

Ganglioside dependent cell attachment and endocytosis of murine polyomavirus-like particles

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Abstract For murine polyomavirus (Py), previous studies suggest the cellular target is a terminal $\alpha 2,3$ -linked sialic acid. Here, we investigate the binding and uptake of mouse polyomavirus-like particles (PyVLP) derived from bacterially expressed VP1. We find that in fibroblast 3T6 cells, binding of PyVLP was substantially reduced by sialidase treatment, but only moderately affected by protease treatment, suggesting glycolipids such as the sialic acid-containing gangliosides mediate cell attachment. We further tested the entry requirement of PyVLP using the ganglioside-deficient GM95 murine cell line, and find PyVLP binding and entry were reduced in these cells. Finally, we find that addition of gangliosides G_{M1} , G_{D1a} , and G_{T1b} to GM95 cells restored cellular PyVLP binding and uptake. Taken together, results indicate that gangliosides function in PyVLP cell attachment and endocytosis.

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Key words: Polyomavirus-like particle; Ganglioside; Endocytosis; Animal virus entry

1. Introduction

To replicate in a host cell, animal viruses have evolved multiple strategies for binding to the cell surface, penetrating cellular membranes and uncoating their encapsulated genome. For the small, non-enveloped murine polyomavirus (Py), binding is receptor mediated, and following endocytosis, leads to the nuclear delivery of a replication-competent genome. Binding of Py requires a direct interaction of the host plasma membrane receptor with surface-exposed domains of the 45 nm viral capsid [1]. The viral capsid shell is constructed of the major structural protein, VP1, where 72 pentons are organized into a $T=7$ icosahedral structure that encases the double-stranded DNA genome and cellular histones [2]. VP1 binds the minor structural proteins VP2 and VP3 [3], which are masked within the capsid shell. The structural integrity of the capsid requires only VP1, as *Escherichia coli*-derived VP1 can be assembled in vitro into virus-like particles (PyVLP) [4].

Evidence by Caruso et al. implicates $\alpha 4, \beta 1$ integrin as a post-attachment receptor for Py [5], whereas biochemical and structural evidence suggests the target for Py cell attachment is a terminal $\alpha 2,3$ -linked *N*-acetyl neuraminic acid, com-

monly known as sialic acid [6–8]. Sialic acid modifications are found on a variety of cell surface glycoproteins, and on a subfamily of glycosphingolipids called gangliosides. Gangliosides are known to be the cellular target for several toxins, including cholera toxin (G_{M1}), tetanus toxin (G_{D1b} , G_{T1b}) and botulinum toxin (G_{T1b} , G_{Q1b}). These glycosphingolipids are composed of ceramide linked by a glycosidic bond to an oligosaccharide chain of hexoses, including glucose, galactose and *N*-acetylgalactosamine, and $\alpha 2,3$ - or $\alpha 2,8$ -linked sialic acid units. Terminal $\alpha 2,3$ -linked sialic acid is found on one of two types of branches. The first branch type, found in G_{M3} , G_{M2} , and G_{M1} , contains linkages to ceramide extending as: ceramide-glucose- $\beta 1,4$ -galactose- $\alpha 2,3$ sialic acid. The second occurs in gangliosides such as G_{D1a} and G_{T1b} on a longer galactose branch, where the linkages are: ceramide-glucose- $\beta 1,4$ -galactose- $\beta 1,4$ -*N*-acetylgalactosamine- $\beta 1,3$ -galactose- $\alpha 2,3$ sialic acid.

Here we investigate the binding and uptake of mouse Py using in vitro assembled virus-like particles as a non-infectious source of Py. Protease-treated cells remain competent for binding PyVLP, whereas cholera toxin and antibodies to G_{M1} function as competitive inhibitors for Py binding, suggesting that glycolipids including ganglioside G_{M1} may function at the level of cell attachment. We investigate the receptor usage and internalization of PyVLP further using the ceramide-glucosyl-transferase-deficient murine cells, GM95, which lack gangliosides. In the GM95 cells, we find that cell association and internalization of PyVLP and cholera toxin is reduced. Finally, upon addition of gangliosides to the GM95 cells, binding and endocytosis of PyVLP is restored in the ganglioside-supplemented cells.

