

# Gangliosides are receptors for murine polyoma virus and SV40

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**Polyoma virus (Py) and simian virus 40 (SV40) travel from the plasma membrane to the endoplasmic reticulum (ER) from where they enter the cytosol and then the nucleus to initiate infection. Here we demonstrate that specific gangliosides can serve as plasma membrane receptors for these viruses, GD1a and GT1b for Py and GM1 for SV40. Binding and flotation assays were used to show that addition of these gangliosides to phospholipid vesicles allowed specific binding of the respective viruses. The crystal structure of polyoma VP1 with a sialic acid-containing oligosaccharide was used to derive a model of how the two terminal sugars (sialic acid- $\alpha$ 2,3-galactose) in one branch of GD1a and GT1b are recognized by the virus. A rat cell line deficient in ganglioside synthesis is poorly infectible by polyoma and SV40, but addition of the appropriate gangliosides greatly facilitates virus uptake, transport to the ER and infection. Lipid binding sites for polyoma are shown to be present in rough ER membranes, suggesting that the virus travel with the ganglioside(s) from the plasma membranes to the ER.**

**Keywords:** endoplasmic reticulum/gangliosides/glycolipids/polyoma virus/SV40

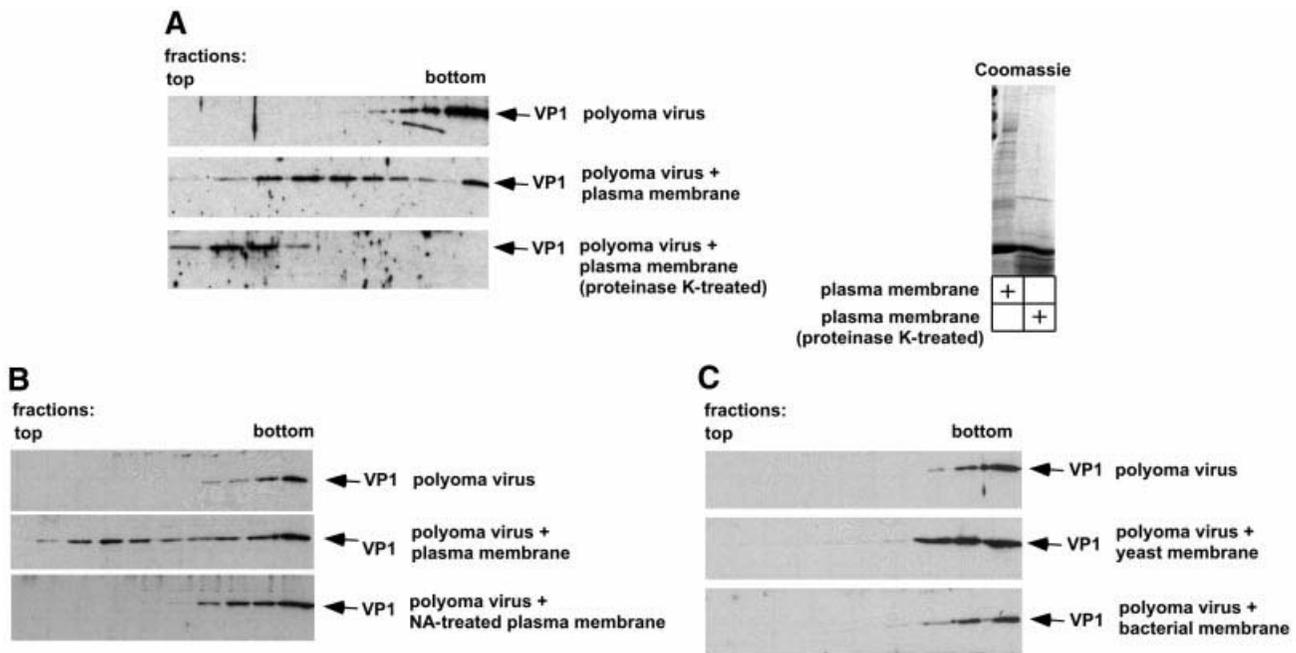
## Introduction

Viruses must deliver their nucleic acid across a cellular membrane into the cytoplasm or nucleus of their target cells to cause infection. The strategy employed by enveloped viruses is relatively simple: the viral membrane fuses either with the plasma membrane or with some intracellular membrane. For example, influenza virus is first taken up by endocytosis and then fuses with the endosomal membrane, delivering the nucleocapsid into the cytosol (reviewed by Poranen *et al.*, 2002). How non-enveloped DNA viruses, particularly those of the polyoma

group, enter the cell, cross a membrane barrier and deliver their DNA to the nucleus is not well understood. Some viruses of this group are taken up in small micropinocytotic vesicles and transported to the endoplasmic reticulum (ER). For example, simian virus 40 (SV40) enters via caveolae, resides initially in caveosomes, a novel organelle enriched in caveolin, and is then transported to the ER (Kartenbeck *et al.*, 1989; Anderson *et al.*, 1996; Pelkmans *et al.*, 2001; Norkin *et al.*, 2002). Transport into the ER is essential, as suggested by the fact that infection can be blocked by treatment of the cells with brefeldin A (Richards *et al.*, 2002). The related murine polyoma virus (Py) is also transported to the ER, where it co-localizes with the luminal protein BiP (Mannova and Forstova, 2003). Although it is not clear how or why these viruses traffic to the ER, or how they would subsequently cross the ER membrane, the overall pathway is similar to that of bacterial toxins, which traffic from the plasma membrane to the ER, from where they exit into the cytoplasm (Hazes and Read, 1997).

The major structural protein VP1 of Py and SV40 forms a pentamer, and 72 copies of the pentamer assemble into an icosahedral viral capsid (Liddington *et al.*, 1991; Stehle *et al.*, 1994). Buried within this capsid are the minor structural proteins VP2 and VP3, which overlap in sequence and differ only at their N-terminus. The identical, hydrophobic C-terminus of VP2 or VP3 associates with the VP1 pentamer in a 1:5 stoichiometry (Barouch and Harrison, 1994; Chen *et al.*, 1998). The longer VP2 protein contains a myristic acid at its N-terminus, which is buried inside the viral particle but may become exposed during the transport of the virus across the ER (Sahli *et al.*, 1993; Chen *et al.*, 1998; Norkin *et al.*, 2002).

VP1 is responsible for attachment to the host cell. SV40 binds to the MHC class I molecule at the plasma membrane (Breau *et al.*, 1992), but also to other, unidentified molecules (Atwood and Norkin, 1989). Antibodies to MHC class I moderately reduce SV40 infection (Atwood and Norkin, 1989; Stang *et al.*, 1997), but the MHC molecule is not endocytosed together with the virus (Anderson *et al.*, 1998). The receptor for Py is also unknown, but sialic acid is an essential component of it (Fried *et al.*, 1981; Cahan *et al.*, 1983). In the crystal structure of Py solved with a carbohydrate ligand the VP1 protein makes contact with sialic acid linked to galactose (sialic acid- $\alpha$ 2,3-Gal) (Stehle *et al.*, 1994), but it is unclear whether variants of this sugar would also bind. Small changes in VP1 affecting the selectivity and avidity of binding to sialic acids have profound effects on viral pathogenicity (Cahan *et al.*, 1983; Freund *et al.*, 1991; Stehle and Harrison, 1996, 1997; Bauer *et al.*, 1999). Efforts to identify a unique receptor by screening for monoclonal antibodies that protect cells from infection have been unsuccessful (Bauer *et al.*, 1999). However,



**Fig. 1.** Py binds to a protease-resistant receptor at the plasma membrane. (A) Purified Py (strain RA) was incubated in the absence of membranes or with plasma membranes pre-treated with or without proteinase K. The samples were floated in a discontinuous sucrose gradient. Fractions were collected from the top of the gradient and analyzed by SDS-PAGE and immunoblotting with a Py VP1 antibody (left panels). The proteins in the non-proteolyzed and proteolyzed plasma membranes were analyzed by SDS-PAGE followed by staining with Coomassie blue (right panel). (B) Purified Py (strain RA) was incubated in the absence of membranes or with plasma membranes pre-treated with or without  $\alpha$ 2,3-neuraminidase (NA). The analysis was performed as in (A). (C) Purified Py was incubated alone, or with yeast or bacterial membranes. The analysis was performed as in (A).

antibody to  $\alpha$ 4 $\beta$ 1 integrin has recently been shown to partially block infection, and a possible role of this integrin as a post-attachment receptor has been suggested (Caruso *et al.*, 2003).

