A photo-reactive derivative of ganglioside GM1 specifically cross-links VIP21-caveolin on the cell surface

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Abstract Previous studies have shown that sphingolipids may be enriched in caveolae, plasmalemmal invaginations implicated in endocytosis and signal transduction. We synthesised a radiolabeled derivative of ganglioside GM1 bearing a photo-reactive cross-linker at the end of its acyl chain. After insertion in the plasma membrane of cultured A431 or MDCK cells and photoactivation, the main protein cross-linked by the GM1 derivative was VIP21-caveolin, an essential structural component of caveolae. This result shows close proximity between GM1 molecules and VIP21-caveolin in the caveolar membrane and strongly implicates sphingolipid segregation in the biogenesis of caveolae.

Key words: Caveolae; Cross-linking; Ganglioside GM1; Sphingolipid; VIP21-caveolin

1. Introduction

Caveolae are specialised plasma membrane invaginations with defined size and morphology [1]. Although traditionally described as non-coated pits, caveolae bear on their cytoplasmic side a coat-like array of filaments revealed by non-conventional electron microscopy techniques [2,3]. In contrast to other coat structures such as the clathrin lattice, the caveolar coat seems insensitive to treatments that strip peripheral components from membranes [4]. VIP21-caveolin, a membrane protein originally identified as a major substrate of the v-Src kinase [5], is localized in caveolae [4,6–8]. Immunofluorescence studies showed that both the N- and the C-terminus of the protein are cytosolic while the central hydrophobic region forms a hairpin-structure within the membrane [8]. This unique topology has been recently confirmed [9]. Several independent observations indicate that VIP21-caveolin plays a structural role in caveolae, probably as a component of their coat. First, VIP21-caveolin has been localised by immunogold staining on the coat filaments detected by freeze-etch electron microscopy [4]. Second, VIP21-caveolin forms high molecular weight complexes resistant to detergent solubilisation [9]. Third, the density of the protein in the caveolar membrane, estimated by immunogold electron microscopy, is extremely high [8,10]. Finally, expression of VIP21-caveolin in lymphocytes lacking the protein induces de novo formation of caveolae [10].

A very interesting feature of caveolae is the organisation of their lipid bilayer, which seems to differ from that of the surrounding plasma membrane. After cell extraction with non-ionic detergents at low temperature, VIP21-caveolin is found in low-density complexes in association with cholesterol and sphingolipids [11,12]. Cholesterol is required for the integrity of caveolae since sterol-binding drugs such as nystatin or filipin disorganise the coat morphology [4]. A concentration of gangliosides in caveolae has been also revealed by electron microscopy studies using tetanus toxin and cholera toxin B subunit (CTB) as specific ligands [13,14]. Interestingly, enrichment of the monosialoganglioside GM1 in caveolae was greatly enhanced by adding the pentavalent CTB to cells [14], probably due to a clustering effect similar to that suggested for GPI-anchored proteins [15].

In this study, we make use of a photoreactive derivative of ganglioside GM1 as a probe to investigate glycolipid-protein interactions within the plasma membrane of living MDCKII and A431 cells.

2. Materials and methods

2.1. Chemicals

Cholera toxin B subunit was purchased from Sigma. Recombinant CTB, free of subunit A, was kindly provided by Dr. R. Rappuoli (Biocine, Siena, Italy). GM1 ganglioside was extracted from bovine brain according to [16] and characterised as previously described [17]. $NaB[^3H]_4$ was purchased from Amersham.

2.2. Synthesis of $[^3H]GM1(N_3)$

The radioactive and photoactivatable derivative of GM1 ganglioside ($[^3H]GM1(N_3)$) was prepared from natural GM1 ganglioside according to the following steps. GM1 was converted into lyso-GM1 by specific alkaline hydrolysis [18]. Lyso-GM1 was then acylated with disuccinimidyl carbonate-activated aminododecanoic acid according to [19] and tritium labeled at position 6 of terminal galactose by the galactose oxidase/$NaB[^3H]_4$ procedure [20] modified as follows. 20 mg of GM1 derivative were suspended in 15 ml Tris-HCl 50 mM, pH 7.4, containing 250 mg Triton X-100. After addition of 1200 U galactose oxidase and overnight incubation at 37°C, the oxidised GM1 derivative was purified on a Kieselgel 100 column (100 × 1.5 cm), equilibrated and eluted with chloroform/methanol/water, 60:35:5 (v/v/v). The elution profile was monitored by TLC. Reduction of the oxidised GM1 derivative with $NaB[^3H]_4$ (500 mCi, 70 Ci/mmol) was performed in 1.5 ml propanol/water, 7:3 (v/v). The reaction mixture containing the tritium labeled GM1 derivative was then applied on a Sep-Pak C18 cartridge, washed with 20 ml water and eluted with methanol. Introduction of the photoactivable nitrophenylazide on the terminal amino group of the acyl chain was performed as described previously [19]. The final specific activity of $[^3H]GM1(N_3)$ was 15 Ci/mmol. Before the cross-linking experiments the solvent was evaporated and the photo-reactive GM1 resuspended in MEM-10 mM HEPES (MEM-H).