2. Materials and methods

2.1. Cell culture

3T6 Swiss albino fibroblasts, and the mouse B16 melanoma mutant cell lines MEB4 and GM95 were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 10% fetal calf serum (FCS) (LabForce AG) and 4 mM glutamax (Gibco) at 37°C in 5% CO₂. For biochemical assays, 3T6, MEB4 and GM95 cells (80% confluent) were removed with 0.8% ethylenediamine tetraacetic acid-phosphate-buffered saline (EDTA-PBS $-/-$). The GM95 and MEB4 cells were a kind gift from H. Sprong [9].

2.2. Antibodies and reagents

Purification of wild-type VP1 and VLP assembly was as described [10]. Fluorescent VLP capsids were prepared by covalent coupling of dye according to the manufacturer's directions. Specifically, Alexa-Fluor-594- or fluorescein isothiocyanate (FITC)-succinimidyl ester derivatives (Molecular Probes) were coupled at a 5-fold molar excess of dye to VP1 protein, and unbound dye was removed by chromatog-

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raphy with a Nap-5 column (Pharmacia). For live cell microscopy, 0.1–0.5 μg PyVLP was added per 1×10^6 cells on a 18 mm coverslip. Unlabeled cholerae toxin β subunit (Sigma) was used at the indicated concentrations for biochemical competition experiments. Cholera toxin β subunit labeled with FITC or AlexaFluor-488 (Sigma) (0.1–1 $\mu\text{g}/\text{ml}$), and transferrin-FITC (5 $\mu\text{g}/\text{ml}$) (Molecular Probes) were used for microscopy. Polyclonal antibodies to G_{M1} (US Biologicals) were used for competition experiments at a final dilution of 1:50, and for immunofluorescence (IF) at 1:200. Highly purified monoclonal antibodies to G_{T1b} and G_{D1a} (US Biologicals) were used for IF at a dilution of 1:100 (10 $\mu\text{g}/\text{ml}$), and did not block uptake of PyVLP under any conditions.

2.3. Enzyme-treated cell preparation

For enzyme-treated cells, neuraminidase (0.1 unit *V. cholerae*/10⁶ cells) (Sigma) pretreatment was at 30°C for 1 h at pH 6.0 to activate the enzyme, cells were washed and pH adjusted to 7.0 for binding. Trypsin (0.1%) (Gibco) pretreatment was at 37°C for 30 min, followed by serum inactivation. Proteinase K pretreatment (5 mg/ml) (Boehringer Mannheim) was for 1 h at 30°C, followed by washing. Cells were counted before processing.

2.4. Glycolipid supplementation assay

Supplementation of glycolipids was as described [11] with gangliosides (Avanti, US Biologicals) and lactosylceramide-C5-bodipy (5 μM) (Molecular Probes). Briefly, gangliosides (20 $\mu\text{g}/\text{ml}$) were incubated as micelles for 12 h at 37°C in serum-free media. Cells were viable for 18 h. After overnight incubation, cells were shifted to 4°C, washed in DMEM containing 25% serum and incubated briefly with trypsin. Cells were washed with FCS-containing media, and with binding buffer before the addition of ligands. For IF of supplemented gangliosides, cells were fixed with 4% final formaldehyde, and antigen was detected with specific antibodies as indicated (antibodies and reagents) in absence of permeabilization.

2.5. ¹²⁵I-PyVLP-biotin-SS attachment and internalization assay

Generation of ¹²⁵I-PyVLP-biotin-SS was as described [12]. For PyVLP, a specific activity of 3.4×10^6 counts per minute (cpm) per μg was determined. For binding and internalization assays 0.3 μg ¹²⁵I-PyVLP-biotin-SS per 0.5×10^6 cells were used. Binding and internalization assay were as described [12]. Radioactivity from cell-associated (binding) or immunoprecipitated biotin-tagged virus (internalization) is expressed as percentage of total input activity, after background subtraction (background was approximately 5%). For antibody and cholera toxin blocking experiments, cells were pretreated for 1 h at 4°C with competitor, and then processed, where the competitor remained in the binding mix.