Here, we report on the identification of gangliosides as functional receptors for Py and SV40. We demonstrate that specific gangliosides mediate virus attachment, and lead to entry into the ER and expression of nuclear T antigen. This viral uptake pathway bears some similarity to that of toxins, which also use gangliosides as carriers from the plasma membrane to the ER.

## Results

### ***Py binds to a protease-resistant receptor at the plasma membrane***

To identify the plasma membrane receptor for Py, we purified total membranes from human erythrocytes. As red cells lack internal organelles, this preparation consists essentially of plasma membranes. The membranes were incubated with purified Py (small plaque RA strain) and then floated in a discontinuous sucrose gradient. After fractionation, the samples were separated by SDS-PAGE and analyzed by immunoblotting with antibodies to the Py VP1 protein (Figure 1A). Whereas in the absence of plasma membranes the virus was found in the bottom fractions (Figure 1A, first panel), in the presence of membranes most of it floated into the middle of the gradient (second panel). Thus, there appear to be binding sites for the virus at the plasma membrane. The fact that the virus was found in different fractions of the gradient is probably explained by the heterogeneity of the vesicle

population. Extensive treatment of the plasma membranes with proteinase K did not reduce binding (Figure 1A, third panel); the virus floated with lighter membranes, consistent with their buoyant density being lowered by proteolysis. The conditions of protease treatment resulted in the disappearance of all Coomassie-stainable bands (right panel), suggesting that the virus binds to a non-protein receptor in the plasma membrane. Pre-incubation of the membranes with  $\alpha$ 2-3 neuraminidase abolished the binding activity (Figure 1B), in agreement with the fact that a similar treatment of cells blocks infection by Py (Helgeland, 1966). Binding appears to be specific, since no interaction was seen with either total yeast or bacterial membranes (Figure 1C, compare first panel with second and third panels).

### ***The Py receptor is a ganglioside***

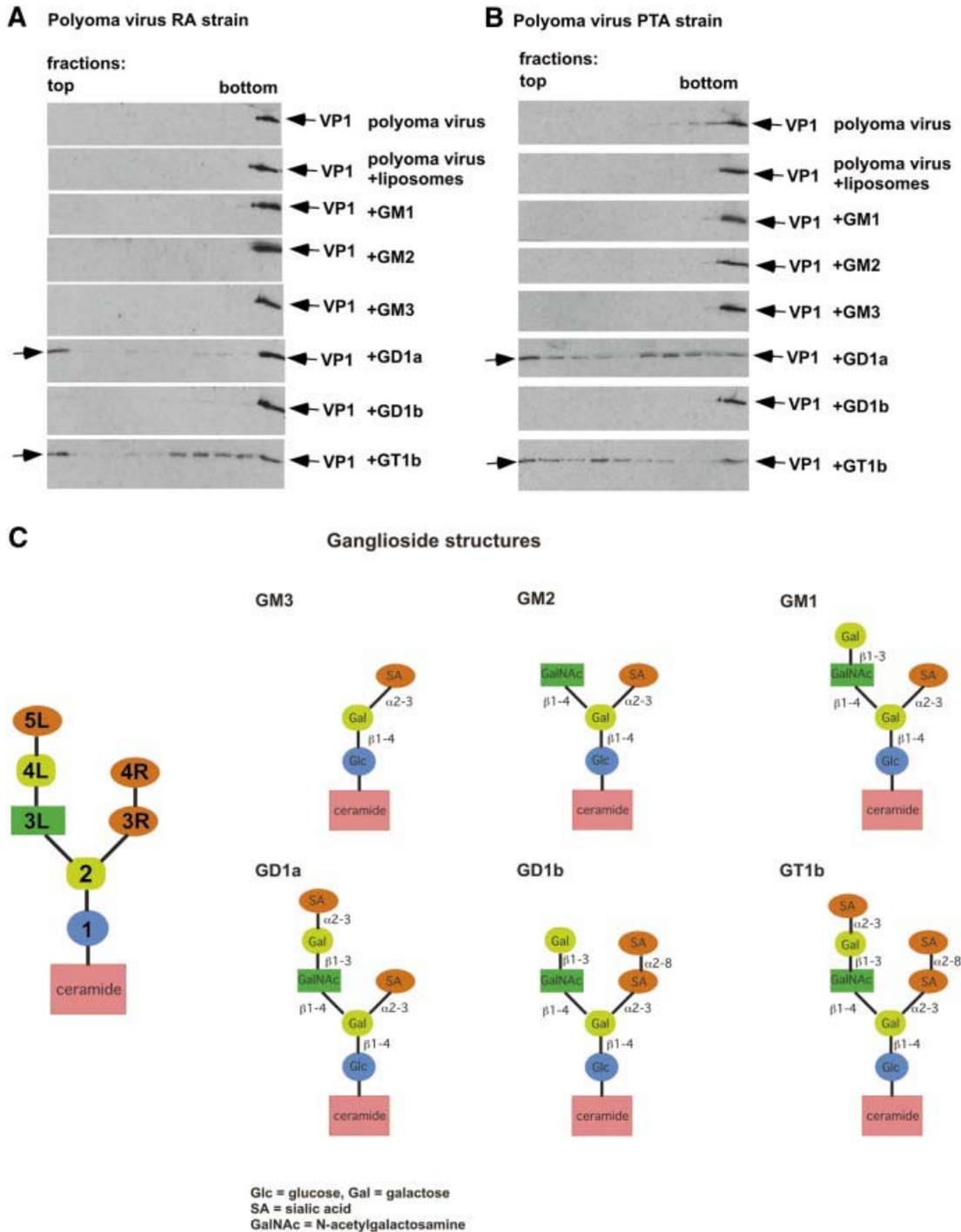
We found that an extract containing total lipids had some binding activity, but that much of the activity was lost during the extraction procedure (data not shown). We therefore suspected that the receptor is a relatively hydrophilic lipid molecule. In agreement with this assumption, vesicles containing all four classes of major phospholipids (phosphatidyl-choline, -ethanolamine, -serine and -inositol) did not bind the virus (Figure 2A and B). Given that Py receptor(s) are known to contain sialic acid, we tested the effects of adding specific gangliosides. Gangliosides are glycolipids that contain a hydrophobic ceramide lipid and a hydrophilic carbohydrate moiety of different structure (see Figure 2C). Small amounts of different gangliosides were added to a phospholipid mixture, which was then dried and resus-

pended in buffer to form vesicles. The small plaque strain RA was added and the vesicles were floated in a sucrose gradient. After fractionation, the samples were analyzed for the presence of VP1. Flotation of Py particles was only seen with gangliosides GD1a or GT1b (Figure 2A, arrows). Similar results were obtained with the more tumorigenic large plaque PTA strain (Figure 2B, arrows). We conclude that GD1a and GT1b gangliosides can function as receptors for both Py strains. The two active gangliosides differ only by a sialic acid residue, which is

present in GT1b in the right branch of the sugar (residue 4R; Figure 2C) but absent in GD1a.

