cells were incubated with TID-GM1 and, after photoactivation, treated with trypsin. The membrane-enriched fraction was prepared, and 2D-EF was performed. As reported in Fig. 4, P55 was still present after this treatment. Immunoprecipitation experiments using monoclonal antibodies (Fig. 4B) demonstrated that the main tubulin isoforms present were α and β.

Successively, the susceptibility of P55 to NH2-OH or to methanol KOH treatment was assessed in order to establish whether or not the cross-linking is occurring with a lipid anchor of the protein (43). The results, reported in Fig. 5, show that NH2-OH treatment did not cause substantial loss of radioactivity from P55, whereas approximately 90% of radioactivity was released after treatment with KOH. No effect was exerted by a mere treatment with methanol.

Further experiments were performed in order to investigate the involvement of DRF in the interaction between GM1 and tubulin. Cells were treated with Triton X-100 at low temperature and spun on a sucrose gradient, and 1.2-ml fractions of the gradient were assayed for radioactivity, protein content, and lipid content. The low density fraction (fraction 4), located at the 5–30% sucrose interface and corresponding to the DRF (40), displayed the highest GM1 enrichment, almost mirroring the distribution of TID-GM1 associated to the cells (Fig. 6A). On the contrary, TID-PC was mostly localized outside the DRF, within the HDF.

A comparison between DRF and HDF showed a higher proportion of sphingomyelin in the detergent resistant-fraction (Fig. 6B). Moreover, DRF was also enriched in cholesterol (Fig. 6C). Western blotting experiments showed that the DRF, al-
ways compared with the HDF, was enriched in signal transduction-related molecules such as Fyn, GAP-43, and Gα (Fig. 7). The bulk of cytoskeleton markers (tubulin, actin, and Tau) were present in the HDF, as expected. Only faint bands were visible in the DRF, corresponding to the α- and acetyl isofoms of tubulin, and even fainter bands, corresponding to β-tubulin and to actin, whereas Tau was not detected in this fraction (Fig. 7).

On the other hand, a comparison of the DRF (Fig. 8A) and the membrane-enriched fraction (Fig. 1C) showed that the protein pattern, detected by silver staining, was much simpler in the detergent resistant fraction. The presence of tubulin in this fraction was confirmed by Western blotting (Fig. 8B). Simple calculations based upon densitometric quantification of the protein bands visible in the two gels indicated that the proportion of tubulin over the total proteins in the DRF (about 18%) was higher than in the membrane-enriched fraction (about 7%). Next, the specific radioactivity of tubulin in the membrane-enriched and in detergent-resistant fraction was assessed, relying on the amount of radioactivity associated and on the amount of tubulin (as protein) in each fraction. Control experiments showed that under the conditions required for tubulin detection by ECL, the film utilized was not sensitized by the low amount of radioactivity associated to the protein.
GM1/protein cross-linking at the plasma membrane is privileged photoactivation at 4 °C. In fact, at this temperature, ganglioside proteins were obtained performing incubation and successive fractionation, a series of proteins become radiolabeled, indicating their light, their remarkable stability under a range of different features of these analogs are the ability to form covalent bonds detection of interacting proteins in cultured neurons. The main fractions prepared from cerebellar granule cells. DRF (fraction 4 and a pool of fractions 8–10 of the gradient described in the label to Fig. 6) was subjected to 2D-EF electrophoresis (7 μg of protein/lane), transferred to nitrocellulose membranes, and immunoblotted with the indicated antibody.

**DISCUSSION**

In the present investigation the GM1 ganglioside analog, TID-GM1, carrying a photoactivable diazirine group and labeled with 125I in the ceramide moiety, has been utilized for the detection of interacting proteins in cultured neurons. The main features of these analogs are the ability to form covalent bonds with neighboring proteins upon photoactivation, their extremely high specific radioactivity, and, in the absence of UV light, their remarkable stability under a range of different chemical and physical conditions (32). After incubation of cerebellar granule cells with TID-GM1 at 37 °C and photoactivation, a series of proteins become radiolabeled, indicating their proximity to the probe. Clues about the localization of these proteins were obtained performing incubation and successive photoactivation at 4 °C. In fact, at this temperature, ganglioside internalization is prevented (46–48), and therefore TID-GM1/protein cross-linking at the plasma membrane is privileged. A protein (P55) with a mass of about 55 kDa and another of about 30 kDa and a pI of about 5 were radiolabeled under all of the experimental conditions adopted. A further confirmation of their cross-linking at the plasma membrane was obtained by experiments carried out with TID-GM1 in the presence of inhibitors of endocytosis (34, 35) and at low temperature, excluding the possibility that part of the probe is endocytosed and interacts with intracellular proteins.