2.6. Microscopy

Confocal images were acquired with an inverted Zeiss LSM 510 meta (100 \times objective, N.A. 1.4, pinhole setting at 1 airy unit). Epifluorescence microscopy was performed using a Zeiss Axiovert microscope with a 100 \times /1.40 N.A. plan-Apochromat lens, and images were collected with a CCD camera (Hamamatsu Inc.). Quantitation of confocal images in Fig. 1A was done using Zeiss LSM 510 analysis tools (Carl Zeiss) and Excel (Microsoft). The average fluorescence intensity per squared micron was calculated from the selected region of interest (ROI) for the two channels (T-1, red; T-2, green) from a 12-bit image, and graphed in arbitrary units (1–4095). Images were exported as 8-bit TIFF's from Openlab LIFF (Improvision) and Zeiss file formats (.lsm), and were processed in Photoshop 6.0 (Adobe).

3. Results

Polyomavirus-like particles (PyVLP) were generated from bacterially derived VP1, and fluorescently labeled with AlexaFluor-594- or FITC-succinimidyl ester (Py594, PyFITC). Throughout this work, cell association (or binding) was analyzed in cells shifted to 4°C to block endocytosis, and uptake (or endocytosis) was evaluated following release from the temperature block with a shift to 37°C. During initial studies, we monitored the cell association of PyVLP and cholera toxin β , which is a well-studied ligand for caveolae-mediated endocy-

tosis [13,14] and perhaps other uptake pathways [14]. AlexaFluor-488-labeled cholera toxin β (Ctx488) and Py594 were simultaneously added to 3T6 cells for 30 min at 4°C. Following fixation, analysis using confocal microscopy showed that for Ctx488 uptake was extremely heterogeneous, and that cell-

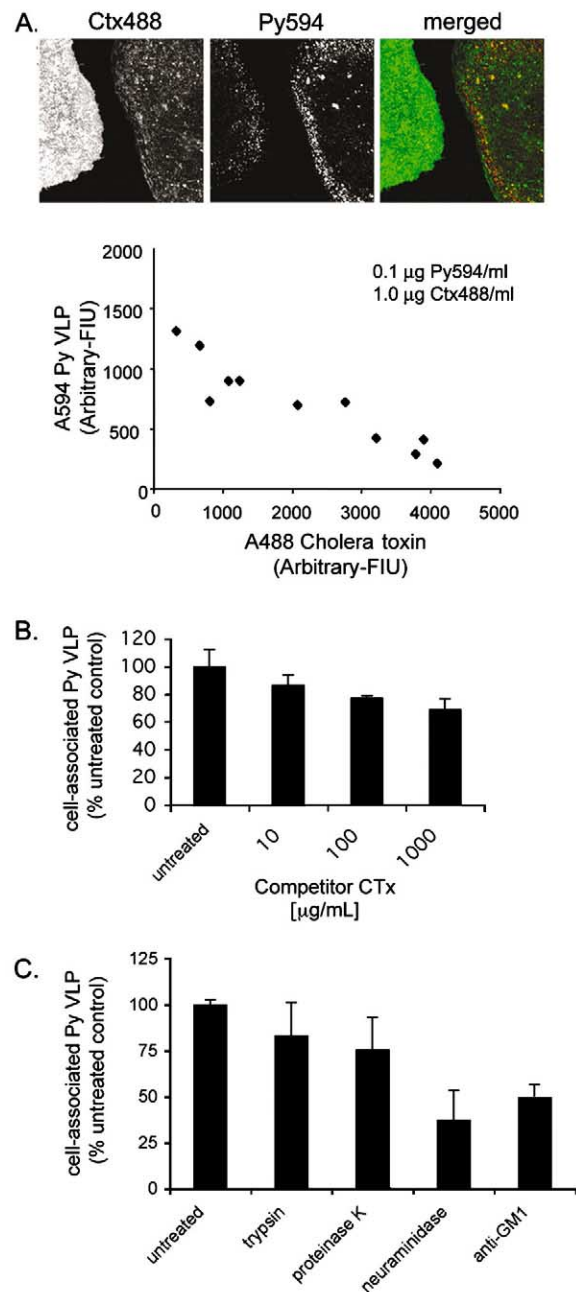


Fig. 1. Cell association of PyVLP is lipid mediated. A: Images: Cholera toxin β -AlexaFluor-488 (Ctx488) and PyVLP-AlexaFluor-594 (Py594) were simultaneously visualized in 3T6 cells with confocal microscopy. Grayscale (left, Ctx488; middle, Py594), and merged (right) images are presented. Graph: Quantitation of average fluorescence intensity per squared micron for ROI ($n=11$) of the two channels (T-1, red, T-2, green) from 12-bit image is graphed in arbitrary units (1–4095). B: Cell-associated cpm of ¹²⁵I-Py-biotin (1 μg virus in 1 ml) in the presence of competitor Ctx is graphed as percent untreated control. Error bars are average of three independent experiments. C: Cell-associated cpm of ¹²⁵I-Py-biotin in protease (trypsin, proteinase K), sialidase (neuraminidase), or anti- G_{M1} pretreated cells is graphed as above.