**Features in the gangliosides recognized by Py VP1**

The crystal structure of Py VP1 in complex with a sialylated oligosaccharide is known at high resolution (Stehle and Harrison, 1997). The oligosaccharide used for complex formation contained a sialic acid- $\alpha$ 2,3-Gal- $\beta$ 1,3-GlcNAc branch, and interactions were seen between VP1 and the sialic acid- $\alpha$ 2,3-Gal moiety only. Comparison

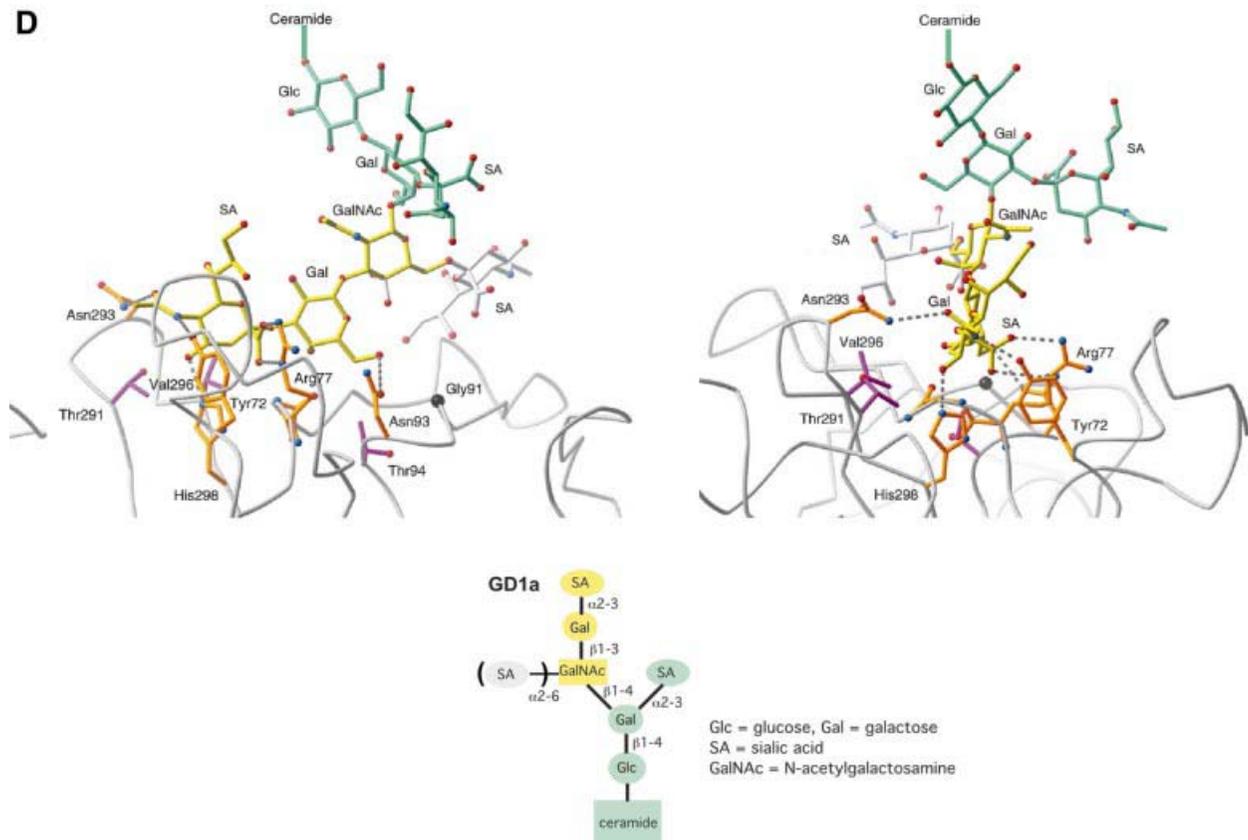


between the binding and non-binding gangliosides suggests that the sialic acid- $\alpha$ 2,3-Gal moieties in the left branches of GD1a and GT1b (residues 5L and 4L; Figure 2C), which are unique to these two gangliosides, must correspond to the sialic acid- $\alpha$ 2,3-Gal moiety recognized by the virus. While all gangliosides contain a second sialic acid- $\alpha$ 2,3-Gal moiety (residues 3R and 2), its proximity to the membrane likely rules out an interaction with VP1 due to steric constraints. This sialic acid-galactose moiety is very close to the lipid, and inspection of the VP1 structure shows that this steric constraint would likely prevent access to the binding pocket of VP1 (not shown). Moreover, in all but GM3, the galactose has a branch on the O4 oxygen, and the structure of the complex clearly shows that additional sugars linked to O4 can not be tolerated by VP1 (Stehle, 1994).

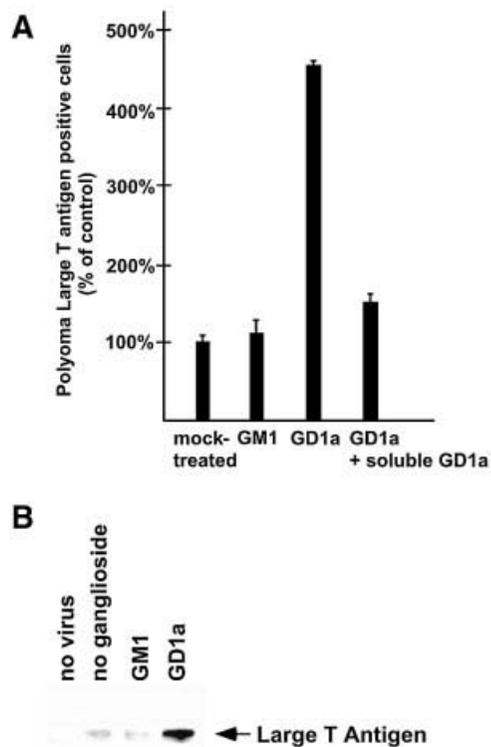
### Modeling of interactions of GD1a and GT1b with VP1

Based on the unique structural features of GD1a and GT1b, we used the crystal structure of the VP1-oligosaccharide complex to derive a model for how

GD1a would bind to VP1 (Figure 2D). This model shows that the sialic acid- $\alpha$ 2,3-Gal moiety of the left branch of GD1a (residues 5L and 4L; Figure 2C) can interact with VP1 exactly as seen in the structures of several complexes (Stehle *et al.*, 1994; Stehle and Harrison, 1996, 1997). The third carbohydrate, GalNAc (residue 3L), can be substituted for the close homolog GlcNAc used in the VP1 complex, without producing any unfavorable contacts. This is not surprising, as the GlcNAc moiety does not contact VP1 in the crystal structure. In its most likely conformation, the remainder of the ganglioside faces away from VP1, with the second sialic acid (residue 3R) and the 'stem' (residues 1 and 2) pointing in opposite directions. The second sialic acid is therefore highly unlikely to engage in contacts with VP1. This explains why the GT1b ganglioside, which has an additional sialic acid attached (residue 4R), also binds VP1, and it suggests that different Py strains bind equally well to both GD1a and GT1b. In the structure of the RA strain there is a binding pocket adjacent to the ganglioside binding site, which is blocked in the strain PTA (the latter contains a Glu instead of a Gly at position 91 of the VP1 protein);



**Fig. 2.** The Py receptor is a ganglioside. (A) Py (RA strain) was incubated in the absence of membranes or with liposomes containing different gangliosides. The samples were floated in a discontinuous sucrose gradient and fractions were analyzed by immunoblotting with antibodies to the polyoma VP1 protein (see Figure 1A). (B) As in (A) except that the more tumorigenic Py PTA strain was used. (C) Structures of the various gangliosides used in (A) and (B). (D) Modeling of interactions between GD1a and polyoma VP1 coat protein. Two orthogonal views are shown. The backbone structure of VP1 is shown as a gray ribbon, with residues involved in carbohydrate contacts shown in ball-and-stick representation (orange for residues with polar interactions, magenta for residues with hydrophobic contacts). VP1 was crystallized with a branched disialylated oligosaccharide (yellow and white) (Stehle and Harrison, 1997). The yellow portion of this oligosaccharide is identical to the longer branch of GD1a (except GlcNAc, which does not contact VP1, is replaced with GalNAc in GD1a), and thus we expect that this portion of GD1a will engage VP1 in the same manner. The remaining three carbohydrate residues of GD1a are attached to the O4 position of GalNAc, and in their most likely conformation they face away from VP1 and are unlikely to engage in contacts (light blue). The second sialic acid binding site of VP1 that accommodates the branched sialic acid (white) is probably not occupied by GD1a, and we therefore expect GD1a to bind equally well to RA and PTA Py strains.



