A Photoreactive Derivative of Radiolabeled GM1 Ganglioside: Preparation and Use To Establish the Involvement of Specific Proteins in GM1 Uptake by Human Fibroblasts in Culture†

Sandro Sonnino,*,‡ Vanna Chigorno,‡ Domenico Acquotti,‡ Marina Pitto,‡ Günther Kirschner,§ and Guido Tettamanti‡

Study Center for the Functional Biochemistry of Brain Lipids, Department of Medical Chemistry and Biochemistry, The Medical School, University of Milan, 20133 Milano, Italy, and Department of Chemistry, FIDIA Research Laboratories, Abano Terme, Italy

Received March 7, 1988; Revised Manuscript Received July 28, 1988

ABSTRACT: A new procedure was used to synthesize a derivative of ganglioside GM1 containing a photoreactive nitrophenyl azide group at the end of the fatty acyl moiety, using deAc-deAcyl-GM1 obtained by deacetylation of the sialic acid and deacylation of the ceramide portion of GM1. This deAc-deAcyl-GM1 was first acylated at the long chain base amino group with 12-aminododecanoic acid, which has the amino group protected by a fluorenyl residue, and tritium labeled at the sialic acid amino group with [^{3}H]acetic anhydride of very high specific radioactivity. The fluorenyl group removed by ammonia treatment was substituted by a nitrophenyl azide group. Cultured human fibroblasts were exposed to mixtures of radioactive photolabeled GM1 and cold natural GM1 (1:10 by mol) for different times and then illuminated and the radioactive protein patterns studied by SDS–PAGE. After 2 h of exposure, the photolabeled GM1 was stably associated to the cells and underwent almost no metabolic processing, behaving exactly as the underivatized natural GM1. Under these conditions very few proteins became radioactive: one, of about 30 kDa, interacted with the ganglioside molecules inserted into the outer membrane layer; three, in the region of 46 kDa, interacted with the portion of associated ganglioside able to be released by trypsin treatment. Thus, it is evident that the ganglioside binding to fibroblasts and insertion into the outer layer of the plasma membrane involve few individual proteins. When the incubation was prolonged to 24 h, photolabeled GM1 underwent extensive metabolic processing and gave origin to the corresponding ganglioside derivatives GM2, GM3, and GD1a. Under these conditions many proteins became radioactive, a consequence of GM1 transfer from the surface to the interior of the cell and of the ready availability of interactions of GM1 and of its metabolites.

Gangliosides appear to be involved in the biotransduction of membrane-mediated information, presumably acting as modulators of some proteins (receptors, ion channels, enzyme regulating proteins, second messenger generating enzymes, protein kinases) instrumental to the process (Brady & Fishman, 1979; Fishman, 1982; Wiegandt, 1985; Hakomori, 1986). A widely used approach in studying the functional implications of gangliosides is their addition to in vitro cellular systems and the determination of the biological effects (Moss et al., 1976; Radsak et al., 1982; Sonderfeld et al., 1985; Facci et al., 1984; Fishman, 1986), which, till now, have been assumed to arise from the preliminary insertion of gangliosides into plasma membranes, thus modifying membrane properties (Moss et al., 1976; Radsak et al., 1982; Facci et al., 1984; Chigorno et al., 1985).

The exogenous ganglioside association process has been studied in detail (Radsak et al., 1982; Schwarzmann et al., 1983; Facci et al., 1984). Gangliosides, presumably in micellar form, become loosely bound to the cell surface and can be removed by treatment with fetal calf serum or albumin solutions, with which they are known to form very tight and stable lipoproteic complexes (Tomasi et al., 1980; Venerando et al., 1982); a smaller portion of gangliosides interacts strongly with proteins protruding from the membrane surface and can be released by trypsin treatment. Finally, an even smaller but well-defined portion of associated gangliosides is made up of individual molecules inserted into the membrane layer, interacting with the components of the membrane as was demonstrated by the use of paramagnetic derivatives of gangliosides (Kanda et al., 1982; Schwarzmann et al., 1983). It has been suggested (Leskawa et al., 1987) that the insertion, which probably proceeds via monomeric gangliosides, is mediated by surface-located membrane-bound proteins. The binding of the monomeric gangliosides to these proteins would facilitate the aggregate to monomeric form transition and provide a continuous source of molecules for insertion.

A question regarding cell surface binding and membrane layer insertion is whether protein mediation is a specific phenomenon. The answer to this question may also open the way to recognizing the proteins, involved in signal reception and transduction, sensitive to ganglioside modulation.

We decided to approach the problem by using photoreactive gangliosides. In fact, photoreactive probes are stable in the dark, where they show no chemical reactivity, light converting them into highly unstable compounds that can covalently link specific groups located nearby. Therefore, derivatives of gangliosides carrying the photoreactive probe in the ceramide

† This work was partially supported by a grant (87.00471.56) from the Consiglio Nazionale delle Ricerche (Progetto finalizzato Medicina Preventiva e riabilitativa, Sottoprogetto Malattie del sistema nervoso), Rome, Italy.

* Address correspondence to this author at the Dipartimento di Chimica e Biochimica Medica, Facoltà di Medicina e Chirurgia, Via Saldini 50, 20133 Milano, Italy.

‡ University of Milan.

§ FIDIA Research Laboratories.

0006-2960/89/0428-0077$01.50/0

portion of the molecule can serve as reagents for labeling the hydrophobic domains of proteins and seem to be paticularly appropriate for recognizing the plasma membrane proteins that specifically bind to the gangliosides after their addition to cells in culture.

Materials and Methods

Reagents. Commercial chemicals were the purest available, common solvents were distilled, and water was doubly distilled. Tetrahydrofuran was depleted of peroxides by passage through an alumina column, dehydrated by refluxing over KOH pellets, and finally refluxed over and distilled from lithium aluminum hydride. The dehydration of acetone was by 4-Å molecular sieve (Merck), of triethylamine and dimethylformamide by shaking and standing over KOH pellets, and of methanol by refluxing over and distilling from metallic magnesium. 12-Aminododecanoic acid and disuccinimidyl carbonate were purchased from Aldrich, 9-fluorenylmethyl chloroformate, Triton X-100, silica gel 100 for column chromatography (0.063–0.2 mm, 70–230 mesh, ASTM), and silica gel precoated thin-layer plates (HPTLC, Kieselgel 60, 20 cm × 20 cm aluminum sheets) from Merck, and trypsin, *N*-acetylneuraminic acid, neuraminic acid β-methylglycoside, and crystalline bovine serum albumin from Sigma. [^{3}H]Acetic anhydride was from Amersham International and 4-fluoro-3-nitrophenyl azide from Fluka AG. En^{3}HanceTM spray was from New England Nuclear. X-Omat SO-282 films were from Kodak. All the solutions for fibroblast culture and washing were from Flow Laboratories. Gangliosides GM3,[1] GM2, GM1, and GD1a were extracted from pig brain (Tettamanti et al., 1973), purified, and characterized (Ghidoni et al., 1980). DeAc-deAcyl-GM1 was prepared by alkaline hydrolysis of GM1 in the presence of tetramethylammonium hydroxide (Sonnino et al., 1985). The reference standards GM3, GM2, GM1 and GD1a were radiolabeled at the long chain base moiety and prepared as the erythro form according to the method of Gazzotti et al. (1984).