associated Py594 seems to be reduced in cells where Ctx488 was robustly bound (Fig. 1A, top panel). We measured the average fluorescence intensity units per square micron for both probes in randomly selected 3T6 cells ($n=11$) and noticed a trend suggesting that binding of PyVLP is reduced where cholera toxin β cell association was most efficient (Fig. 1A, bottom panel). We further observed the reciprocal reduction in cell-associated Ctx in the presence of excess PyVLP (data not shown).

To quantify whether there was, indeed, a dose-dependent inhibition of PyVLP in the presence of Ctx, we generated radio-iodinated PyVLP for use in a biochemical uptake assay [12]. The iodinated PyVLP was additionally biotin labeled with a compound that contained a disulfide bond in the spacer region, sulfo-NHS-SS-biotin, to generate ^{125}I -Py-biotin. Cell association can thus be distinguished from internalization upon treatment with a membrane-impermeable reducing agent where only intracellular virus maintains a biotin tag. The intracellular virus can then be specifically immunoprecipitated with an anti-biotin antibody. 3T6 cells were incubated with increasing concentrations of Ctx for 30 min at 4°C and ^{125}I -Py-biotin was added to the cells in the presence of competitor Ctx for an additional 1 h at 4°C to allow for virus–cell attachment. Total cell-associated radioactivity expressed as cpm was compared between samples relative to the untreated control (Fig. 1B). Results indicate that in the presence of excess cholera toxin, cell association of PyVLP is reduced (maximal reduction = $31.1 \pm 7.9\%$).

Cholera toxin β uses ganglioside G_{M1} [15], a glycolipid that contains one α 2,3-linked sialic acid residue, as a specific receptor. We speculated that the observed reductions in cell-associated PyVLP in the presence of cholera toxin (Fig. 1A and B) was due to a direct competition at the cell surface for G_{M1} . This suggested the possibility that a glycolipid functions as a cellular receptor for PyVLP.

To test this hypothesis, we first asked if PyVLP cell association was altered when plasma membrane proteins are removed with proteases. Cell-associated radioactivity (cell-associated cpm) following binding at 4°C for 1 h of ^{125}I -Py-biotin in trypsin (cell-associated cpm = $83.2 \pm 18.3\%$) or proteinase K (cell-associated cpm = $75.6 \pm 17.7\%$) pretreated cells was partially reduced compared to that of untreated cells (cell-associated cpm = 100%). We next verified that cell attachment was, indeed, dependent upon sialic acid. When 3T6 cells were treated with the sialidase, called neuraminidase, cell-associated cpm of ^{125}I -Py-biotin was extensively reduced (cell-associated cpm = $37.4 \pm 15.2\%$) (Fig. 1C). Finally, we find that binding of ^{125}I -Py-biotin was also reduced in the presence of a polyclonal antibody to G_{M1} (cell-associated cpm = $51 \pm 6.8\%$) (Fig. 1C). Using confocal microscopy, anti- G_{M1} antibody competition was also observed to inhibit Py594 binding to 3T6 cells (data not shown). Taken together, these data suggest that the bulk of cell attachment of PyVLP in 3T6 cells is lipid mediated, and that the ganglioside G_{M1} is a candidate target.

To test the requirement for gangliosides in the binding and endocytosis of PyVLP, we obtained a murine B16 melanoma cell line mutagenized subclone, GM95, which lacks the ceramide-glucosyl-transferase gene and has no glycolipids [9,16,17]. We visualized the cell-associated Py594 and Ctx488 in 3T6 and GM95 cells with epifluorescence microscopy. We observed no cell-associated Ctx488 (Fig. 2A, panels C, D) in GM95 cells, and that cell-associated PyVLP (Fig. 2A, panels