**Fig. 3.** Binding of Py to ganglioside GD1a is required for infection. (A) Quantification of Py LTA<sub>g</sub> expression. C6 glioma cells lacking gangliosides more complex than GM3 (see Figure 2C) were incubated with or without gangliosides GM1 or GD1a as indicated. The cells were washed and incubated with Py (RA strain) in the absence or presence of GD1a. Expression of Py LTA<sub>g</sub> was monitored after immunostaining in a fluorescence microscope. The data are presented as the percentage of total nuclei (duplicates of about 500 nuclei) that were Py LTA<sub>g</sub> positive, normalized to 100%. (B) As in (A) except expression of Py LTA<sub>g</sub> in C6 cells was analyzed by SDS-PAGE followed by immunoblotting with Py LTA<sub>g</sub> antibodies.

Freund *et al.*, 1991). Modeling shows that this additional binding pocket in the RA strain is unlikely to be involved in the interaction with gangliosides (Figure 2D). It may, however, contribute to the binding of glycoproteins (see below).

#### **Binding of Py to ganglioside GD1a is required for efficient infection**

To test whether Py requires gangliosides to infect cells, we used a rat cell line (C6 glioma) that is deficient in ganglioside synthesis. C6 cells make detectable levels of GM3 but little or none of the more complex gangliosides such as GM1 or GD1a (Sottocornola *et al.*, 1999) (Figure 2C). However, the missing gangliosides can be added to the cells simply by incubating them with glycolipid micelles (Moss *et al.*, 1976). Rat cells in general are non-permissive for Py and SV40, but can be non-productively infected by each of these viruses. Such infection can readily be monitored by immunostaining of cells with antibodies to the large T antigen using standard fluorescence microscopy. When untreated C6 cells were infected with Py at a high multiplicity of infection (m.o.i.) (>100 p.f.u./cell), roughly 12% of the cells (64/536) became infected (normalized to 100% in Figure 3A). Similarly, when cells were pre-incubated with purified GM1 ganglioside for 24 h, washed and then challenged

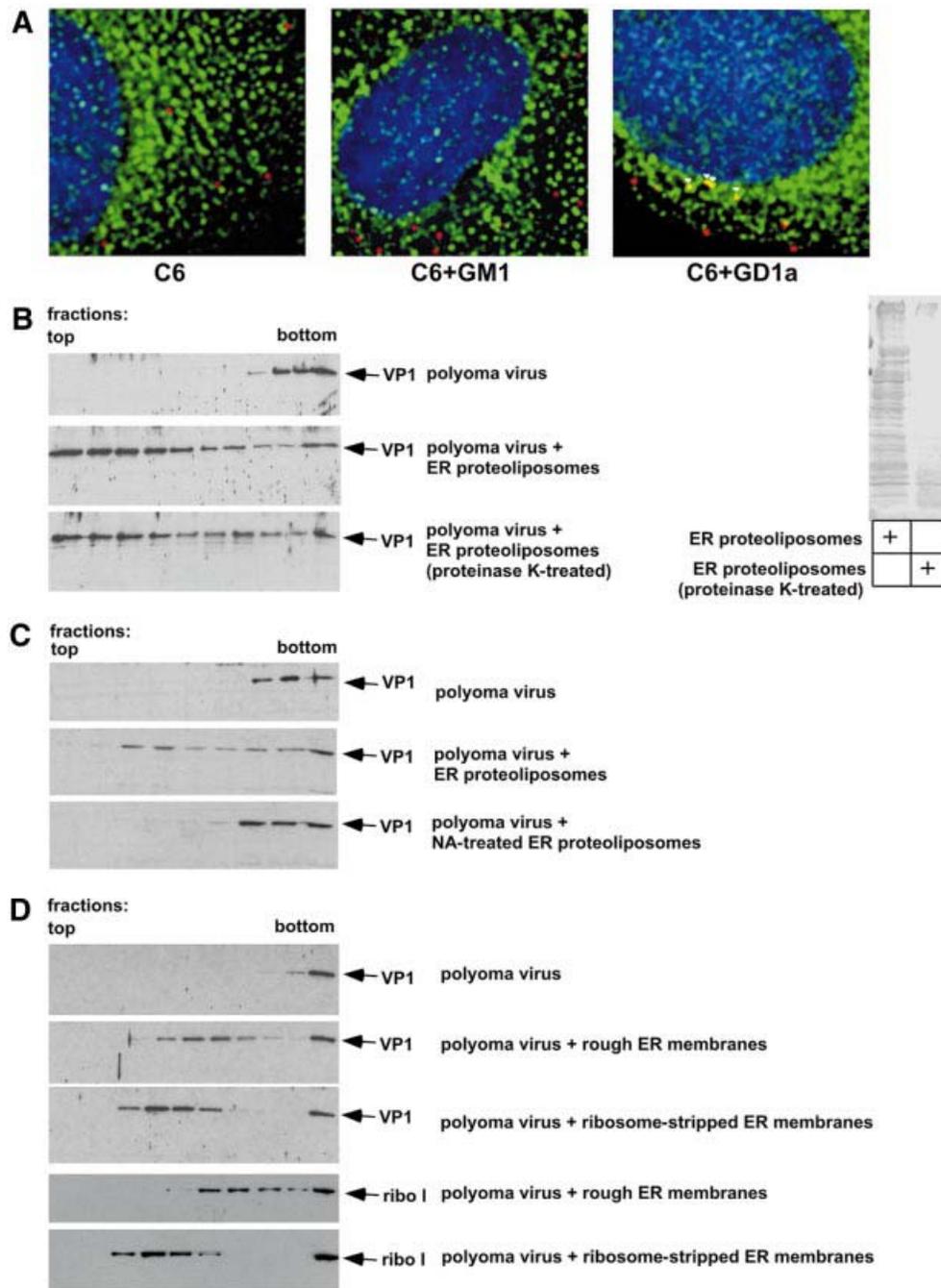
with virus, little infection was observed. However, when the cells were pre-incubated with purified GD1a, the simpler of the two active gangliosides, the number of infected cells increased drastically (242/516). In repeated experiments, the increase in infection with added GD1a varied between 4-fold, shown here, to 15-fold. This increase was largely prevented when GD1a was present during the incubation with the virus (Figure 3A, '+ soluble GD1a'). Successful infection with added GD1a and not GM1 was confirmed by immunoblotting for large T (Figure 3B). These results show that binding of Py to ganglioside GD1a (or the similar GT1b) is required for efficient infection of these cells.

#### **Transport of Py to the ER by ganglioside GD1a**

To test whether ganglioside GD1a is required for the transport of Py to the ER, we pre-treated C6 glioma cells with different gangliosides, infected them with virus labeled with a fluorescent dye, and examined the localization of the virus by deconvolution fluorescence microscopy. Arrival of the virus in the ER was monitored by its co-localization with the ER luminal protein BiP (Figure 4A: the red color shows the virus, the green color BiP and the yellow color co-localization). Little co-localization was observed in the absence of added ganglioside or upon addition of ganglioside GM1 (left and middle panel). However, upon addition of ganglioside GD1a, significant co-localization was seen (right panel; about 17–20% of the virus co-localized with BiP after a 4 h incubation).