2.3. Cross-linking experiments

A431 and MDCKII cells were cultured as previously described [8]. For the incorporation of the ganglioside analogue into the plasma membrane, 80–90% confluent cells in 10-cm dishes were washed with

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Abbreviations: BSA, bovine serum albumin; CTB, cholera toxin B subunit; GPI, glycosylphosphatidylinositol; MDCK, Madin-Darby canine kidney cell line; PBS, phosphate-buffered saline.

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MEM-H and incubated for 3 h at 8°C with 2.5 ml of the same medium containing 6 μM [^{3}H]GM1(N_3). After removal of the unincorporated ganglioside, unspecific binding was minimised by washing the cells for 30 min at 8°C with MEM-H/0.2% BSA [21]. When indicated, cells were further treated at 8°C with 3 μg/ml CTB in MEM-H/0.2% BSA. All the above incubations were performed in the dark. The cells were then washed with ice-cold PBS, irradiated for 10 min on ice with an UV lamp at 366 nm, scraped and homogenised by sonication in 500 μl Tris-HCl 10 mM, pH 7.4. The cell homogenate was brought to 1.5 M sucrose, transferred to a centrifuge tube and overlayed with 8.5 ml of 1.2 M and 2.5 ml of 0.8 M sucrose in Tris-HCl 10 mM, pH 7.4. After flotation for 18 h at 250,000 × g, 4°C, membranes at the 0.8/1.2 M sucrose interface fraction were diluted two-fold and pelleted for 1 h at 230,000 × g. The pellet was either treated for immunoprecipitation as below or directly solubilised in lysis buffer [22] for 2D gel analysis.

For the in vitro cross-linking of the GM1 probe, 200 μl of 6 μM [^{3}H]GM1(N_3) in MEM-H, either alone or in the presence of 3 μg/ml CTB, were photo-activated at 366 nm for 10 min. After precipitation with 90% ice-cold acetone, the pellet was solubilised for 30 min at 37°C in lysis buffer for 2D gel analysis.

2.4. Immunoprecipitation of VIP21-caveolin

Membranes prepared as described above were solubilised at 37°C for 20 min in 500 μl of the following buffer: 25 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% gelatin, and a mixture of protease inhibitors containing 20 μg of chymostatin, leupeptin, antipain and pepstatin. The lysate was centrifuged at 3000 × g for 10 min and then incubated at 4°C for 1 h with affinity-purified rabbit anti-VIP21 antibodies against the N-terminal peptide 14–33 [8]. After Protein A Sepharose (PAS) was added, incubation was prolonged for 2 h at 4°C. The PAS beads were then washed and eluted at 37°C for 30 min in the lysis buffer for 2D gel analysis.

2.5. 2D gel electrophoresis

2D gels analysis was performed using the BioRad Mini-protean II 2D system according to the manufacturer's recommendations, except for the first dimension mixture that was prepared as described in [22]. 15% gels were used for the second dimension. For fluorography gels were enhanced with Entensify (Dupont) and exposed to X-omat films (Kodak).

3. Results and discussion

Our experimental strategy was designed to specifically investigate ganglioside-protein interactions occurring within the plasma membrane. A radio-labeled photo-reactive GM1 derivative ([^{3}H]GM1(N_3)) was prepared by modifying the synthesis of an analogue previously used to probe for interacting proteins in fibroblasts and cerebellar granule cells [23,19]. In order to obtain a higher specific radioactivity, the reagent used here was labeled on the terminal galactose (Fig. 1).