Chemical Synthesis of Photoreactive Tritium-Labeled GM1 Ganglioside. The photoreactive derivative of GM1, tritium labeled at the level of the sialic acid acetyl group, was prepared according to the scheme of reactions presented in Figure 1. The reaction intermediates were numbered I–VI.

Preparation of Product I. A solution of 9-fluorenylmethyl chloroformate (2.6 g, 11.2 mmol) in tetrahydrofuran (350 mL) was added under continuous magnetic stirring to 150 mL of 1% aqueous $NaHCO_3$ containing 12-aminododecanoic acid (2 g, 9.3 mmol). The constantly stirred mixture was maintained a further 30 min at room temperature, and then the precipitate, corresponding to unreacted 12-aminododecanoic acid, was filtered off. The solution was partitioned by adding 250 mL of ethyl ether and shaking; the aqueous phase was withdrawn and washed with a further 250 mL of ethyl ether, and the organic phase that formed was added to the previous one. Evaporation of the solvent gave product I; this was dissolved in 10 mL of tetrahydrofuran and precipitated by adding 100 mL of 1 N HCl. The white precipitate was washed with water and crystallized from hexane/toluene, 80:20 v/v. The yield of product I, gravimetrically determined, was about 54%.

Preparation of Product II. Under continuous magnetic stirring a solution of disuccinimidyl carbonate (0.7 g, 2.7 mmol) in dry acetone (100 mL) was used to dissolve product I (1 g, 2.3 mmol) and added to dry triethylamine (0.4 mL, 4.6 mmol) dissolved in 150 mL of dry tetrahydrofuran. The reaction mixture, after 18 h under stirring at room temperature, was evaporated under vacuum. The product formed (II) was purified by crystallization from ethyl acetate. The yield of product II, gravimetrically determined, was about 85%.

Preparation of Product III. Compound II (175 mg, 350 μmol) dissolved in 2.5 mL of dry tetrahydrofuran was added under continuous magnetic stirring to 1.5 mL of dimethylformamide containing deAc-deAcyl-GM1 (100 mg, 80 μmol), Triton X-100 (1 mL), and dry triethylamine (15 μL). The reaction mixture was allowed to stand under continuous stirring for 24 h at room temperature. The mixture was evaporated under vacuum to 1 mL, and 25 mL of ethyl acetate was added. The precipitate was purified by silica gel 100 column (100 cm × 2 cm) chromatography, using chloroform/methanol/water, 60:35:8 v/v/v, as eluting solvent. The elution profile was monitored by TLC (see below), and the fractions containing a single spot showing a chromatographic R_f of 0.22 and corresponding to product III were collected, pooled, and evaporated to dryness. The residue was dissolved in 1 mL of water and precipitated by adding 10 mL of ethyl acetate. The yield of product III, evaluated by weighing and determining the bound neuraminic acid, was about 45%.

Preparation of Product IV. [^{3}H]Acetic anhydride (25 mCi, 8.2 Ci/mmol, sample under vacuum) was added to 1 mL of methanol containing 3 mg of product III following Amersham International recommendations accompanying the sample (method A—transfer under vacuum) for opening break-seal ampules. The reaction mixture was kept under stirring for 1 h at room temperature. The product formed (IV) was purified by silica gel 100 column (40 cm × 1 cm) chromatography using chloroform/methanol/water, 60:35:8 v/v/v, as eluting solvent. The elution profile was monitored by TLC followed by fluorography (see below). The fractions containing a single spot showing a chromatographic R_f of 0.55 and corresponding to product IV were collected, pooled, and dried by evaporation. The residue was dissolved in 1 mL of methanol. The yield of product IV, determined by TLC densitometric quantification, was about 60%.

Preparation of Product V. The final methanolic solution of product IV was allowed to stand at room temperature under an ammonia flow for 2 h. The reaction tube was then saturated with ammonia, tightly closed, and, under continuous stirring, maintained overnight at room temperature. The reaction mixture was dried under vacuum and the residue dissolved and stored in 10 mL of propan-1-ol/water, 7:3 v/v. The yield of product V, by TLC densitometric quantification, was over 95%.

Preparation of Product VI (Photoreactive Tritium-Labeled GM1). All the operations involving the use of azide and azide-containing gangliosides were carried out in the dark. The above propan-1-ol/water solution of product V was dried under vacuum and the residue dissolved in 400 μL of dimethyl

[1] Abbreviations: NeuAc, *N*-acetylneuraminic acid; Neu, neuraminic acid; Cer, ceramide; lcb, long-chain base; GM1, II^{3}NeuAcGgOse$_4$Cer, Galβ1–3GalNAcβ1–4(NeuAcα2–3)Galβ1–4Glcβ1–1′Cer; deAc-deAcyl-GM1, II^{3}NeuGgOse$_4$lcb, Galβ1–3GalNAcβ1–4(Neuα2–3)Galβ1–4Glcβ1–1′lcb; GD1a, IV^{3}NeuAcII^{3}NeuAcGgOse$_4$Cer, NeuAcα2–3Galβ1–3GalNAcβ1–4(NeuAcα2–3)Galβ1–4Glcβ1–1′Cer; GM2, II^{3}NeuAcGgOse$_3$Cer, GalNAcβ1–4(NeuAcα2–3)Galβ1–4Glcβ1–1′Cer; GM3, II^{3}NeuAcLacCer, NeuAcα2–3Galβ1–4Glcβ1–1′Cer; LacCer, Galβ1–4Glcβ1–1′Cer; GlcCer, Glcβ1–1′Cer; ^{1}H NMR, proton nuclear magnetic resonance; DMSO-d_6, deuteriated dimethyl sulfoxide; TLC, thin-layer chromatography; EMEM, Eagle's minimum essential medium with Earle's salts; PBS, Dulbecco phosphate buffer saline solution without calcium and magnesium; FCS, fetal calf serum; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis. Gangliosides and allied glycosphingolipids nomenclature is in accordance with Svennerholm (1970) and IUPAC–IUB Recommendations (1977, 1982).

sulfoxide and supplemented with 8 μL of triethylamine and 482 μL of an ethanolic solution of 4-fluoro-3-nitrophenyl azide (18 μmol), under stirring. The reaction mixture, after standing at 80 °C for 12 h, was reduced to a small volume by evaporation under vacuum and poured onto a silica gel 100 column (10 cm × 1 cm), equilibrated and eluted with chloroform/methanol/water, 60:35:8 v/v/v. The elution profile was monitored by TLC followed by fluorography. The fractions containing a single spot showing a chromatographic R_f of 0.37 and corresponding to product VI were collected, pooled, and evaporated to dryness under vacuum. The product obtained (VI) was dissolved in 20 mL of propan-1-ol/water, 7:3 v/v, and stored at 4 °C. The yield of product VI, determined by TLC followed by radioscanning quantification, was over 95%.