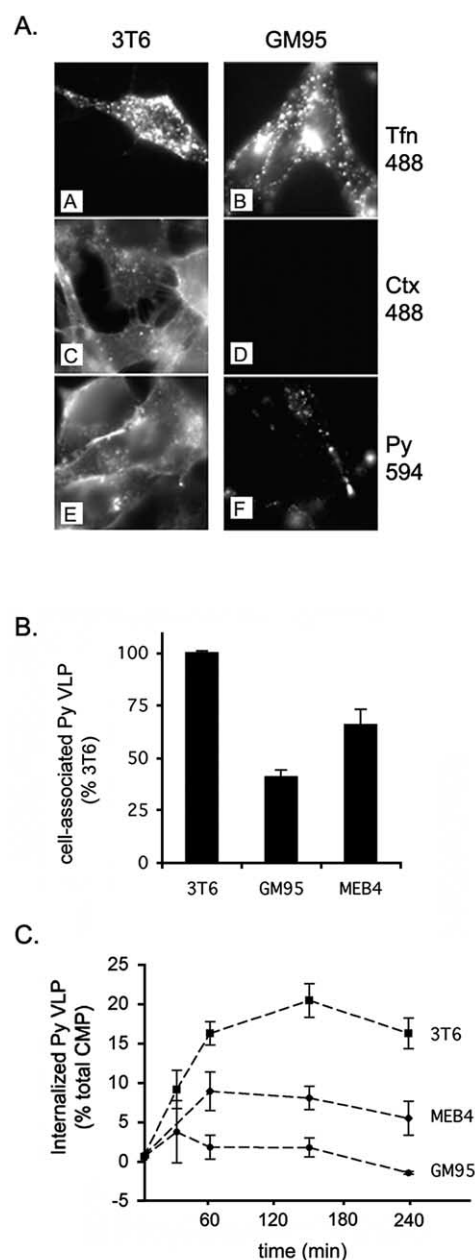


Fig. 2. Binding and endocytosis of PyVLP is reduced in the glycolipid-deficient cells, GM95. A: Epifluorescent micrographs of transferrin-AlexaFluor-488 (Tfn488), cholera toxin β -AlexaFluor-488 (Ctx488), and PyVLP-AlexaFluor-594 (Py594) in 3T6 (A, C, E, respectively) and in GM95 (B, D, F, respectively) cells. B: Cell-associated cpm of ^{125}I -Py-biotin in 3T6, GM95 and MEB4 cells is graphed as a percent of 3T6 binding (100%). C: Timecourse of ^{125}I -Py-biotin uptake in 3T6 (■), MEB4 (◆) and GM95 (●) cells, where the internalized fraction of virus is graphed as a percent of total virus input following background subtraction.

E, F) was reduced in the GM95 cells relative to the 3T6 cells. A substrate for clathrin-mediated uptake, AlexaFluor-488-transferrin (Tfn488), was internalized with similar efficiency in 3T6 and GM95 cells (Fig. 2A, panels A, B).

Using the biochemical uptake assay, we quantified the levels of cell-associated ^{125}I -Py-biotin following binding at 4°C for 1 h for three murine cell lines: 3T6, GM95 and MEB4 cells. MEB4 cells are a subclone of B16 melanoma cells containing a functional ceramide-glucosyl-transferase gene and, unlike

GM95 cells, the MEB4 cells contain glycolipids, principally G_{M3} and glucosylceramide [16]. We find that in GM95 cells, cell-associated cpm is reduced to $40.9 \pm 3.4\%$, when compared to that of 3T6 cells (100%), while for the MEB4 cells binding was reduced to $65.8 \pm 7.2\%$ relative to 3T6 cells (Fig. 2B). Confocal microscopy of MEB4 and GM95 cells confirms that binding and uptake of PyVLP is enhanced in the MEB4 cells in comparison to GM95 cells (data not shown).

To measure endocytosis, ^{125}I -Py-biotin was bound at 4°C for 1 h, and samples were then shifted to 37°C to allow for internalization in the presence of excess unbound virus. At varying time points, samples were removed, endocytosis was inhibited with a temperature block, and cells were treated with Tris-(2-carboxyethyl) phosphine (TCEP), a membrane-impermeable reducing agent, to specifically cleave the biotin residue from the extracellular virus particles. Cells were lysed and the internalized virus was immunoprecipitated with an anti-biotin antibody. Uptake of virus in the 3T6 cells was rapid and without an observable lag phase in the first 30 min (Fig. 2C), where $20 \pm 2.2\%$ of total radioactivity was internalized at 150 min. For the MEB4 cells, uptake was less efficient where $8 \pm 0.8\%$ of total radioactivity was internalized at 150 min (Fig. 2C). In contrast to 3T6 and MEB4 cells, virus internalization was remarkably inefficient in GM95 cells ($1.7 \pm 1.5\%$ total cpm at 150 min) (Fig. 2C). Taken together, fluorescence microscopy and biochemical data provide evidence that gangliosides play a functional role in the endocytosis of PyVLP. As cell association was also reduced in GM95 cells, gangliosides may be cellular receptors that stimulate uptake of PyVLP.