Our attention was attracted by P55 because it displayed electrophoretic features similar to those of tubulin, present as a major spot and recognized by amino acid sequencing in the endogenous protein pattern of the membrane-enriched fraction.

Immunoprecipitation and Western blotting experiments showed that P55 is indeed the product of cross-linking between the photoactivated ganglioside and α-, β-, and acetylated tubulin isoforms. The existence of a covalent bond between the two molecules, typical of photoreagents of this class (32), is further strengthened by the following considerations: (a) 2D electrophoresis and autoradiography were carried out on membrane-enriched fractions after delipidization, removing lipids that are not covalently bound. In addition, under the EF conditions, unbound lipids dissociate from proteins and run at the front (as discussed in Ref. 17). (b) Tubulin immunoprecipitation was carried out on membrane-enriched fractions after lysis in detergent-containing buffer at 37 °C. At this temperature, membrane domains, detergent-resistant only at low temperature (10, 40, 56), are disrupted. Therefore, immunoprecipitation of membrane rafts, containing tubulin along with noncovalently bound TID-GM1 or other cross-linked proteins, is prevented, reducing the possibility of artifacts.

Taken together, these results suggest that TID-GM1 cross-links tubulin at the plasma membrane of cerebellar granule cells. Even if microtubules, in which tubulin is the main component, are intracellular structures and gangliosides are typical membrane components, their apparent interaction has an explanation in the long known existence of membrane-associated tubulin (49–53). However, the topology of membrane tubulin is not completely understood. For instance, it has been postulated that tubulin-like, trypsin-sensitive proteins can be present at the cell surface in neurons (54). It has also been suggested that membrane-associated tubulin is an integral membrane protein (50, 51). Other investigations claim that its hydrophobicity arises from the interaction with other membrane components (52). Recently, Caron (53) showed that tubulin is palmitoylated and ascribed its hydrophobic behavior at least in part to this feature.

Experiments with trypsin, to which P55 was resistant, on the one hand ruled out that cross-linking was occurring with tubulin at the plasma membrane surface, and on the other hand gave additional information. In fact, cell-associated exogenous gangliosides that are not removed by trypsin treatment (the so-called trypsin-stable form of ganglioside association with cells) are considered to be correctly inserted with the ceramide moiety in the hydrophobic core of the bilayer (55). Because the ganglioside photoactivatable group is located at the end of the ceramide fatty acid moiety, these results indicate that ganglioside-tubulin interaction is taking place within the hydrophobic core of the plasma membrane bilayer.

Therefore, we inspected the possibility that TID-GM1 was cross-linked with a lipid anchor of tubulin. In this case, a release of radioactivity from P55 would occur after treatment with hydroxylamine or KOH, which are able to selectively remove different protein lipid anchors (43). On the contrary, if cross-linking was occurring with an amino acid residue of the protein, the radioactivity would be retained. The experiments

**FIG. 7.** Characterization of proteins in detergent-resistant fractions prepared from cerebellar granule cells. DRF and HDF (fraction 4 and a pool of fractions 8–10 of the gradient described in the label to Fig. 6, respectively), were separated on 10% SDS-polyacrylamide gel electrophoresis (7 μg of protein/lane), transferred to nitrocellulose membranes, and immunoblotted with the indicated antibody.

**FIG. 8.** Characterization of proteins in detergent-resistant fractions prepared from cerebellar granule cells. DRF (fraction 4 of the gradient described in the label to Fig. 6) was subjected to 2D-EF followed by silver staining (A) or by Western blotting using anti-tubulin antibodies (B). The specific radioactivity of tubulin was 2.1 arbitrary units in the DRF and 0.28 arbitrary units in the membrane-enriched fraction.
showed that the radioactivity was released only by KOH, consistent with the hypothesis that upon photoactivation, TID-GM1 is cross-linked with a fatty acid anchor that is linked with an ester-linkage to membrane-associated tubulin.