Cell Culture Conditions and Subcellular Fractionation of Human Skin Fibroblasts. Skin biopsies were obtained by the punch technique from normal young individuals, and fibroblast cultures were initiated and maintained (Leroy et al., 1972) by using 75-cm^2 Corning plastic flasks. Subcultures were made on 28 cm^2 culture dishes by using 5 mL of EMEM containing 10% FCS. Fibroblast cultures in monolayers were used at confluence (130–150 μg of cell protein/dish). Rupture, homogenization, and subcellular fractionation of fibroblasts were carried out according to the method of Chigorno et al. (1986). In particular, a P_2 fraction and a P_2 light subfraction (plasma membrane enriched) were prepared. Characterization of subcellular fractions was carried out by enzyme marker techniques (Chigorno et al., 1986). Fibroblast viability was assayed by the Trypan Blue absorption method (Phillips, 1973). The morphology of cultured fibroblasts was examined by using a phase contrast microscope.

Treatment of Fibroblasts and of Fibroblast Subcellular Fractions with Photoreactive and Tritium-Labeled GM1 (All Operations Carried Out in the Dark). A given amount of product VI was pipetted into a sterile tube and dried under a stream of nitrogen and the residue dissolved in 0.1 mL of chloroform/methanol, 2:1 v/v, containing 10 times the quantity of cold natural GM1. This was done to decrease the probability that photolabeled ganglioside molecules were near each other in the lipid membrane layer (see below) and thus to decrease self-quenching reactions during illumination (Reiser et al., 1968). The mixture was evaporated to dryness and the residue dissolved in an appropriate volume of EMEM to obtain a final 10^{-4} M ganglioside concentration. After removal of the original medium followed by rapid washing with EMEM, 2 mL of the ganglioside-containing medium was added to each culture dish that contained fibroblasts at confluence. Cells, in the presence of the ganglioside-containing medium, were incubated at 37 °C for up to 24 h. At the end of incubation and after removing the ganglioside-containing EMEM, the cells were washed three times with 3 mL (each time) of Hank's solution to remove unbound GM1. They were then maintained at 37 °C for 30 min with 2 mL of 10% FCS–EMEM to remove any GM1 loosely adhering to the fibroblasts. To remove GM1 firmly attached to membrane proteins protruding on the surface, the cells were treated with 2 mL of PBS solution containing 0.1% trypsin (Schwarzmann et al., 1983; Chigorno et al., 1985). This ensures that the ganglioside molecules still stably fixed after trypsin treatment are actually inserted into the external lipid layer (Kando et al., 1982; Schwarzmann et al., 1983).

Cells subjected to the above treatment or to part of it underwent UV illumination (360 nm) for 20 min and were then harvested by scraping with a rubber policeman and centrifuged (1000*g*, 10 min). In parallel experiments, 500 μg, as protein, of the plasma membrane enriched fraction homogeneously suspended in 50 mM Tris-HCl buffer and 0.5 mM EDTA, pH 7.5, was suspended with 2 mL of 10^{-4} M ganglioside (cold natural GM1/photolabeled and tritium-labeled GM1, 10:1 by mol) and kept at 4 °C under gentle stirring and moderate nitrogen pressure. At the end of incubation, the suspension was centrifuged at 105000*g* for 1 h, the pellet being washed once with the above buffer containing 10% FCS and then with buffer alone. The final pellet was suspended in 0.1 mL of 10 mM potassium phosphate buffer, pH 6.8, and illuminated as above.

Pelleted cells or cell subfractions were subjected to lipid extraction in order to remove the remaining unbound photolabeled material that underwent self-quenching after illumination and endogenous lipids. The extraction was performed according to the method of Tettamanti et al. (1973) with 1 mL of solvent mixture tetrahydrofuran/10 mM potassium phosphate buffer, 4:1 v/v, pH 6.8, per 100–150 μg of protein. The delipidized pellet was suspended in 2% SDS solution and submitted to sonication, and the proteins were separated under reducing conditions on a 7–15% linear gradient slab gel SDS–PAGE at 75 V (Laemmli, 1970) overnight. Proteins were stained by means of the Coomassie blue procedure (Fazekas de St. Groth et al., 1963). In preliminary experiments the delipidized pellet was subjected to *Vibrio cholerae* sialidase treatment to determine the protein-bound radioactive sialic acid, as reported by Ghidoni et al. (1987).

Metabolic Experiments. Following the above procedures cells were incubated at 37 °C with 2 mL of 10^{-4} M ganglioside (cold natural GM1/photolabeled GM1, 10:1 by mol) containing EMEM. In routine experiments cells were incubated for 2 and 24 h; they were then washed with 10% FCS–EMEM and directly subjected to ganglioside extraction (Tettamanti et al., 1973) and the individual gangliosides separated out by TLC (see below) and subjected to quantitative radiochromatoscanning analysis (Ghidoni et al., 1983). The whole procedure was carried out in the dark. In the control experiments cells were incubated with a medium containing tritium-labeled GM1 instead of photolabeled and tritium-labeled GM1.

Nuclear Magnetic Resonance Spectroscopy. Samples were carefully dried under high vacuum and dissolved in DMSO-d_6/D_2O, 98:2 v/v, and spectra recorded at 30 °C. ^{1}H NMR spectra were performed at 500 MHz on a AM Bruker instrument equipped with an Aspect 3000 computer and operating in the Fourier transform mode with quadrature detection.

Thin-Layer Chromatography. The followup of the chemical reactions to prepare photoreactive tritium-labeled GM1, the elution profile of the various products from chromatographic columns, and the assessment of homogeneity of products III–VI were carried out by TLC using 6 cm long HPTLC plates developed at room temperature, the solvent system, chloroform/methanol/0.2% aqueous $CaCl_2$, 50:42:11 v/v/v, reaching the top edge of the plate. Ganglioside spots, made visible by treatment with a *p*-(dimethylamino)benzaldehyde spray reagent followed by heating at 120 °C for 10 min (Chigorno et al., 1982), were quantified by densitometric scanning of the plate using a Camag TLC densitometer equipped with a IBM data system (Chigorno et al., 1982; Sonnino et al., 1986). Azide-containing tritium-labeled ganglioside derivatives were chromatographed in the dark and, after exposure to light, were visible as red spots. Quantification of radioactive spots was by radiochromatographic scanning of the plate using a Berthold TLC linear analyzer LB282 equipped with an Apple II data system (Ghidoni et al., 1983).