To directly test a panel of gangliosides for their ability to

rescue binding and stimulate endocytosis of PyVLP, we supplemented GM95 cells with purified, exogenous gangliosides. Exogenous lipids are incorporated into live cells through incubation with lipid micelles, where lipids in the plasma membrane spontaneously exchange with the exogenous pool [11]. However, because we cannot determine the moles of individual gangliosides that properly insert into the plasma membrane during the supplementation assay, comparisons can only be made relative to the untreated control.

We supplemented the GM95 cells with G_{D1a} and G_{T1b} , which contain a long branch terminal $\alpha 2,3$ -linked sialic acid galactose, and G_{M1} with a short terminal $\alpha 2,3$ -linked sialic acid galactose branch. The insertion of lipids into the plasma membrane of GM95 cells was monitored using antigen-specific antibodies against each ganglioside (data not shown). To test endocytosis using live cell confocal microscopy, we followed the internalization of FITC-labeled PyVLP (Py-FITC) in ganglioside-supplemented GM95 cells following binding at 4°C for 1 h, and a temperature shift for 1 h to 37°C . We specifically visualized the intracellular fraction of Py-FITC using an acidified medium, thereby quenching the extracellular FITC fluorescence signal. We find G_{M1} , G_{D1a} and G_{T1b} were each sufficient to rescue uptake (Fig. 3A). The uptake of FITC-cholera toxin β was specifically stimulated only in G_{M1} -supplemented cells (Fig. 3A). We determined the timecourse for uptake of ^{125}I -Py-biotin in GM95 cells following ganglioside addition. Results indicated that in ganglioside-supplemented GM95 cells, internalization is efficiently stimulated relative to the untreated control (Fig. 3B). Lactosylceramide-supplemented cells, lacking sialic acid, did not stimulate internalization of PyVLP (Fig. 3B). Ganglioside-dependent