To further test whether gangliosides might function as carriers for virus and to confirm that lipid binding sites for the virus are present in the ER, rough ER membranes were purified from rat liver and solubilized in detergent, and the detergent was then removed to generate proteoliposomes. The latter contain only membrane proteins and lipids, but no peripheral or luminal proteins. When Py was incubated with the ER proteoliposomes, most of the virus floated with the membranes in a sucrose gradient (Figure 4B), similar to the results found with plasma membranes (Figure 1A) or phospholipid micelles containing GD1a or GT1b (Figure 2A and B). When the ER proteoliposomes were incubated with a high concentration of proteinase K, resulting in almost complete disappearance of all Coomassie-stainable material (Figure 4B, right panel), the proteolyzed membranes bound the virus with unreduced efficiency (Figure 4B, left bottom panel). On the other hand, treatment of the ER proteoliposomes with  $\alpha$ 2,3-neuraminidase abolished binding activity (Figure 4C), consistent with an interaction with a sialic acid-containing ganglioside. Some binding activity could be recovered after lipid extraction (data not shown). Lipid binding sites for the virus are therefore present in the ER of uninfected cells, consistent with a possible transport pathway involving gangliosides from plasma membranes to ER in normal cells.

To rule out the possibility that binding was to contaminating membranes rather than ER, we examined whether the virus-interacting membranes contain bound ribosomes, a characteristic of rough ER membranes. For these experiments we used microsomes, because preliminary experiments showed that they bind Py with about the same efficiency as proteoliposomes. Presumably, a



**Fig. 4.** Transport of Py to the ER by ganglioside GD1a. (A) C6 glioma cells lacking gangliosides more complex than GM3 (see Figure 2C) were incubated with GM1 or GD1a and then infected with Texas Red-labeled Py (RA strain). After 4 h the cells were fixed and immunostained for the ER luminal protein BiP using Oregon Green-labeled secondary antibodies (green). Nuclei were labeled with DAPI. Yellow points (white arrow head) indicate co-localization. (B) Purified Py (strain RA) was incubated in the absence or presence of ER proteoliposomes pre-treated or not with proteinase K. The samples were floated in a sucrose gradient and fractions were analyzed by immunoblotting with antibodies to the Py VP1 protein (left panels). The proteins in the non-proteolyzed or proteolyzed membranes were analyzed by SDS-PAGE followed by staining with Coomassie blue (right panel). (C) Purified Py (strain RA) was incubated in the absence or presence of ER proteoliposomes pre-treated or not with  $\alpha$ 2,3-neuraminidase (NA). The samples were analyzed as in (A). (D) Purified Py (strain RA) was incubated in the presence or absence of rough ER membranes or ribosome-stripped ER membranes. The samples were analyzed as in (A). An antibody against ribophorin I was used to monitor ER membranes in the fractions.

certain fraction of the vesicles is not fully sealed, allowing the virus to bind to the luminal side of the membrane presumed to contain gangliosides. The membranes were treated with puromycin and high salt to strip off bound ribosomes before addition of the virus. In the subsequent flotation, the virus fractionated with the ribosome-stripped membranes at a lower density than seen with the original

membranes (Figure 4D, second versus third panels). A similar shift in density was observed with the ER membrane protein ribophorin I (fourth versus fifth panel). Thus, the virus is able to bind to rough ER membranes. Together, these results show that gangliosides, which receive their sugar moiety in the Golgi, must be able to go back to the ER.

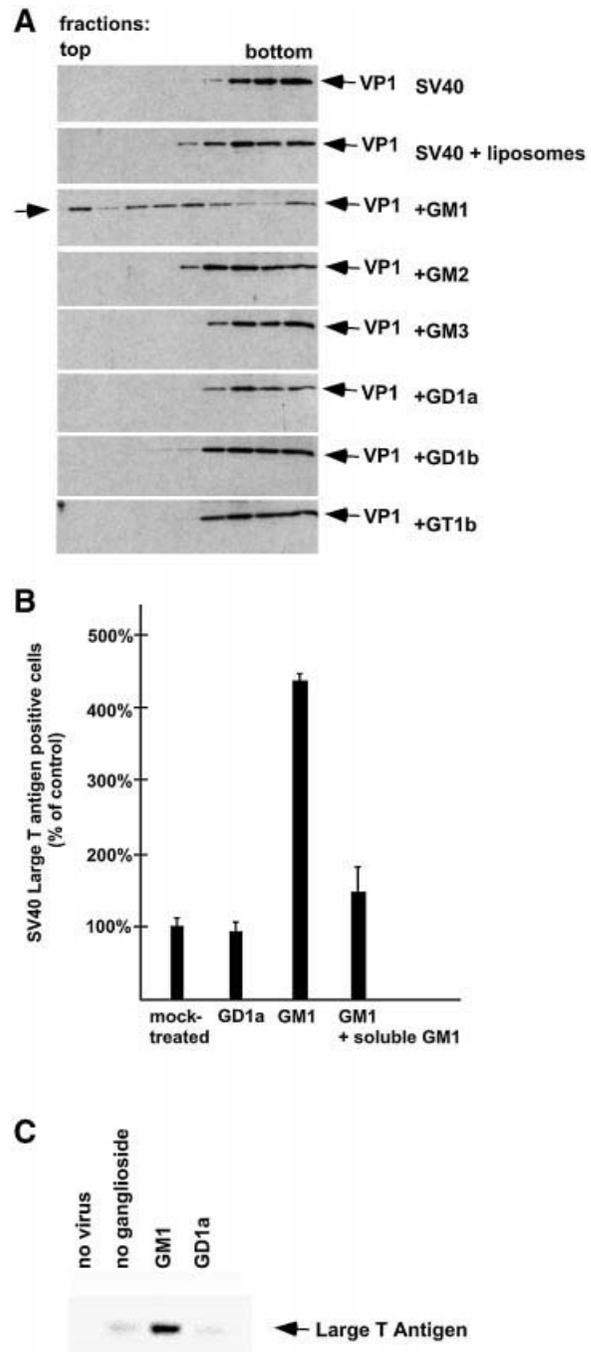
### SV40 binds to ganglioside GM1 to infect cells

SV40 is structurally related to Py (Liddington *et al.*, 1991) and also travels to the ER (Kartenbeck *et al.*, 1989; Pelkmans *et al.*, 2001; Norkin *et al.*, 2002). To determine whether it also binds to a ganglioside, phospholipid vesicles were spiked with small amounts of different gangliosides and their interaction with SV40 was tested by flotation of the membranes in a sucrose gradient and immunoblotting for VP1. Binding of SV40 was only seen with the ganglioside GM1 (Figure 5A, arrow). To test whether GM1 is required for SV40 infection of cells, we infected C6 cells that were either untreated or pre-incubated with GM1, and the infection was monitored by immunostaining for SV40 large T antigen (Figure 5B). Few cells expressing SV40 large T were observed when the incubation was performed without ganglioside or with ganglioside GD1a (~4 out of 500 cells). However, upon addition of GM1 a substantial increase in the number of infected cells was observed (19 out of 522 cells). This increase was not apparent if GM1 was present in the medium during the incubation with the virus. Immunoblotting for SV40 large T confirmed the specificity of GM1 in mediating infection (Figure 5C). We conclude that ganglioside GM1 can serve as a functional receptor for SV40.

### Discussion

Our results show that the gangliosides GD1a/GT1b and GM1 serve as receptors for Py and SV40, respectively. The two viruses recognize the carbohydrate moieties of the glycolipids. Molecular modeling shows that in the case of Py, the known sialic acid-binding pocket of VP1 accommodates the two terminal sugar residues in the left branch of GD1a and GT1b (see Figure 2C). The binding of Py and SV40 to specific gangliosides promotes transport to the ER and infection of cells. Lipid binding sites for Py were also found in ER membranes, suggesting the possibility that the virus may travel on gangliosides from the plasma membrane into the ER.