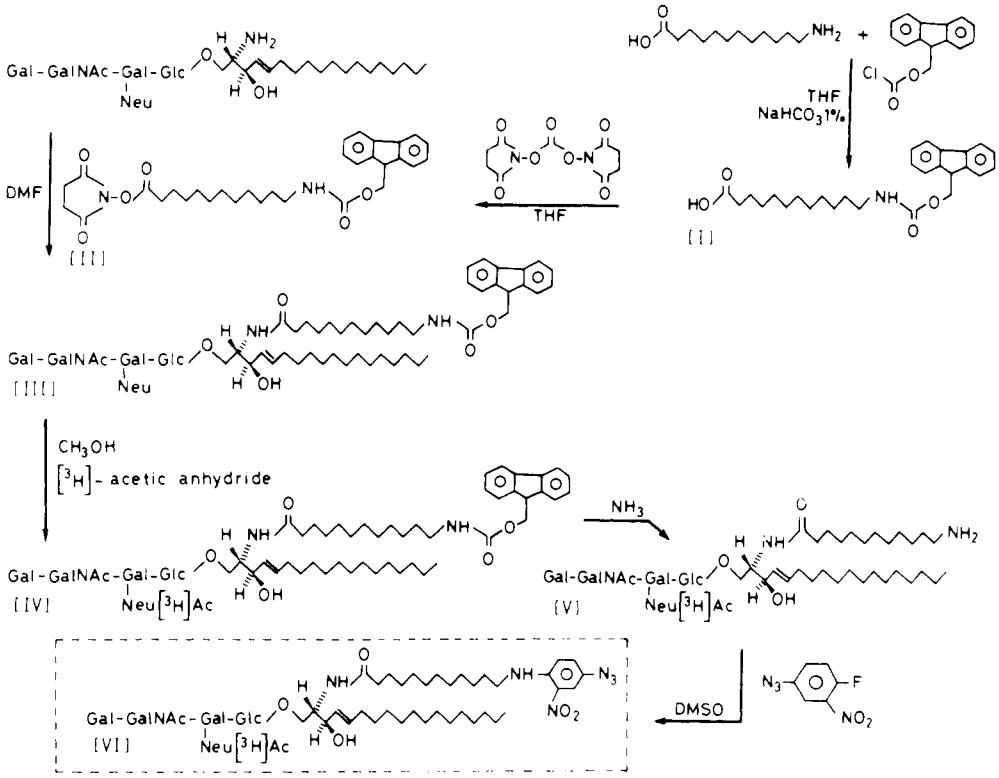

FIGURE 1: Scheme of the reactions for preparing photoreactive derivatives of tritium-labeled GM1.

Radioactive areas on the plates were located by fluorography. The plates were sprayed with a surface autoradiographic enhancer and exposed to films for different times (2–24 h) at −80 °C.

Colorimetric Methods. DeAc-deAcyl-GM1 and products III–V were assayed as bound neuraminic acid or *N*-acetylneuraminic acid by the resorcinol–HCl method (Svennerholm, 1957), pure Neu β-methylglycoside NeuAc being used as the reference standard. Control experiments showed that the extinction coefficients of Neu β-methylglycoside NeuAc were similar. Protein content was determined in solubilized cell pellets according to Peterson's modification (Peterson, 1977) of the method of Lowry et al. (1951), bovine serum albumin being used as the reference standard.

Results and Discussion

Preparation of Tritium-Labeled and 12-[(4-Azido-2-nitrophenyl)amino]dodecanoyl-GM1. Previous experiments show that deAc-deAcyl-GM1 is a suitable substrate for the preparation of ganglioside derivatives carrying fluorescent, paramagnetic, and radioactive probes on their fatty acyl moiety (Sonnino et al., 1985, 1986; Acquotti et al., 1986): deAc-deAcyl-GM1 is first acylated at the level of the long chain base amino group by using an acyl chain carrying the proper probe and then at the level of neuraminic acid amino group. Attempts were first made to apply this procedure to the preparation of a photoreactive derivative of GM1 by using a presynthesized azide-containing acyl chain; however, the reaction was not quantitative since the adoption of several chromatographic steps was needed to purify the photoreactive compound. These operations had to be carried out in darkness to avoid the formation of undesirable compounds (Bisson & Montecucco, 1985); this poses obvious difficulties. Moreover, photoreactive lipids to be used for biological experiments have to be prepared in the radioactive form and with very high specific activity. In this case it is possible to decrease the quantity of photolabeled compound that can be diluted in the natural one, with a consequent decrease in the self-quenching reaction that occurs during illumination when the azide groups are near. In our case a good radiolabeling method appeared to be acetylation of the amino group of neuraminic acid with the use of highly radioactive tritiated acetic anhydride (Chigorno et al., 1985). Commercial acetic anhydride possessing a high specific radioactivity is, however, supplied under vacuum, and its handling in the dark is very dangerous; to overcome these difficulties we devised a new procedure in which the insertion of the nitrophenyl azide group was the last operational step, accomplished after tritium labeling of the sialic acid moiety (Figure 1). The key compound, product III, contains an aminolauryl residue linked to the long chain base, the amino group being protected by a fluorenyl group. Prior to product III's preparation, the amino and carboxyl groups of 12-aminododecanoic acid were protected and activated according to well-established procedures (Staab, 1962; Carpino & Han, 1972). The resulting derivative of 12-aminododecanoic acid, product II, was insoluble in the reaction medium suitable for acylation of deAc-deAcyl GM1; it could, however, be dispersed by adding a nonionic detergent such as Triton X-100. After one day at room temperature in the presence of detergent, the yield of the acylation reaction was substantially good, about 55%, as determined by TLC densitometric quantification. After chromatographic purification, the final yield of product III, determined by weighing and neuraminic acid assay, was 45%, still good. Figure 2 presents the TLC chromatographic behavior of the major products obtained. Figure 3 shows the ^1H NMR spectrum of product III. Besides the large series of signals belonging to the basic structure of ganglioside, the spectrum displays (a) the aromatic spin system of the fluorenyl group centered at 7.35 and 7.85 ppm, indicating the incorporation of the fluorenyl group into the molecule, (b) the amide proton of the ceramide and the hexosamine at 7.40 and 7.44 ppm and a triplet deriving from the amide proton of the fluorenyl group centered at 7.15 ppm, indicating that the ganglioside backbone did not undergo any

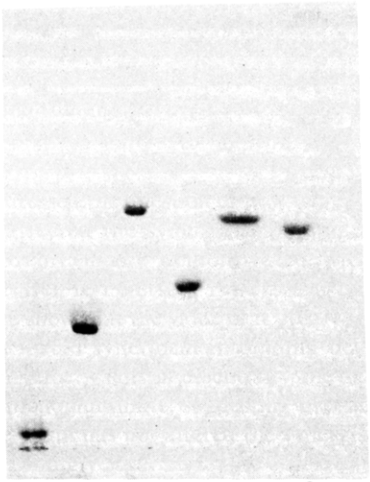

FIGURE 2: TLC of several products presented in the scheme of Figure 1. (Lane 1) deAc-deAcyl-GM1; (lane 2) product III; (lane 3) product IV; (lane 4) product V; (lane 5) product VI; (lane 6) natural GM1, as reference.