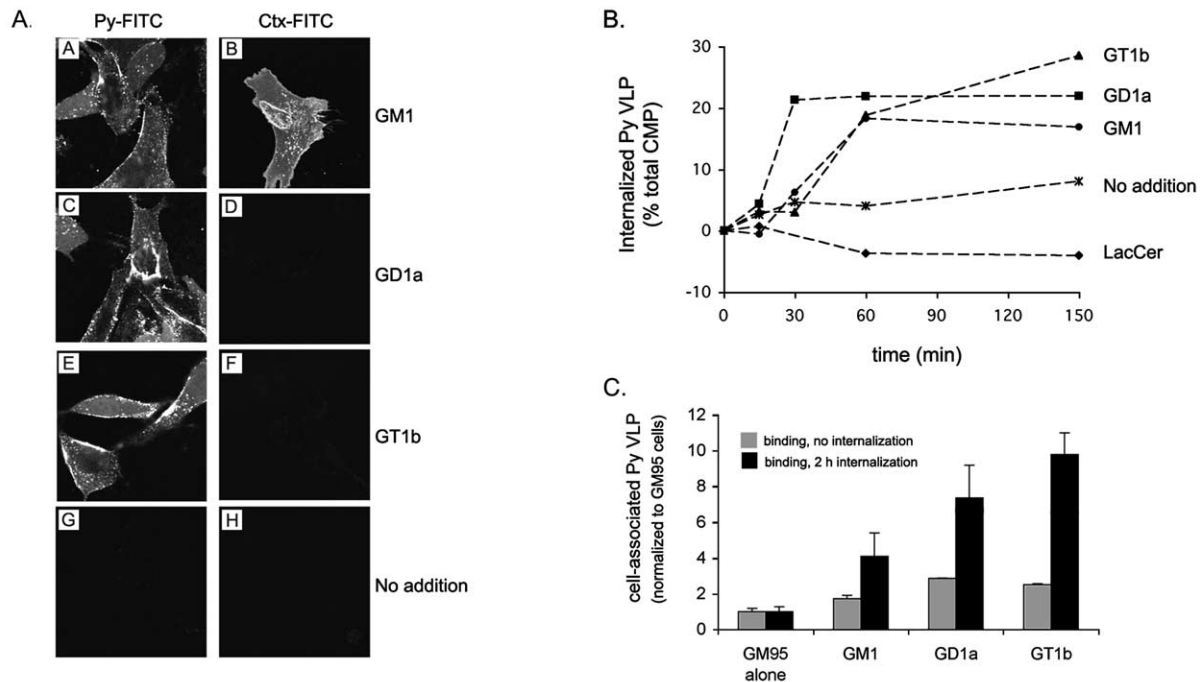


Fig. 3. Addition of gangliosides restores PyVLP cell attachment and internalization in GM95 cells. A: Confocal micrographs in ganglioside-supplemented GM95 cells of cholera toxin β -FITC (Ctx-FITC) or PyVLP-FITC (Py-FITC) where G_{M1} (A, B), G_{D1a} (C, D), G_{T1b} (E, F) or no ganglioside (G, H) was added. Micrographs are of live cells in acidic media to quench extracellular FITC signal. B: Timecourse of ^{125}I -Py-biotin uptake in GM95 cells where cells were supplemented with G_{M1} (●), G_{D1a} (■), G_{T1b} (▲), LacCer (◆), or untreated (*). Internalized fraction of virus is graphed as a percent of total virus input. C: Cell-associated cpm of ^{125}I -Py-biotin in GM95 cells supplemented with G_{M1} , G_{D1a} , or G_{T1b} where total bound fraction at $t=0$ (bound, no internalization) and $t=2$ h at 37°C (bound, 2 h internalization) is normalized to untreated control.

cell association in GM95 cells of ^{125}I -Py-biotin was determined, and G_{M1} -, G_{D1a} -, and G_{T1b} -supplemented cells all showed increased binding activity when compared to untreated control samples (Fig. 3C). The cell-associated cpm accumulated over time during endocytosis for 2 h in ganglioside-supplemented cells, but only poorly in the untreated control cells. Taken together, these data suggest that multiple gangliosides function in cell attachment as receptors and that these glycolipids are required for endocytosis of PyVLP.

4. Discussion

In summary, the major conclusion of this work is that glycolipids function in cell attachment and endocytosis of PyVLP. We showed that glycolipids are preferentially targeted over glycoproteins for cell attachment of PyVLP in 3T6 cells (Fig. 1C), and that supplementation of gangliosides containing terminal $\alpha 2,3$ -linked sialic acid-galactose to a cell line devoid of glycolipids was sufficient to restore binding and efficient uptake (Fig. 3). PyVLP can therefore engage multiple gangliosides for binding and internalization. The availability of gangliosides and the presence of alternative glycoprotein targets are likely major determinants for cellular tropism. The G_{M3} -positive MEB4 cells are an example where only one ganglioside family member is present, and PyVLP endocytosis occurs.

Recent evidence provided by Tsai et al. [18] suggests that gangliosides G_{D1a} and G_{T1b} are required for Py infection. Here, we show that G_{M1} - and G_{M3} -dependent mechanisms for internalization exist for PyVLP in murine cell lines, although uptake may not lead to productive infection [19]. Interestingly, the virus-like particles that bound to GM95 cells in a ganglioside-independent manner lacked endocytic potential (Fig. 2), implicating ganglioside function in a post-attachment process, perhaps via signal transduction. One attractive possibility is that PyVLP endocytosis involves ligand-induced, ganglioside-mediated signal transduction from the outer plasma membrane leaflet to the cytosolic endocytic machinery. It will be important to determine if signaling pathways engaged by PyVLP through various gangliosides differ, and identify determinants for pathogenicity.

Finally, pathogens including virus, bacteria, and their toxins are part of a growing class of ligands that make use of lipids for binding and endocytosis [20]. Recent evidence for cholesterol-dependent, lipid raft-mediated endocytic pathways

indicates that some pathogens have evolved to exploit lipid rafts during infection. Further study of the mechanisms by which pathogens use these organized lipid membrane domains are likely to yield insight into the biological role of lipid rafts in endocytosis and membrane trafficking.

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