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Formation of a cytosolic ganglioside-protein complex following administration of photoreactive ganglioside GM1 to human fibroblasts in culture

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A GM1 ganglioside derivative bearing a photoreactive nitrophenyl azide group and tritium labeled at the acetyl group of *N*-acetylneuraminic acid, has been administered to cultured human fibroblasts. With photolabeling experiments we found that a portion of the ganglioside in the cell cytosol was associated with a soluble protein of about 30 kDa.

Photoreactive GM1; Cytosolic complex; Ganglioside-protein interaction

1. INTRODUCTION

Gangliosides are normal constituents of the external layer of the plasma membranes of vertebrate cells [1]. Although the main site of ganglioside deposition is the plasma membrane, small amounts of gangliosides occur in soluble form in the brain of different animals and in cultured cells [2–7]. In calf [3] and rat [6] brain cytosolic gangliosides were shown to be present as lipoprotein complexes having varying ganglioside and protein composition. A role for ganglioside-protein complexes as intracellular 'carriers' to and from the plasma membrane or between different intracellular membranes has been suggested [2,5,6].

Addition of gangliosides to the culture medium leads to their insertion into the plasma membrane of cells [8,10–13] followed by penetration into the cell interior. When we exposed cultured human fibroblasts to a radioactive derivative of GM1 ganglioside bearing a photoreactive group in the lipid moiety [13], we observed that the uptake of ganglioside molecules by the cells was mediated by specific membrane proteins. In the present work we administered the same derivative of GM1 to cultured human fibroblasts and showed that a portion of the administered ganglioside reached the cytosol where it was associated with a soluble protein of about 30 kDa.

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2. MATERIALS AND METHODS

2.1. *Reagents*

Commercial chemicals were the purest available. Trypsin and Neu5Ac were from Sigma. *Vibrio cholerae* sialidase was from Behringwerke. All the solutions for fibroblast cultures and washing were from Flow Laboratories. GM1 ganglioside, II^{3}Neu5AcGgOse$_4$Cer, β-Gal-(1--3)-β-GalNAc-(1--4)-[α-Neu5Ac-(2--3)]-β-Gal-(1--4)-β-Glc-(1--1)-Cer, was extracted from pig brain [15], purified over 99% and characterized [16]. [^{3}H]GM1(N$_3$), β-Gal-(1--3)-β-GalNAc-(1--4)-{α-Neu5[^{3}H]Ac-(2--3)}-β-Gal-(1--4)-β-Glc-(1--1)-{2-[12-(2-nitro-4-azidophenyl)amino-dodecanoyl]-amino-3-hydroxy-octadec/eicos-4*t*-ene} (4.2 Ci/mmol), was prepared from GM1 [13]. Human fibroblast cells were prepared and maintained in culture as reported [13].

2.2. *Treatment of fibroblasts with [^{3}H]GM1(N$_3$) and preparation of the fibroblast cytosolic fraction*

[^{3}H]GM1(N$_3$) and GM1 in a molar ratio of 1:10 were dissolved in an appropriate volume of Eagle's minimum essential medium with Earle's salts (EMEM) to obtain a final 10^{-4} M ganglioside concentration. Cells were incubated at 37°C in 2 ml of the ganglioside-containing medium for up to 24 h, and then washed and treated as reported [9,10,13]. All the above-described operations were carried out in the dark.

After UV illumination (360 nm) for 40 min, the cells were pelleted and homogenized in 200 μl of 0.25 M sucrose containing 0.1 mM bisodium EDTA and 1 mM potassium phosphate buffer [16]. The suspension was then centrifuged at 150000 × *g* for 1 h at 4°C. The upper third of the supernatant (cytosolic fraction) was accurately siphoned, the remaining supernatant being disregarded. The collected supernatant was dialyzed, frozen, and lyophilized. The lyophilized material was suspended (1 mg/ml) in 0.01 N potassium-phosphate buffer, pH 7.0, and lipids not covalently linked to the proteins [13] were extracted [14]. An aliquot of the delipidized residue was submitted to liquid scintillation counting, a second aliquot was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [13]. In preliminary experiments the delipidized pellet was

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subjected to *Vibrio cholerae* sialidase treatment to determine the protein-bound radioactive sialic acid.

In further experiments the GM1 derivative was incubated directly with the cytosolic fraction of the fibroblast cell prepared as above and the incubation mixture, after illumination, was treated as reported for radioactive protein analyses.

2.3. *Colorimetric methods*

GM1 ganglioside was assayed as bound Neu5Ac by the resorcinol-HCl method [17,18], pure Neu5Ac being used as the reference standard. The protein content was determined in solubilized pellets and cytosolic fraction [19,20], with bovine serum albumin as standard.

3. RESULTS AND DISCUSSION

The use of photoreactive ganglioside [^{3}H]GM1(N_3) demonstrated that gangliosides, after binding to surface membrane proteins, entered the cell interior and generated metabolic products, thus giving rise to a large number of labeled proteins [13]. The aim of this work was to test if [^{3}H]GM1(N_3) is present in the cell cytosol and is able to bind to cytosolic proteins.