As expected from the similar structures of the Py and SV40 VP1 proteins, the ganglioside receptors are also structurally related. The critical sialic acid- $\alpha$ 2,3-Gal moiety of the Py receptors GD1a/GT1b (residues 5L and 4L; Figure 2C) is also present on the short arm of GM1 (residues 3R and 2), but too close to the ceramide to bind Py. In GM1, it is presumably the disaccharide lacking sialic acid (residues 4L and 3L) that binds SV40. This is consistent with previous observations that infection of cells by Py, in contrast to SV40, can be prevented by treatment with neuraminidase (Helgeland, 1966). It is also consistent with results from the structural comparison of Py and SV40 (Stehle and Harrison, 1996). While the binding pockets of both viruses are quite similar, SV40 VP1 lacks the specific residues corresponding to those that contact sialic acid in Py, and therefore it would not be expected to bind sialic acid in the same manner as Py. On the other hand, the general similarity of the Py and SV40 VP1 structures, and especially the presence of equally spaced pockets on the VP1 surfaces, indicates that both bind carbohydrates. The GD1b ganglioside retains all of the features of GM1 but has an extra sialic acid attached to its right branch (residue 4R). Our observation that GM1



**Fig. 5.** SV40 binds to ganglioside GM1 to infect cells. (A) SV40 was incubated in the absence or presence of liposomes containing different gangliosides, as indicated. The samples were floated in a sucrose gradient and fractions were analyzed by immunoblotting with antibodies directed against Py VP1, VP2 and VP3 proteins. (B) Quantification of SV40 LTA<sub>g</sub> expression. C6 glioma cells lacking gangliosides more complex than GM3 (see Figure 2C) were incubated with or without gangliosides GM1 or GD1a, as indicated. The cells were washed and incubated with SV40 in the absence or presence of GM1. Expression of SV40 LTA<sub>g</sub> was monitored after immunostaining in a fluorescence microscope. The data are presented as the percentage of total nuclei (duplicates of about 500 nuclei) that were SV40 LTA<sub>g</sub> positive, normalized to 100%. (C) As in (B) except expression of SV40 LTA<sub>g</sub> in C6 cells was analyzed by SDS-PAGE followed by immunoblotting with SV40 LTA<sub>g</sub> antibodies.

**Table I.** ER-directed toxins and viruses and their receptors (see text)

Bacterial/plant toxins	Host cell receptor
Cholera toxin	GM1
LT type I	GM1
LT IIa	GD1b
LT IIb	GD1a
Shiga toxin	GB3
Verotoxin	GB3
Exotoxin	$\alpha$ 2 macroglobulin receptor
Ricin	Galactose-lipid/protein?
Non-envelope viruses	
Py	GD1a/GT1b
SV40	GM1

binds SV40, whereas GD1b does not, suggests that this second sialic acid may interfere sterically with binding. This would imply that the Gal- $\beta$ 1,3-GalNAc moiety binds to SV40 in a conformation that brings the first sialic acid in close proximity to the virus, and does not tolerate the presence of an additional sialic acid attached to it.

The identification of glycolipids as receptors for Py and SV40 suggests obvious similarities with bacterial toxins, such as cholera toxin, Shiga toxin and ricin (for a more complete list, see Table I). These toxins are transported from the plasma membrane back to the ER, where they cross the membrane into the cytosol and induce cytotoxicity by various mechanisms. For most, and perhaps all, toxins of this class, glycolipids appear to be the receptor on the plasma membrane. The best studied case is cholera toxin. Each of its B subunits in a pentameric ring binds to the ganglioside GM1. Association of GM1 with lipid rafts is required for retrograde transport of cholera toxin via the Golgi to the ER (Wolf *et al.*, 2002; Y.Fujinaga, A.A.Wolf, C.Rodighiero, H.Wheeler, B.Tsai, L.Allen, J.Jobling, T.A.Rapoport, R.K.Holmes and W.I.Lencer, submitted). The holotoxin is disassembled by protein disulfide isomerase in the ER, and the toxic A1 fragment of the A subunit is subsequently transported across the ER membrane (Tsai *et al.*, 2001). The structurally related toxin LTIIb binds to and travels on ganglioside GD1a (van den Akker *et al.*, 1996). Forward transport of glycolipids has long been established: the ceramide moiety is synthesized in the ER and the carbohydrate chain in the Golgi, and the mature molecules then travel on to the plasma membrane (Kolter *et al.*, 2002). Our data now suggest that a fraction of the glycolipids can go backwards and serve as carrier for viruses and toxins. Viruses, however, unlike the toxins, may bypass the Golgi, and follow an alternate route to the ER, as suggested for SV40 (Pelkmans *et al.*, 2001). To date, no co-localization of Py with Golgi markers has been seen in GD1a-supplemented C6 cells.

The identification of GD1a/GT1b as receptors for Py, together with the known crystal structure, indicates that there is surprisingly little specificity in the interaction; the VP1 protein recognizes only the sialic acid-galactose terminal sugars. This structure is also present on glycoproteins. For example, *N*-glycosylated proteins with a complex type of sugar contain the terminal sialic acid-galactose-GlcNAc chain, which differs from that in

ganglioside GD1a only by the third sugar (GalNAc). Similarly, *O*-linked sugars on glycoproteins can have the sialic acid-galactose-GalNAc chain that is found on gangliosides. Thus, one would expect Py to bind to these glycoproteins as well. Indeed, we have observed strong binding of Py to rat C6 cells lacking GD1a/GT1b (data not shown). The previously observed binding of SV40 to MHC class I molecules (Breau *et al.*, 1992) could also be explained by interaction with an attached sugar chain, although one would expect other glycoproteins to bind as well. Interaction of the viruses with glycoproteins does not lead to efficient infection of rat C6 cells. However, the low level of infection in these cells lacking gangliosides could be mediated by glycoproteins.

In mouse cells, it is possible that glycoprotein receptors may play a more prominent role as Py receptors.  $\alpha$ 4 $\beta$ 1 integrin for example has been implicated as a receptor in mouse cells (Caruso *et al.*, 2003). Infection of mouse cells by Py can be inhibited by tunicamycin (Chen and Benjamin, 1997). However, as an inhibitor of *N*-glycosylation, tunicamycin may act upstream of both glycoprotein and glycolipid synthesis. This is because many glycosyltransferases, including some sialyltransferases, are themselves *N*-glycosylated and are known to fold and function poorly in the absence of glycosylation (J.Paulson, personal communication). Since Py can bind to different glycoproteins carrying the appropriate carbohydrate chain, many of these proteins may bind the virus or even internalize it without causing infection. This may contribute to the known low efficiency of infection. It is also conceivable that glycoproteins serve as primary receptors from which the virus is transferred to the gangliosides, although there is no apparent driving force at the plasma membrane for the directed transfer of the virus from one receptor to the other, and no reason why the virus could not bind directly to the ganglioside. In addition, electron microscopic studies of SV40 and Py infection shows that the viral membrane is in extremely close apposition with the cellular membrane, both for virus at the surface and inside the cell (Mackay and Consigli, 1976; Maul *et al.*, 1978; Stang *et al.*, 1997), as would be expected from a high avidity binding to glycolipid receptor molecules that extend only a short distance from the plane of the lipid bilayer.

The existence of proteinaceous 'decoys' or pseudo-receptors in the mouse may also explain the observed differences in the infectivity of Py strains (Bauer *et al.*, 1999). The crystal structure shows that strain RA, in contrast to PTA, has a secondary binding pocket, which can accommodate a sialic acid residue that branches off from a GlcNAc or GalNAc at the third terminal position (Stehle *et al.*, 1994; Stehle and Harrison, 1997). This type of branch occurs in *O*-glycosylated proteins, such as glycophorin A (Cahan *et al.*, 1983). The binding of the RA strain to these glycoproteins may explain why it is less infectious in the mouse than the non-binding PTA strain (Dubensky *et al.*, 1991; Freund *et al.*, 1991). The RA virus may be diverted to a non-productive pathway and prevented from spreading efficiently in the mouse.