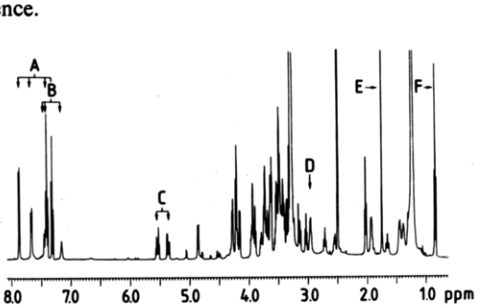

FIGURE 3: 500-MHz ^1H NMR spectrum of a solution of product III in DMSO-d_6:D_2O, 98:2 v/v. (A) Aromatic protons; (B) amide protons; (C) olefinic protons; (D) CH_2 in position 12 of protected aminolauryl chain; (E) acetyl group of *N*-acetylgalactosamine; (F) methyl group of long-chain base.

degradation during the reaction, (c) the multiplet at 2.95 ppm that derives from the methylene protons near the NH group of the fluorenyl-protected aminododecanoic chain, suggesting that the fluorenyl group is linked to the amino group of 12-aminododecanoic acid, and (d) the signals in unitary ratio of methyl groups located at 0.83 and 1.74 ppm and belonging to the alkyl chain and to the hexosamine acetyl group. At 1.87 ppm (Koerner et al., 1983), the peak of the acetyl group of sialic acid is lacking, as expected. This signal appeared after acetylation of product III (Chigorno et al., 1985), in agreement with the structure proposed for product III and presented in Figure 1. With regard to the acetylation of neuraminic acid, employed for radiolabeling the molecule, the conditions used (anhydrous methanol as solvent with labeled acetic anhydride dissolved in toluene) are known to provide a quantitative yield (Chigorno et al., 1985). Control experiments performed under these conditions with cold acetic anhydride stored under vacuum and following the operational suggestions of the producers for its transfer never led to a yield better than 75% for the acetylation process; presumably our ampules contained more acetic acid than expected. The radioactive product IV was purified by column chromatography and analyzed for chemical and radiochemical purity by TLC followed by colorimetric revealing and fluorography, with 60% yield and fairly good homogeneity (Figure 2). Densitometric quantification of sialic acid and liquid scintillation counting of a scraped-off spot showed a specific radioactivity of 4.1 Ci/mmol, as expected; removal of the fluorenyl group and insertion of the azide residue into the molecule (see Figure 1) gave a quantitative yield. This product (VI) was purified by column chromatography and freed of excess fluoronitrophenyl azide. The TLC behavior and homogeneity of products V and VI are shown in Figure 2. It is evident that, under the experimental conditions employed, the photoreactive derivative of GM1 is faster moving than natural GM1, an expression of its higher degree of hydrophobicity. After 4 months of storage in propan-1-ol/water, 7:3 v/v, at 4 °C and in the dark, the tritium-labeled GM1 containing nitrophenyl azide maintained complete stability.

GM1 Binding to Cultured Human Fibroblasts and Insertion into the Outer Lipid Layer of the Membrane: Involvement of Specific Proteins. Illumination of compounds containing nitrophenyl azide at or over 360 nm leads to the formation of nitrene derivatives that react preferentially with electron-rich reactive groups such as amino or thiol groups (Staros, 1980). In the deep core of the membrane these groups are carried only by proteins. Therefore, once radioactive gangliosides, carrying a nitrophenyl azide group at the end of the lipid moiety, are inserted into the membrane and subjected to illumination, the formation of radiolabeled proteins indicates that these proteins were so close to gangliosides as to be involved in the photosensitive reaction.

The radioactive, azide-labeled GM1 derivative (compound VI) was diluted 1:10 with cold natural GM1 and used for photolabeling experiments employing cultured human fibroblasts. The protocol for ganglioside–fibroblast interaction has already been published (Chigorno et al., 1985). Following this protocol a total ganglioside concentration of 10^{-4} M led to an association, after a 2-h incubation, of 20 nmol of gangliosides/mg of protein, 10% and 15% in the trypsin-removable and trypsin-stable forms, in agreement with the data reported by Chigorno et al. (1985). Moreover, the viability of fibroblasts after exposure to the above mixture of photolabeled and natural GM1 for up to 24 h was good and was identical with that of the cells exposed to equal concentrations of the natural ganglioside alone. The occurrence of metabolic processing of added radioactive and photolabeled GM1 was ascertained under the condition used by Chigorno et al. (1985) for radioactive GM1, taking the obvious precaution of carrying out the whole experiment in the dark. As shown in Figure 4, after 2 h of incubation, a preponderant radioactive band corresponding to the derivative of GM1 was present. Two faint faster moving bands were also present, accounting for 2–3% of all the radioactivity present and probably corresponding to the derivatives of GM2 and GM3. This means that after 2 h, the major part of the added ganglioside was still at the membrane level and presumably had not yet undergone internalization into cells and been metabolically processed. Prolongation of incubation to 24 h was followed by a marked increase in the bands corresponding to GM2 and GM3 derivatives, accounting for about 25% of the total radioactivity, and by the appearance of a faint but reproducible band (about 1% of total radioactivity) most likely corresponding to the GD1a derivative. Concomitantly, the GM1 derivative underwent a marked relative decrease. A parallel experiment using natural radiolabeled GM1 gave very similar results (see Figure 4), indicating that the mode (mainly degradation and some sialylation) and apparent rate of metabolic processing of the ganglioside carrying a photoreactive probe were the same as that typical of exogenous natural ganglioside [see Chigorno et al. (1985) and Sonderfeld et al. (1985)]. All this evidence clearly shows that the behavior of the photoreactive derivative of GM1, with regard to cell association, internalization into cells, and intracellular metabolic processing, is quite similar to that of natural GM1. This makes it reasonable to assume

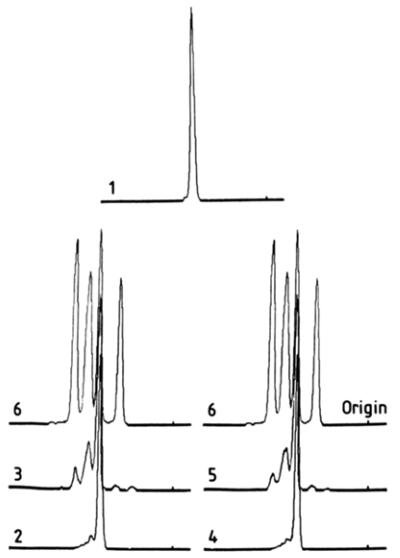

FIGURE 4: Metabolic processing of natural tritiated GM1 and photolabeled tritiated GM1 following incubation of fibroblast cells with gangliosides. After incubation, gangliosides were extracted and separated by TLC, and the plate was submitted to radiochromatographic scanning. (1) Starting photoreactive tritium labeled GM1; (2) radioactive ganglioside pattern after 2-h incubation with the mixture photoreactive GM1/cold natural GM1; (3) radioactive ganglioside pattern after 24-h incubation with the mixture photoreactive GM1/cold natural GM1; (4) radioactive ganglioside pattern after 2-h incubation with natural GM1; (5) radioactive ganglioside pattern after 24-h incubation with natural GM1; (6) standard tritium-labeled gangliosides, from the left, GM3, GM2, GM1, and GD1a.