Since the cell surface distribution of endogenous GM1 had been thoroughly investigated by electron microscopy in A431 fibroblasts [24,14], these cells were selected for the present study. Binding of [^{3}H]GM1(N_3) to cells and cross-linking were performed at 8°C to minimise endocytosis of the probe. No internalisation of GM1 was detected by electron microscopy at this temperature [14]. By using 6 μM [^{3}H]GM1(N_3) in the medium, 125 pmol photo-reactive GM1/mg protein were incorporated over a 3 h incubation. After photoactivation of the probe, an enriched light membrane fraction was prepared which included both the plasma membrane and intracellular smooth membranes [12]. Analysis of the cross-linked molecules contained in this fraction by 2D gel electrophoresis demonstrated a remarkably simple pattern (Fig. 2a). Three radio-labeled proteins were visible in the 20–25 kDa range and another species was detectable in the upper right-end region of the gel. The same pattern was obtained when cells were incubated with CTB (Fig. 2b), a treatment known to favour localisation of GM1 in caveolae [14]. The cross-linked proteins were not visible when the GM1 suspension was photo-activated in vitro, either in the absence (Fig. 2c) or in the presence of CTB (Fig. 2d). Many low molecular weight products accumulated at the gel front, while high molecular weight material, including cross-linked CTB, did not enter the second dimension gel. A very similar pattern was observed in a subsequent experiment in which A431 fibroblasts were compared to MDCKII epithelial cells (Fig. 3).

The main cross-linked proteins in the 20–25 kDa range, indicated by arrowheads in Fig. 3, migrated as the VIP21-caveolin isoforms previously described in MDCKII cells [6]. To confirm the identity of these proteins, membranes derived from a cross-linking experiment performed on A431 cells were solubilised in Triton X-100 at 37°C and immunoprecipitated with anti-VIP21-caveolin antibodies. Indeed, the immunoprecipitated material analysed by 2D gel electrophoresis (Fig. 4) consists of the two VIP21-caveolin isoforms as well as of a VIP21-caveolin

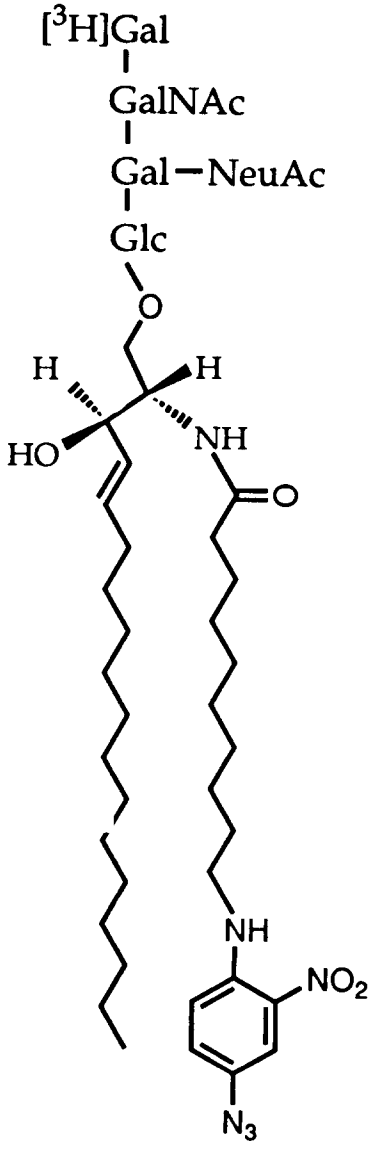

Fig. 1. Structure of [^{3}H]GM1(N_3). Note the tritium labeling on the terminal galactose and the cross-linker position at the end of the acyl chain.