Py has an extremely broad host range in terms of the number of different cell types it infects in its natural host (Dawe *et al.*, 1987). SV40 likewise can infect a variety of different cell types. Whether these viruses indeed utilize

both glycoproteins and glycolipids as functional receptors in the intact host, or whether in a given cell type they rely uniquely on one or the other class, and how they might traffic differently depending on the receptor, are important issues that remain for further study.

## Materials and methods

### Materials

Purified gangliosides were purchased from Matreya, purified phosphatidyl-choline,-ethanolamine, -serine and -inositol from Avanti, proteinase K-agarose beads and 4',6'-diamidino-2-phenylindole (DAPI) from Sigma,  $\alpha$ 2,3-neuraminidase, puromycin, antibodies against BiP and SV40 large T antigen from Calbiochem, C6 glioma cell line from ATCC, Oregon Green anti-rabbit antibodies from Molecular Probes, and rhodamine-labeled goat anti-rat antibodies from Jackson Laboratories. Antibodies against the Py VP1 protein, VP1, -2 and -3 proteins and Py large T antigen were generated in the Benjamin laboratory. Antibodies against ribophorin I were generated in the Rapoport laboratory.

### Purification of Py and SV40

Purification of Py and SV40 was performed as described by Gilbert *et al.* (2003). Purified polyoma virus was labelled with the FluoReporter Texas Red protein labeling kit (Molecular Probes; Gilbert *et al.*, 2003).

### Preparation of membranes

Human erythrocytes from Dr B.Tsai (Harvard Medical School) were centrifuged at 500 *g* for 10 min. The cells were washed with a buffer containing 150 mM NaCl and 50 mM HEPES (pH 7.8), lysed in water, and total membranes collected by centrifugation (12 000 *g* for 10 min). The membranes, hereafter referred to as plasma membrane, were resuspended in a buffer containing 50 mM HEPES (pH 7.6), 250 mM sucrose, 150 mM NaCl and 2 mM MgOAc<sub>2</sub>. Rough ER membranes were isolated from canine pancreas. The generation of ribosome-stripped ER membranes and ER proteoliposomes were as previously described (Görllich and Rapoport, 1993). Yeast total membranes were as described previously (Tsai and Rapoport, 2002), and *Escherichia coli* membranes were a generous gift from Dr W.Wickner (Dartmouth Medical School, Dartmouth, NH).

### Sucrose flotation assay

Approximately 15  $\mu$ g of plasma membrane or ER proteoliposomes were incubated with 50 ng of Py for 30 min at 30°C. Eighty microliters of a 73% sucrose solution was added and mixed thoroughly with the sample and placed at the bottom of a Beckman centrifuge tube (7  $\times$  20 mm). Fifty microliters of a 45% sucrose solution was layered over the sample, followed by 50  $\mu$ l of a 25% sucrose solution. The sample was centrifuged in a Beckman TLA100 rotor for 1 h at 100 000 r.p.m. Samples (20  $\mu$ l) were sequentially removed from the top of the gradient and analyzed by SDS-PAGE. For testing Py and SV40 binding to liposomes spiked with various gangliosides, 1  $\mu$ l of purified ganglioside (1 mM) was mixed with 19  $\mu$ l of phosphatidyl-choline (10 mg/ml), 5  $\mu$ l of phosphatidyl-ethanolamine (10 mg/ml), 3  $\mu$ l of phosphatidyl-inositol (10 mg/ml) and 1  $\mu$ l of phosphatidyl-serine (10 mg/ml), all dissolved in chloroform. The mixture was dried under vacuum, and resuspended in a buffer containing 50 mM HEPES (pH 7.6), 250 mM sucrose, 150 mM NaCl and 2 mM MgOAc<sub>2</sub>. The resuspended vesicles were sonicated in a water sonicator bath for 30 min at room temperature and incubated at 4°C overnight. For analyzing Py binding, 1  $\mu$ l of Py (50 ng/ $\mu$ l) was added to 19  $\mu$ l of ganglioside-containing lipid vesicles. Eighty microliters of a 73% sucrose solution was then added and mixed thoroughly with the sample and placed at the bottom of a Beckman centrifuge tube (7  $\times$  20 mm). The sample was processed as above. To analyze SV40 binding, 15  $\mu$ l of SV40 was added to 25  $\mu$ l of ganglioside-containing lipid vesicles. Sixty microliters of a 73% sucrose solution was then added and mixed thoroughly with the sample and placed at the bottom of a Beckman centrifuge tube (7  $\times$  20 mm). The sample was processed as above.

### Proteinase K digestion

Plasma membrane and ER proteoliposomes were incubated with 10  $\mu$ l of proteinase K-agarose beads (10 mg/ml) for 30 min at room temperature. The protease was removed from the membranes by pelleting. The proteolyzed membranes were tested for their ability to bind to the virus as above.

### Infectivity assay

Infection of C6 glioma cells by Py and SV40 was performed as previously described in Gilbert and Benjamin (2000). Cells ( $5 \times 10^4$ ) were plated on 12 mm glass coverslips and let settle for 3 h at 37°C in a CO<sub>2</sub> incubator. Cells were pre-incubated with either media, 3.2  $\mu$ M GM1 or 2.7  $\mu$ M GD1a for 24 h at 37°C. Prior to infection, cells were washed three times with media. Cells were infected at an m.o.i. of 500 for Py and 2000 for SV40. For ganglioside competition experiments, either 3.2  $\mu$ M GM1 or 2.7  $\mu$ M GD1a was added with the virus. Successful entry was assessed by nuclear expression of Py and SV40 large T antigen by standard fluorescence microscopy.

### Immunoblotting for large T antigen

Cells were plated in 24-well dishes at  $5 \times 10^4$  per well. Cells were pre-incubated with either media, 3.2  $\mu$ M GM1 or 2.7  $\mu$ M GD1a for 24 h at 37°C. Cells were washed three times and infected with m.o.i. of 400 for either Py or SV40 and let incubated for 32 h. Cells were rinsed twice and lysed in RIPA buffer with protease inhibitors. Samples were analyzed by SDS-PAGE and immunoblotted with either antibodies against Py LTAG or SV40 LTAG.

### Immunofluorescence and co-localization assay

Co-localization of Py and BiP was performed using deconvolution microscopy, as previously described in Gilbert *et al.* (2003). Briefly, cells were plated at  $5 \times 10^4$  per well on 12 mm glass coverslips. Media was changed at  $t = 3$  h to media alone, a media containing 3.2  $\mu$ M GM1 or 2.7  $\mu$ M GD1a. After 24 h at 37°C in a CO<sub>2</sub> incubator, cells were rinsed three times and placed on ice. Texas Red-labeled Py was added and allowed to bind for 1 h at 4°C. Cells were rinsed twice and new media was added. Cells were fixed in 4% paraformaldehyde at 4 h post-infection. Cells were permeabilized in 0.5% Triton X-100 and then BiP was immunostained with a rabbit anti-BiP antibody. Anti-rabbit antibodies were detected with goat anti-rabbit secondary Oregon Green-labeled antibodies. Cells were washed, mounted in Mowiol and examined using a Nikon Eclipse microscope with an apochromatic Plan 60  $\times$ 1.4 oil objective equipped with a Delta Vision optical sectioning system employing SoftWoRx software with 0.2- $\mu$ M thick step Z sections.

### Modeling of GD1a onto the VP1 protein of Py

The existing structure of the sialylated oligosaccharide (Stehle and Harrison, 1997) was modified as follows. The terminal sialic acid  $\alpha$ 2-3 Gal moiety was left unaltered. The third sugar, GlcNAc, was replaced with GalNAc. This only involved switching the O4 oxygen from the equatorial to the axial position. The  $\alpha$ 2-6-linked sialic acid was deleted. The additional sugars of GD1a were modeled onto the GalNAc residue in conformations that have favorable staggered dihedral angles and do not result in steric clashes. Other conformations are of course possible; however, none of these conformations would result in contacts of these additional sugars with VP1 residues.