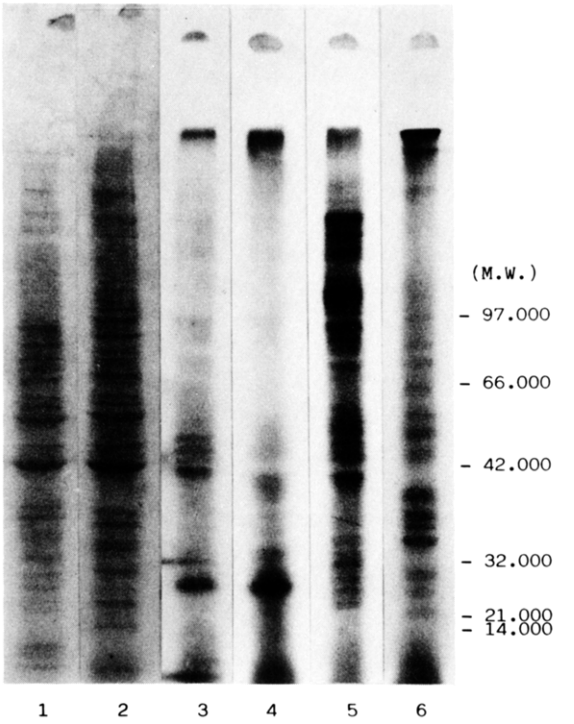

FIGURE 5: SDS–PAGE separation of fibroblast proteins. (Lane 1) Protein pattern of cultured fibroblasts, 100 μg of proteins, Coomassie blue staining; (lane 2) protein pattern of cultured fibroblasts after 2-h treatment with the mixture photoreactive GM1/cold natural GM1 followed by washing with 10% FCS–EMEM (the same result was obtained after 24-h treatment), 100 μg of proteins, Coomassie blue staining; (lane 3) fibroblast protein pattern after treatment as in lane 2, 100 μg of proteins, 75 000 dpm, autoradiographic detection; (lane 4) protein pattern of cultured fibroblasts after 2-h treatment with the mixture photoreactive GM1/cold natural GM1 followed by washing with 10% FCS–EMEM and treatment with trypsin, 100 μg of proteins, 80 000 dpm, autoradiographic detection; (lane 5) protein pattern of cultured fibroblasts after 24-h treatment with the mixture photoreactive GM1/cold natural GM1 followed by washing with 10% FCS–EMEM, 100 μg of proteins, 230 000 dpm, autoradiographic detection; (lane 6) protein pattern of fibroblast plasma membrane enriched fraction (see Materials and Methods for its preparation) after 2-h treatment with the mixture photoreactive GM1/cold natural GM1 followed by washing with 10% FCS–EMEM, 40 μg of proteins, 220 000 dpm, autoradiographic detection.

that, also with regard to the interactions with membrane-bound proteins, the behavior of the photoreactive derivative of GM1 mimics that of natural GM1.

Experimental results of protein involvement in the ganglioside association to fibroblasts are presented in Figure 5. The PAGE protein patterns of cultured fibroblasts before and after treatment with the mixture of the photoreactive GM1/cold natural GM1, followed by washing with 10% FCS–EMEM, are shown in lanes 1 and 2 of Figure 5 and are practically identical, as expected; some were radioactive. Sialidase treatment led to the release of only 1% for the 2-h incubation and 3% for the 24-h incubation of the total incorporated radioactivity released, indicating that recycling of sialic acid and sialic acid derivatives, under experimental conditions, occurs to a very low extent. Thus, the radioactivity carried by proteins must come from the covalent stable association with the photolabeled ganglioside derivative. The radioactive patterns, after 2 and 24 h of treatment (lanes 3 and 5 of Figure 5), are quite different from that obtained by Coomassie blue staining. After 2 h of incubation, a very small number of radioactive bands were present. A group of bands, centered at the reference value of 46 kDa, and one band at 30 kDa covered about 60% of the total radioactivity carried by protein material subjected to PAGE analysis. Substances of small molecular weight, presumably corresponding to self-quencing ganglioside not completely removed after the lipid extraction procedure, were also present in the front area. Since the occurrence of nonspecific interactions between ganglioside and proteins would have led to the general labeling of a large number of proteins, it should be inferred that ganglioside association to the cell surface is a selective phenomenom involving few membrane proteins. When incubation (2 h) was followed by washing with 10% FCS–EMEM, treatment with trypsin, and illumination (lane 4 of Figure 5), the intensity of the 46-kDa protein was markedly decreased, whereas the band at 30 kDa underwent a relative increase, being by far the most prominent band of the pattern. Since trypsinization does not remove the ganglioside molecules inserted into the outer lipid layer of the membrane (Schwarzmann et al., 1983; Chigorno et al., 1985), it can be suggested that the 30-kDa protein specifically interacts with these gangliosides, possibly in a well-defined microenvironment of the membrane. On the other hand, the proteins in the 46-kDa area, which diminish after trypsinization, may be exposed on the cell surface and responsible for the initial steps of firm association of ganglioside to cells. Also in this interaction the proteins involved are very few. All this suggests that the process of ganglioside binding to cells and insertion into the outer membrane layer are selective phenomena guided by proteins with a particular binding affinity for gangliosides.

The pattern of radioactive proteins after 24 h of incubation was characterized by the presence of a large number of bands (lane 5 of Figure 5). It should be remembered that after 24 h of incubation, photoreactive GM1 has undergone a process of extensive metabolic processing (see Figure 4). This implied transfer of GM1 from the cell membrane to the lysosomal apparatus, where photolabeled GM1 was degraded to photolabeled GM2 and photolabeled GM3, and also, to a lesser extent, to the Golgi apparatus, where a glycosylation (sialylation) process occurred to form photolabeled GD1a, analogous

to the events described for natural GM1 (Sonderfeld et al., 1985; Ghidoni et al., 1986).