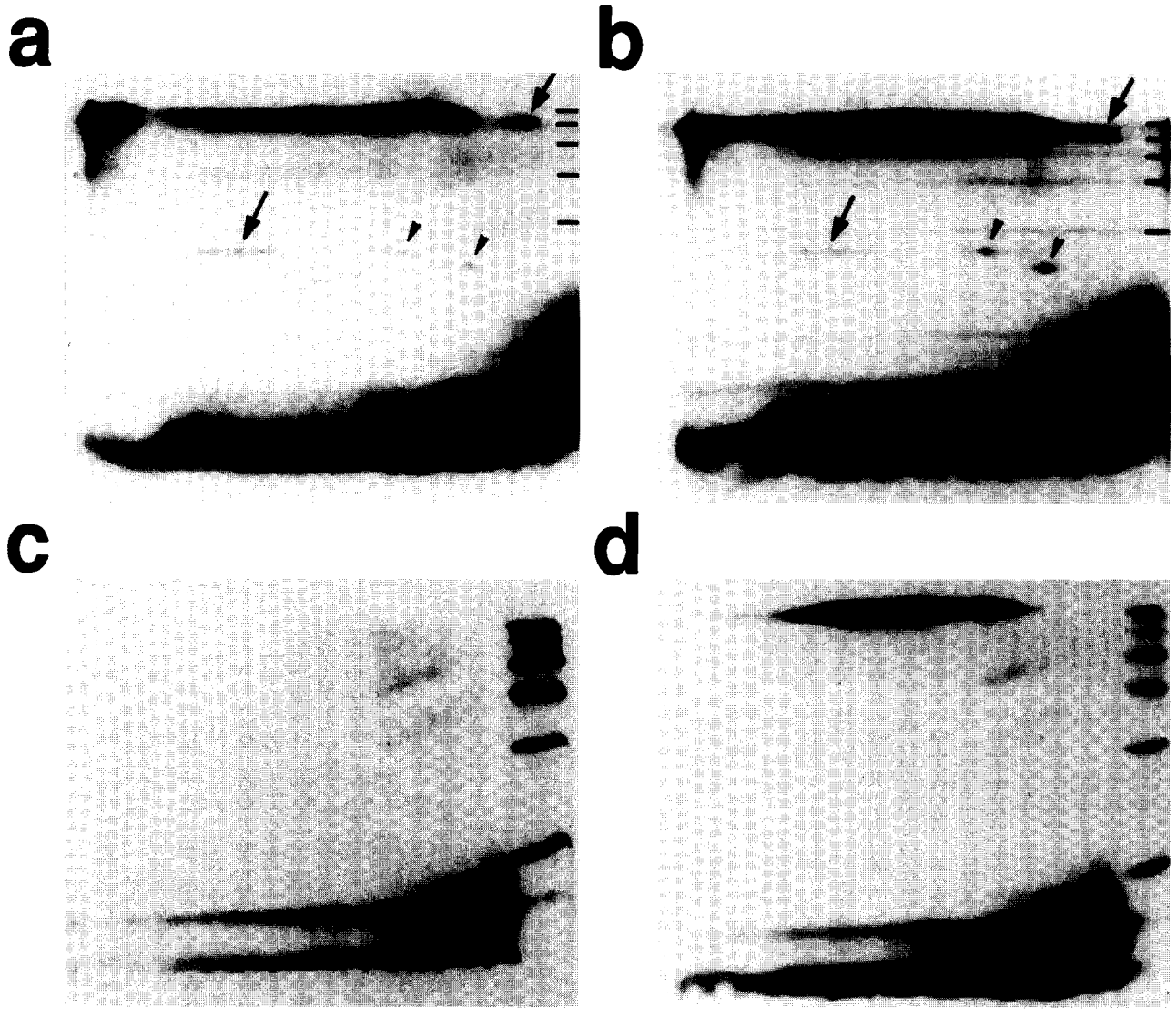

Fig. 2. 2D gel analysis of proteins cross-linked by [^{3}H]GM1(N_3) in A431 cells, either in the absence (panel a) or in the presence of CTB (panel b). In vitro cross-linking of [^{3}H]GM1(N_3), either alone (panel c) or with CTB (panel d), is presented for comparison. Specific cross- linked products are marked by arrows and arrowheads. Molecular weight markers: myosin, 200 kDa; phosphorylase B, 97.4 kDa; bovine serum albumine, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; lysozyme, 14.3 kDa.

oligomer of over 200 kDa molecular weight. This oligomer, partially resistant to SDS treatment, has been described previously [9]. As expected for the cross-linked proteins, the GM1-conjugated VIP21-caveolin forms are shifted to a higher molecular weight and a more acidic pI as compared to unmodified VIP21-caveolin.

The selective cross-linking of VIP21-caveolin by the GM1 analogue demonstrates a direct interaction of a ganglioside with VIP21-caveolin within the plasma membrane. Cholera toxin treatment seems to promote, but is not strictly required, for the cross-linking of GM1 to VIP21-caveolin. Since GM1 is also present in the plasma membrane areas between caveolae one has to assume that photoreative GM1 is being cross-linked randomly to other plasma membrane proteins as well but that these do not show up in the 2D gel analysis because none of the proteins are sufficiently enriched in the vicinity of GM1 molecules.

Previous studies have suggested that sphingolipids are enriched in caveolae on the basis of co-purification of VIP21-caveolin with light detergent-insoluble complexes containing preferentially sphingolipids and cholesterol, as well as GPI-anchored proteins [6,11,12]. However, these studies did not specify whether the different components were in the same or distinct complexes. In fact the data available suggest that the detergent-insoluble complexes derive from different cellular compartments. It is also important to point out that similar complexes have been isolated from cells lacking VIP21-caveolin and caveolae [25].