The time course of radioactivity incorporation in the soluble fraction obtained from fibroblasts during incubation with [^{3}H]GM1(N_3) and GM1 is presented in Fig. 1. A measurable amount of radioactivity was already present in the cytosol after one hour of incubation. It increased with time, reaching a value of 720000 dpm/mg cytosolic proteins after 24 h, and at all incubation times the radioactivity carried by the cytosolic fraction was about 4% of the total radioactivity incorporated by the cells. Extraction of free lipids from cytosol diminished the radioactivity content, and the protein-bound radioactivity was about 42000 dpm/mg after 24 h (see Fig. 1). Sialidase treatment of the cytosolic proteins led to the release of 3–4%, for the 24 h incubation, of the total radioactivity, indicating that under the experimental conditions, recycling of sialic acid for cytosolic glycoprotein biosynthesis occurred to a very low extent, and that the radioactivity carried by the proteins is derived from the covalently linked ganglioside.

The SDS-PAGE analysis of cell cytosol after 24 h incubation of cells with ganglioside followed by illumination is presented in Fig. 2. The profile obtained by the Coomassie blue staining procedure is compared with that obtained by fluorography. Few of the proteins present in the cytosol were labeled by the ganglioside and one in particular carried about 70% of the total protein-bound radioactivity. This protein corresponds to a band which shows a molecular weight of about 30 kDa. A band carrying a large amount of radioactivity and corresponding to lipid material is present at the lower edge of the SDS-PAGE. The observation that only a small number of cytosolic proteins became labeled indicates that the binding of gangliosides to cytosolic proteins might be a rather specific process and supports a previous suggestion indicating the occurrence of specific ganglioside-protein complexes in the cytosol

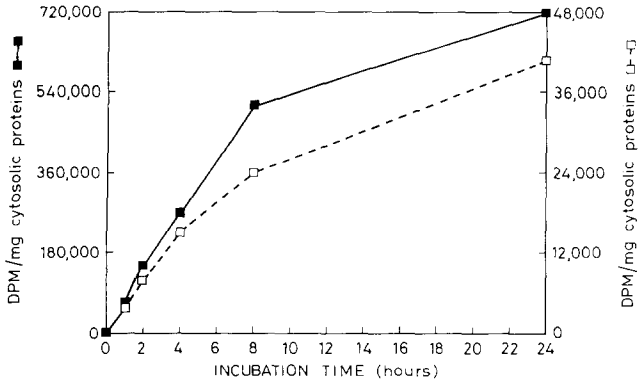

Fig. 1. Time course of the radioactivity incorporation into the cytosol of human fibroblast cells, after cell incubation with the mixture GM1 and [^{3}H]GM1(N_3). Solid line, total incorporated radioactivity; broken line, radioactivity linked to proteins.

[6]. This was reinforced by further experiments in which the cytosolic fraction of fibroblasts was incubated with GM1 and [^{3}H]GM1(N_3) for 2 h. The fluorographic SDS-PAGE protein profile (gangliosides were extracted before protein analyses) is presented in Fig. 2. In this experiment the quantity of GM1 linked to proteins was higher, although the ganglioside-protein interactions differ from those occurring using

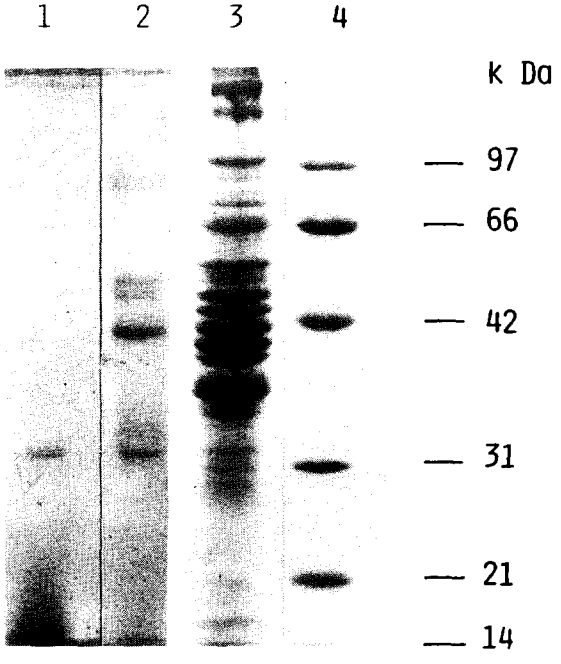

Fig. 2. SDS-PAGE separation of human fibroblast cytosolic proteins. (Lane 1) Cytosol of cultured fibroblasts after 24 h incubation of the cells with the mixture GM1 and [^{3}H]GM1(N_3), 4.3 μg of proteins, 3100 dpm, autoradiographic detection; (lane 2) protein pattern after 2 h of incubation of GM1 and [^{3}H]GM1(N_3) with the cytosol of cultured fibroblasts and lipid extraction, 5 μg of proteins, 5500 dpm, autoradiographic detection; (lane 3) protein pattern of the cytosol of cultured fibroblasts, 15 μg of proteins, Coomassie blue staining; (lane 4) mixture of standard proteins, 20000 dpm, autoradiographic detection.

intact cells. Only a small number of bands is present; one band showing a molecular weight of about 30 kDa is still evident and is a major component of the radioactive protein pattern.

Characterization of the soluble 30 kDa protein and verification of its functional implications require further study.

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