Internalization of membrane-bound gangliosides and the intracellular traffic that follows may involve cytosolic (Sonnino et al., 1979, 1984; Ledeen et al., 1987) and endosomic proteins (Sandhoff et al., 1987), both potentially selective with regard to gangliosides. Metabolic processing also involves enzymes capable of selective recognition of photolabeled GM1 and its metabolic derivatives. Therefore, it is obvious that once photolabeled GM1 has entered the cell a number of proteins, cytosolic or membrane-bound transfer proteins, soluble or membrane-bound glycosidases and glycosyltransferases, and activator proteins (Li & Li, 1982) can become radiolabeled by illumination owing to specific interaction with photolabeled gangliosides. This view is further supported by data obtained after 2 h of incubation of the photolabeled GM1/natural GM1 mixture with the light fibroblast subfraction enriched by intracellular and pericellular membrane structures. The experiments were carried out at 4 °C to avoid a possible protein degradation. It is known that the binding at 4 °C of GM1 to mouse fibroblast cells in monolayer cultures is one-third that at 37 °C (Radsak et al., 1982), and binding at 0 °C of a fluorescent derivative of GM1 to human fibroblasts is significantly decreased in comparison to that determined at 37 °C (Spiegel et al., 1984). This behavior probably derives from different physicochemical features of the membranes at the two temperatures, e.g., different fluidity, with consequent different ganglioside–protein interactions. When a membrane-enriched fibroblast subfraction was treated for 2 h at 4 °C with gangliosides, the protein-specific radioactivity was 5 times higher than that obtained by incubating for the same time fibroblast cells and ganglioside, and at the same time a number of protein bands (lane 6 of Figure 5) appear radioactive, reflecting the multiplicity of proteins interacting with GM1. Bearing in mind all the above findings and the small number of radioactive bands that we found after 2 h of incubation with intact viable cells, the high number of radioactive bands observed here further underlines and reflects the potential role played by some proteins in the association of exogenous gangliosides to membranes under different experimental conditions.

Acknowledgments

We express our gratitude to Dr. Giovanni Fronza (Polytechnic, Milan) and Prof. Cesare Montecucco (University of Padua) for their help and friendly discussions in preparing the manuscript and to Riccardo Casellato for his skillful technical assistance.

Registry No. I, 116912-29-9; II, 116912-30-2; III, 116926-92-2; IV, 116912-31-3; V, 116926-93-3; VI, 116926-94-4; GM3, 89678-50-2; GM2, 104443-57-4; GM1, 104443-62-1; GD1a, 104443-59-6; deAc-deAcyl-GM1, 94458-46-5; 3-NO_2-4-F-$C_6H_3N_3$, 28166-06-5; 12-aminododecanoic acid, 693-57-2; 9-fluorenylmethyl chloroformate, 28920-43-6; [^{3}H]acetic anhydride, 25962-31-6; disuccinimidyl carbonate, 74124-79-1.

References

Acquotti, D., Sonnino, S., Masserini, M., Casella, L., Fronza, G., & Tettamanti, G. (1986) *Chem. Phys. Lipids 40*, 71–86.

Bisson, R., & Montecucco, C. (1985) in *Progress in Protein–Lipid Interactions* (Watts & De Pont, Eds.) pp 259–287, Elsevier, Amsterdam.

Brady, R. O., & Fishman, P. H. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 50*, 303–323.

Carpino, L. A., & Han, G. X. (1972) *J. Org. Chem. 37*, 3404–3409.

Chigorno, V., Sonnino, S., Ghidoni, R., & Tettamanti, G. (1982) *Neurochem. Int. 5*, 397–403.

Chigorno, V., Pitto, M., Cardace, G., Acquotti, D., Kirschner, G., Sonnino, S., Ghidoni, G., & Tettamanti, G. (1985) *Glycoconjuate J. 2*, 279–291.

Chigorno, V., Cardace, G., Pitto, M., Sonnino, S., Ghidoni, R., & Tettamanti, G. (1986) *Anal. Biochem. 153*, 283–294.

Facci, L., Leon, A., Toffano, G., Sonnino, S., Ghidoni, R., & Tettamanti, G. (1984) *J. Neurochem. 42*, 299–305.

Fazekas de St. Groth, S., Webster, R. G., & Datyner, A. (1963) *Biochim. Biophys. Acta 71*, 377–391.

Fishman, P. H. (1982) *J. Membr. Biol. 69*, 85–98.

Fishman, P. H. (1986) *Chem. Phys. Lipids 42*, 137–151.

Gazzotti, G., Sonnino, S., Ghidoni, R., Orlando, P., & Tettamanti, G. (1984) *Glycoconjugate J. 1*, 111–121.

Ghidoni, R., Sonnino, S., Tettamanti, G., Baumann, N., Reuter, G., & Shauer, R. (1980) *J. Biol. Chem. 255*, 6990–6995.

Ghidoni, R., Trinchera, M., Venerando, B., Fiorilli, A., Sonnino, S., & Tettamanti, G. (1986) *Biochem. J. 237*, 147–155.

Ghidoni, R., Trinchera, M., Sonnino, S., Chigorno, V., & Tettamanti, G. (1987) *Biochem. J. 247*, 157–164.

Hakomori, S. I. (1986) in *Gangliosides and Neuronal Plasticity* (Tettamanti, G., Ledeen, R. W., Sandhoff, K., Nagai, Y., & Toffano, G., Eds.) pp 201–214, Liviana, Padua, Italy.

IUPAC–IUB Recommendations for Lipid Nomenclature (1977) *Lipids 12*, 455–468. (1982) *J. Biol. Chem. 257*, 3347–3351.

Kanda, S., Inoue, K., Nojima, S., Utsumi, H., & Wiegandt, H. (1982) *J. Biochem. (Tokyo) 91*, 2095–2098.

Koerner, T. A. W., Prestegard, J. H., Demov, P. C., & Yu, R. K. (1983) *Biochemistry 22*, 2676–2687.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–687.

Ledeen, R. W., Aquino, D. A., Sbaschnig-Agler, M., Gammon, C. M., & Vaswani, K. K. (1987) in *Gangliosides and Modulation of Neuronal Functions* (Rahmann, H., Ed.) NATO ASI Series Vol. H7, pp 259–274, Springer-Verlag, Berlin.

Leroy, J. G., Ho, M. W., MacBrinn, M. C., Rielke, K., Jacob, J., & O'Brien, J. (1972) *Pediatr. Res. 6*, 752–757.

Leskawa, K. C., Erwin, R. E., & Hogan, E. L. (1987) in *New Trends in Ganglioside Research: Neurochemical and Neurogenerative Aspects* (Hogan, E., Ledeen, R. W., Li, Y. T., Neff, N., Tettamanti, G., Yates, A., & Yu, R. K., Eds.) Abstracts, p 67, Satellite Symposium of ISN–ASN Joint Meeting, June 9–12, Puerto La Cruz, Venezuela.

Li, Y. T., & Li, S. C. (1982) *Adv. Carbohydr. Chem. Biochem. 40*, 235–286.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Moss, J., Fishman, P. H., Manganello, V. C., Vaughan, N., & Brady, R. O. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1034–1037.

Peterson, G. L. (1977) *Anal. Biochem. 83*, 346–356.

Phillips, H. J. (1973) in *Tissue Culture Methods and Applications* (Kruse, P. F., Jr., & Patterson, M. K., Jr., Eds.) Academic, New York.

Radsak, K., Schwarzmann, G., & Wiegandt, H. (1982) *Hoppe Seyler's Z. Physiol. Chem. 363*, 263–272.