As a further step, we investigated the possible involvement of specialized domains of the neuronal plasma membrane. In fact, in recent years, substantial progress has been made in understanding the biological role of glycolipid-enriched, detergent-resistant membrane domains in eukaryotic cells (11, 35, 56). However, the neuron, which perhaps represents one of the greatest challenges to research on membrane traffic and function, has only been partially investigated (13, 42, 57). Therefore, we prepared DRF from a lysate of cerebellar granule cells after incubation with TID-GM1 and cross-linking. This fraction was found to be enriched in endogenous GM1 (and TID-GM1), sphingomyelin, and cholesterol, in analogy with other cellular systems (10, 11, 13). Also signal transduction molecules were enriched in DRF, again in analogy with other neuronal and nonneuronal cell types (10–12, 42, 58). Although the bulk of cytoskeleton proteins were localized within the HDF, some of them were also detected in trace amounts in DRF. This finding is somewhat expected, because the presence of actin, in particular, in membrane domains has been already reported (11, 59). However, the neuronal microtubule-associated protein Tau (60) was not detected in DRF, reducing the possibility of a mere contamination of DRF by the cytoskeleton. According to the above reported results, tubulin in DRF is present in very small amounts in comparison with HDF, which contains the bulk of cell tubulin. On the contrary, upon comparison of DRF with membrane-enriched fractions (which contain all of the tubulin associated to the membrane), it turns out that membrane tubulin is enriched in detergent-resistant membrane domains.

Next, we assessed the presence of radioactive, GM1-cross-linked, tubulin within DRF. Radioactive tubulin was detected, indicating that ganglioside-enriched detergent-resistant domains do contain lipid-anchored tubulin. The presence of lipid-anchored tubulin in DRF could explain why cell treatment with nucodazole does not affect the protein cross-linking with TID-GM1; in fact, it is conceivable that tubulin molecules associated to the membrane with their lipid anchor are insensitive to this microtubule-depolymerizing drug. However, the evaluation of this hypothesis deserves further investigation.

The specificity of the interaction between tubulin and TID-GM1 could be debated. In this particular case, at least two types of specificity may be involved: (a) tubulin interaction with ganglioside with respect to other lipids, and (b) TID-GM1 interaction with DRF tubulin in comparison with tubulin in the bulk membrane. Concerning the first type, experiments were carried out with the phosphatidylcholine analogue TID-PC. TID-PC was localized in the bulk membrane, and cross-linking experiments suggested that tubulin interacts specifically with GM1. Concerning the second type of interaction (of GM1 with DRF tubulin in comparison with tubulin in the bulk membrane) the higher specific radioactivity in DRF suggests that TID-GM1 is more specific toward tubulin associated with domains. However, this latter interaction is unlikely to be driven by a mutual recognition, because it is occurring between lipids (ganglioside ceramide moiety and palmitoyl residues of the protein). Instead, the apparent interaction likely depends on co-segregation and enrichment of both molecules—in particular ganglioside—within the same domain. In this sense, we propose to name it a “domain-specific interaction.” In this view, TID-GM1 is instrumental to ascertaining which proteins are present within DRF, in this case, it detects tubulin anchoring to glycolipid-enriched detergent-resistant domains of the neuronal plasma membrane.

The functional implications of the presence of lipid-anchored tubulin within detergent-resistant domains could be debated. First, it is likely that such localization is important for structural remodeling of the plasma membrane, as it occurs during cell mitosis or axon extension in neurons. Glycolipid-enriched domains could play a key role as sites where microtubules, via lipid-anchored tubulin, come into contact with the plasma membrane and contribute to physically driving its changes. Second, the presence of lipid-anchored tubulin within DRF could play a role in signal transduction. In fact, domains are enriched in signal-transducing molecules, G-protein families included (10–12), and it is known that tubulin is involved in G-protein-mediated signal transduction in a variety of systems (61). The interaction between these two protein families inside domains can affect signal transduction. GM1-tubulin interactions may participate in the modulation of this process.

In conclusion, herein we have shown that some tubulin molecules are associated with a lipid anchor to detergent-resistant glycolipid-enriched domains of the plasma membrane. This novel feature of glycolipid-enriched domains increases the evidence of their proven or postulated participation in the evidence of a important cell functions (10–12) and provides a key for a better understanding of their biological role in the plasma membrane.