It has been shown that (glyco)sphingolipids and cholesterol undergo lateral phase separation in lipid bilayers (reviewed in [26]). This segregation phenomenon could play a crucial role in caveolar biogenesis. Glycolipid clustering, known to strongly decrease membrane fluidity [27–29], could induce sphingomyelin to co-segregate [30]. Given the known preference of sphingomyelin for cholesterol [31], this latter lipid would also associate with the clusters. Proteins with a preference for one of these

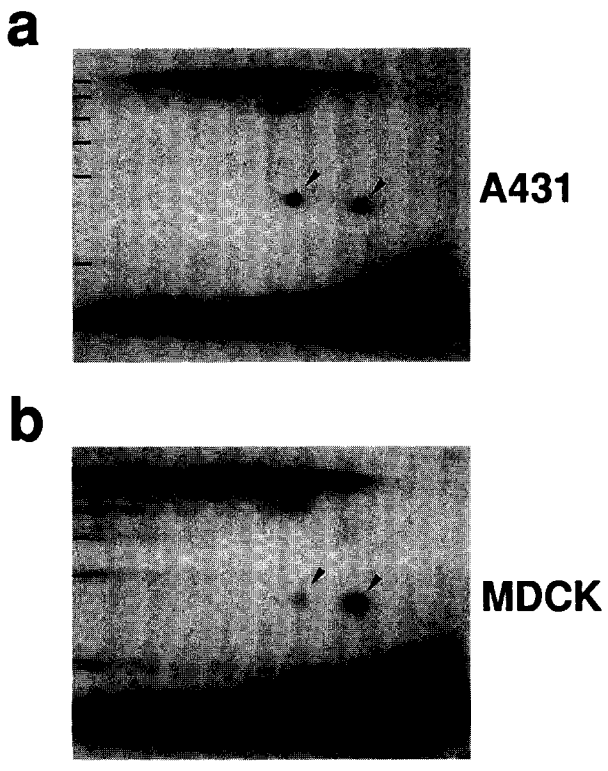

Fig. 3. 2D gel analysis of proteins cross-linked by [^{3}H]GM1(N_3) in A431 cells (panel a) compared to MDCKII cells (panel b), in the presence of CTB. The two spots indicated by arrowheads are putatively derived from cross-linking of isoforms of VIP21-caveolin. Molecular weight markers are as in Fig. 2.

lipids could facilitate clustering and stabilize the lipid domain. Recent results demonstrate that VIP21-caveolin is a cholesterol-binding protein and may act as a stabilizer [32]. The protein-bound cholesterol would associate with sphingolipids including GM1 and thus increase the concentration of the latter lipids around VIP21-caveolin [33].

Finally, the detection of other labeled proteins beside VIP21-caveolin opens the possibility that these are also present in caveolae and the cross-linking procedure described here may prove valuable for further analysis of caveolar components.

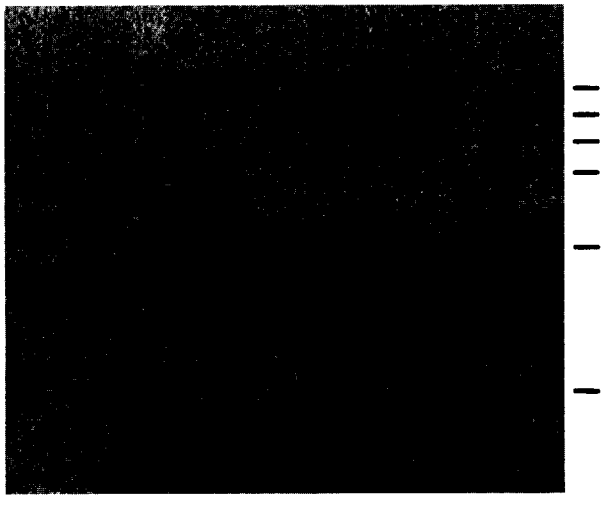

Fig. 4. 2D gel analysis of VIP21-caveolin cross-linked by [^{3}H]GM1(N_3), immunoprecipitated from A431 cells by specific antibodies. The monomeric isoforms as well as the VIP21-caveolin oligomer are marked by arrowheads. Molecular weight markers are as in Fig. 2.

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