Reiser, A., Willets, F. W., Terry, G. C., Williams, V., & Marley, R. (1968) *Trans. Faraday Soc. 64*, 3265–3275.

Sandhoff, K., Schwarzmann, G., Sarmientos, F., & Conzelmann, E. (1987) in *Gangliosides and Modulation of Neuronal Functions* (Rahmann, H., Ed.) NATO ASI Series Vol. H7, pp 231-250, Springer-Verlag, Berlin.
Schwarzmann, G., Hoffmann-Bleihauer, P., Shubert, J., Sandhoff, K., & Marsh, D. (1983) *Biochemistry 22*, 5041-5048.
Sonderfeld, S., Conzelmann, E., Schwarzmann, G., Burg, J., Hinrichs, U., & Sandhoff, K. (1985) *Eur. J. Biochem. 149*, 247-255.
Sonnino, S., Ghidoni, R., Marchesini, S., & Tettamanti, G. (1979) *J. Neurochem. 33*, 117-121.
Sonnino, S., Ghidoni, R., Fiorilli, A., Venerando, B., & Tettamanti, G. (1984) *J. Neurosci. Res. 12*, 193-204.
Sonnino, S., Kirshner, G., Ghidoni, R., Acquotti, D., & Tettamanti, G. (1985) *J. Lipid Res. 26*, 248-257.
Sonnino, S., Acquotti, D., Riboni, L., Giuliani, A., Kirschner, G., & Tettamanti, G. (1986) *Chem. Phys. Lipids 42*, 3-26.
Spiegel, S., Schlessinger, J., & Fishman, P. H. (1984) *J. Cell Biol. 99*, 699-704.
Staab, H. A. (1962) *Angew. Chem. 74*, 407-410.
Staros, J. V. (1980) *Trends Biochem. Sci. (Pers. Ed.) 5*, 320-322.
Svennerholm, L. (1957) *Biochim. Biophys. Acta 13*, 856-858.
Svennerholm, L. (1970) in *Handbook of Neurochemistry* (Lajta, A., Ed.) pp 425-452, Plenum, New York.
Tettamanti, G., Bonali, F., Marchesini, S., & Zambotti, V. (1973) *Biochim. Biophys. Acta 296*, 160-170.
Tomasi, M., Roda, G., Ausiello, C., D'Agnolo, G., Venerando, B., Ghidoni, R., Sonnino, S., & Tettamanti, G. (1980) *Eur. J. Biochem. 111*, 315-324.
Venerando, B., Roberti, S., Sonnino, S., Fiorilli, A., & Tettamanti, G. (1982) *Biochim. Biophys. Acta 692*, 18-26.
Wiegandt, H. (1985) *New Compr. Biochem. 10*, 199-260.

Structure and Negative Transcriptional Regulation by Glucocorticoids of the Acute-Phase Rat α_1-Inhibitor III Gene[†,‡]

Wolfgang Northemann, Brian R. Shiels,[§] Todd A. Braciak, and Georg H. Fey*
Department of Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037
Received March 24, 1988; Revised Manuscript Received August 5, 1988

ABSTRACT: DNA clones representing the negative acute-phase gene coding for the plasma proteinase inhibitor α_1-inhibitor III were isolated from a rat genomic library. Structural analysis established the existence of at least four different members of the α_1-inhibitor III gene family. Partial DNA sequence analysis of the 5′-terminal regions was performed for the α_1-inhibitor III gene and the related α_1-inhibitor IV gene. The transcription start site of the α_1-inhibitor III gene was located by S1 mapping and primer extension. No stable α_1-inhibitor IV mRNA was detected in rat liver. In an experimentally induced acute-phase reaction, the transcription rate of the α_1-inhibitor III gene was reduced 12.7-fold at 6 h after stimulation. Four hours after injection of a high dose of dexamethasone into rats, the transcription rate of this gene was reduced 9-fold. Thus, glucocorticoids alone are capable of causing a strong transient down-regulation of the transcription of this gene in rats, independent of other inflammatory mediators. An inverted consensus glucocorticoid responsive element (5′GG$^{A}_{C}$A$^{C}_{A}$A$^{T}_{A}$T3′) shared with the glucocorticoid-regulated α_1-fetoprotein, α_{2u}-globulin, and α_1-acid glycoprotein genes was detected by computer-assisted sequence analysis in the promoter proximal 5′-flanking region of the α_1-inhibitor III gene.

With normal concentrations of 6-10 mg/mL, α_1-inhibitor III (α_1I3)[1] is the second most abundant plasma globulin in rats after albumin (Gauthier & Ohlsson, 1978; Esnard & Gauthier, 1980; Esnard et al., 1985; Lonberg-Holm et al., 1987). It is a thiol ester protein, and together with the related α_1-macroglobulin (α_1M) and α_2-macroglobulin (α_2M), it is part of a protein family that also includes complement components C3, C4 and C5 (Sottrup-Jensen et al., 1985; Sottrup-Jensen, 1987; Gehring et al., 1987; Braciak et al., 1988). While α_2M is the most dramatically increased acute-phase protein in rats, α_1M and C3 are increased only about 2-fold during an acute-phase response, and α_1I3 is among the most strongly down-regulated acute-phase proteins in rats. During the first few days of an acute-phase response, α_1I3 concentrations fall to 1-2 mg/mL, and during chronic inflammations, they further decline to less than 0.5 mg/mL (Lonberg-Holm et al., 1987).

We have recently isolated and sequenced α_1I3 cDNA clones and deduced the α_1I3 protein sequence (Braciak et al., 1988). The mature α_1I3 polypeptide is 1453 amino acids in length

[†] This work was supported by Grants AI22166 and AI19651 from the National Institute of Allergy and Infectious Diseases and by an award from the United Liver Association (to G.H.F.). Short-term fellowships from the North Atlantic Treaty Organization (to W.N.) and the Fulbright Commission (to B.R.S.) provided additional support. This is Publication No. 5056-IMM from the Department of Immunology, Research Institute of Scripps Clinic.
[‡] The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J03552.
* Correspondence should be addressed to this author at the Department of Immunology, IMM14, Research Institute of Scripps Clinic, 10666 North Torrey Pines Rd., La Jolla, CA 92037.
[§] Present address: Welcome Unit of Molecular Parasitology, Department of Veterinary Parasitology, Bearsden Rd., Glasgow G61 1QH Scotland, United Kingdom.

[1] Abbreviations: α_1I3, α_1-inhibitor III; α_1I4, α_1-inhibitor IV; α_2M, α_2-macroglobulin; α_1M, α_1-macroglobulin; α_1AGP, α_1-acid glycoprotein; α_1FP, α_1-fetoprotein; SDS, sodium dodecyl sulfate; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); bp, base pair(s); kbp, kilobase pair(s); HSF, hepatocyte stimulating factor; IL-6, interleukin 6; GRE, glucocorticoid receptor element.

0006-2960/89/0428-0084$01.50/0