REFERENCES
1. Karlsson, K.-A. (1986) Chem. Phys. Lipids. 42, 153–172
2. Hakomori, S.-I. (1981) Annu. Rev. Biochem. 50, 733–764
3. Bremer, E. G., Hakomori, S., Bowen-Pope, D. G., Raines, E., and Ross, R. (1984) J. Biol. Chem. 259, 6818–6825
4. Fishman, P. H. (1986) in New Trends in Ganglioside Research: Neurochemical and Neurodegenerative Aspects (Ledeen, R. W., Hogan, E. L., Tettamanti, G., Yates, A. J., and Yu, R. K., eds) Fidia Research series, Vol. 14, pp. 183–201, Liviana Press, Padova
5. Hakomori, S.-I. (1990) J. Cell Biol. 165, 1873–18716
6. Rebbau, A., Hurth, J., Yamamoto, H., Kersey, D. S., and Bremer, E. G. (1996) Glycobiology 6, 399–406
7. Fueshko, S. M., and Schengrund, C.-L. (1992) J. Neurochem. 59, 527–535
8. Tiemeyer, M., Yasuda, Y., and Schnaar, R. L. (1989) J. Biol. Chem. 264, 1671–1681
9. Pittu, M., Mutoh, T., Kuriyama, M., Ferrarotto, A., Palestini, P., and Masserini, M. (1998) FEBS Lett. 439, 93–96
10. Simons, K., and Ikonen, E. (1997) J. Lipid. Res. 38, 569–572
11. Anderson, R. G. W. (1998) Annu. Rev. Biochem. 67, 199–225
12. Lisanti, M. P., Scherer, P. E., Tang, Z., and Sargiacomo, M. (1994) Trends Cell Biol. 4, 221–225
13. M. Masserini, P. Palestini, and M. Pittu (1999) J. Neurochem. 73, 1–11
14. Kasahara, K., Watanabe, Y., Yamamoto, T., and Sanai, Y. (1997) J. Biol. Chem. 272, 29947–29953
15. Iwahuchi, K., Handa, K., and Hakomori, S. (1996) J. Biol. Chem. 271, 13103–13108
16. Iwahuchi, K., Yamamura, S., Prinetti, A., Handa, K., and Hakomori, S. (1998) J. Biol. Chem. 273, 33766–33773
17. Fra A, Masserini, M. Palestini, P. Sonnino, S. Simons, K. (1995) FEBS Lett. 375, 11–14
18. Sonnino, S., Chiogno, V., Acquotti, D., Pittu, M., Kirschner, G., and Tettamanti, G. (1998) Biochemistry 28, 77–84
19. Sonnino, S., Chiogno, V., Valsecchi, M., Pittu, M., and Tettamanti, G. (1992) Neurochem. Int. 20, 315–321
20. Gardner, J., Durrer, P., Kitchen, J., Brunner, J., and Crooke, R. (1998) J. Biological Chem. 273, 5167–5173
21. Eicher, J., Brunner, J., and Wickener, W. (1997) EMBO J. 16, 2188–2196
22. Shapiro, R. E., Specht, C. D., Collins, B. E., Woods, A. S., Cotter, R. J., and Schnaar, R. L. (1997) J. Biol. Chem. 272, 30349–30356
23. Kuhn, C. S., Lehmann, J., and Sandhoff, K. (1992) Bioconjugate Chem. 3, 230–233
24. Tettamanti, G., and Riboni, L. (1994) in Progress in Brain Research (Svennerholm, L., Asbury, A. K., Reisfeld, R. A., Sandhoff, K., Suzuki, K., and Tettamanti, G., eds) Vol. 101, pp. 77–84, Elsevier Science BV, Amsterdam
25. Tettamanti, G., Bonali, F., Marchesini, S., and Zambotti, V. (1973) Biochim. Biophys. Acta 296, 160–170
26. Ghidoni, R., Sonnino, S., Masserini, M., Orlando, P., and Tettamanti, G. (1982) J. Lipid. Res. 23, 1286–1295
27. Sonnino, S., Ghidoni, R., Gazzotti, G., Kirschner, G., Galli, G., and Tettamanti, G. (1984) J. Lipid. Res. 25, 620–629
28. Gallo, V., Ciotti, M. T., Coletti, A., Aloisi, F., and Levi, G. (1982) Proc. Natl.
Tubulin Anchoring to Glycolipid-enriched, Detergent-resistant Domains of the Neuronal Plasma Membrane
Paola Palestini, Marina Pitto, Gabriella Tedeschi, Anita Ferraretto, Marco Parenti, Joseph Brunner and Massimo Masserini

*J. Biol. Chem.* 2000, 275:9978-9985.
doi: 10.1074/jbc.275.14.9978

Access the most updated version of this article at [http://www.jbc.org/content/275/14/9978](http://www.jbc.org/content/275/14/9978)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 60 references, 25 of which can be accessed free at [http://www.jbc.org/content/275/14/9978.full.html#ref-list-1](http://www.jbc.org/content/275/14/9978.full.html#ref-list-1)