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## References

- Anderson,H.A., Chen,Y. and Norkin,L.C. (1996) Bound simian virus 40 translocates to caveolin-enriched membrane domains, and its entry is inhibited by drugs that selectively disrupt caveolae. *Mol. Biol. Cell*, **7**, 1825–1834.
- Anderson,H.A., Chen,Y. and Norkin,L.C. (1998) MHC class I molecules are enriched in caveolae but do not enter with simian virus 40. *J. Gen. Virol.*, **79**, 1469–1477.
- Atwood,W.J. and Norkin,L.C. (1989) Class I major histocompatibility proteins as cell surface receptors for simian virus 40. *J. Virol.*, **63**, 4474–4477.

- Barouch,D.H. and Harrison,S.C. (1994) Interactions among the major and minor coat proteins of polyomavirus. *J. Virol.*, **68**, 3982–3989.
- Bauer,P.H., Cui,C., Stehle,T., Harrison,S.C., DeCaprio,J.A. and Benjamin,T. (1999) Discrimination between sialic acid-containing receptors and pseudoreceptors regulates polyomavirus spread in the mouse. *J. Virol.*, **73**, 5826–5832.
- Breau,W.C., Atwood,W.J. and Norkin,L.C. (1992) Class I major histocompatibility proteins are an essential component of the simian virus 40 receptor. *J. Virol.*, **66**, 2037–2045.
- Cahan,L.D., Singh,R. and Paulson,J.C. (1983) Sialyloligosaccharide receptors of binding variants of polyoma virus. *Virology*, **130**, 281–289.
- Caruso,M., Belloni,L., Sthandier,O., Amati,P. and Garcia,M.I. (2003)  $\alpha 4\beta 1$  integrin acts as a cell receptor for murine polyomavirus at the postattachment level. *J. Virol.*, **77**, 3913–3921.
- Chen,H.M. and Benjamin,T.L. (1997) Roles of *N*-glycans with  $\alpha 2,6$  as well as  $\alpha 2,3$  linked sialic acid in infection by polyoma virus. *Virology*, **233**, 440–442.
- Chen,X.S., Stehle,T. and Harrison,S.C. (1998) Interaction of polyomavirus internal protein VP2 with the major capsid protein VP1 and implications for participation of VP2 in viral entry. *EMBO J.*, **17**, 3233–3240.
- Dawe,C.J., Freund,R., Mandel,G., Ballner-Hofer,K., Talmage,D.A. and Benjamin,T.L. (1987) Variations in polyoma virus genotype in relation to tumor induction in mice: Characterization of wild type strains with widely differing tumor profiles. *Am. J. Pathol.*, **127**, 243–261.
- Dubensky,T.W., Freund,R., Dawe,C.J. and Benjamin,T.L. (1991) Polyomavirus replication in mice: influences of VP1 type and route of inoculation. *J. Virol.*, **65**, 342–349.
- Freund,R., Calderone,A., Dawe,C.J. and Benjamin,T.L. (1991) Polyomavirus tumor induction in mice: effects of polymorphisms of VP1 and large T antigen. *J. Virol.*, **65**, 335–341.
- Fried,H., Cahan,L.D. and Paulson,J.C. (1981) Polyoma virus recognizes specific sialyloligosaccharide receptors on host cells. *Virology*, **109**, 188–192.
- Gilbert,J.M. and Benjamin,T.L. (2000) Early steps of polyomavirus entry into cells. *J. Virol.*, **74**, 8582–8588.
- Gilbert,J.M., Goldberg,I.G. and Benjamin,T.L. (2003) Cell penetration and trafficking of polyomavirus. *J. Virol.*, **77**, 2615–2622.
- Görlich,D. and Rapoport,T.A. (1993) Protein translocation into proteoliposomes reconstituted from purified components of the endoplasmic reticulum membrane. *Cell*, **75**, 615–630.
- Hazes,B. and Read,R.J. (1997) Accumulating evidence suggests that several AB-toxins subvert the endoplasmic reticulum-associated protein degradation pathway to enter target cells. *Biochemistry*, **36**, 11051–11054.
- Helgeland,K. (1966) Polyoma virus. 3. On the nature of the virus receptors on the mouse embryo cells. *Acta Pathol. Microbiol. Scand.*, **68**, 439–444.
- Kartenbeck,J., Stukenbrok,H. and Helenius,A. (1989) Endocytosis of simian virus 40 into the endoplasmic reticulum. *J. Cell Biol.*, **109**, 2721–2729.
- Kolter,T., Proia,R.L. and Sandhoff,K. (2002) Combinatorial ganglioside biosynthesis. *J. Biol. Chem.*, **277**, 25859–25862.
- Liddington,R.C., Yan,Y., Moulai,J., Sahli,R., Benjamin,T.L. and Harrison,S.C. (1991) Structure of simian virus 40 at 3.8 Å resolution. *Nature*, **354**, 278–284.
- Mackay,R.L. and Consigli,R.A. (1976) Early events in polyoma virus infection, attachment, penetration and nuclear entry. *J. Virol.*, **19**, 620–636.
- Mannova,P. and Forstova,J. (2003) Mouse polyoma virus utilizes recycling endosomes for a traffic pathway independent of COPI vesicle transport. *J. Virol.*, **77**, 1672–1681.
- Maul,G.G., Rovera,G., Vorbrodt,A. and Abramczuk,J. (1978) Membrane fusion as a mechanism of simian virus 40 entry into different cellular compartments. *J. Virol.*, **28**, 936–944.
- Moss,J., Fishman,P.H., Manganiello,V.C., Vaughan,M. and Brady,R.O. (1976) Functional incorporation of ganglioside into intact cells: induction of cholera responsiveness. *Proc. Natl Acad. Sci. USA*, **73**, 1034–1037.
- Norkin,L.C., Anderson,H.A., Wolfrom,S.A. and Oppenheim,A. (2002) Caveolar endocytosis of simian virus 40 is followed by brefeldin A-sensitive transport to the endoplasmic reticulum, where the virus disassembles. *J. Virol.*, **76**, 5156–5166.
- Pelkmans,L., Kartenbeck,J. and Helenius,A. (2001) Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular transport pathway to the ER. *Nat. Cell Biol.*, **3**, 473–483.
- Poranen,M.M., Daugelavicius,R. and Bamford,D.H. (2002) Common principles in viral entry. *Annu. Rev. Microbiol.*, **56**, 521–538.
- Richards,A.A., Stang,E., Pepperkok,R. and Parton,R.G. (2002) Inhibitors of COP-mediated transport and cholera toxin action inhibit simian virus 40 infection. *Mol. Biol. Cell*, **13**, 1750–1764.
- Sahli,R., Freund,R., Dubensky,R., Garcea,R., Bronson,R. and Benjamin,T.L. (1993) Defect in entry and altered pathogenicity of a polyoma virus mutant blocked in VP2 myristylation. *Virology*, **192**, 142–153.
- Sottocornola,E., Colombo,I., Vergani,V., Tarabozetti,G. and Berra,B. (1999) Increased tumorigenicity and invasiveness of C6 rat glioma cells transfected with the human  $\alpha 2,8$  sialyltransferase cDNA. *Invasion Metastasis*, **18**, 142–154.
- Stang,E., Kartenbeck,J. and Parton,R.B. (1997) Major histocompatibility complex class I molecules mediate association of SV40 with caveolae. *Mol. Biol. Cell*, **8**, 47–57.
- Stehle,T. and Harrison,S.C. (1996) Crystal structures of murine polyomavirus in complex with straight-chain and branched-chain sialyloligosaccharide receptor fragments. *Structure*, **4**, 183–194.
- Stehle,T. and Harrison,S.C. (1997) High-resolution structure of a polyomavirus VP1-oligosaccharide complex: implications for assembly and receptor binding. *EMBO J.*, **16**, 5139–5148.
- Stehle,T., Yan,Y., Benjamin,T.L. and Harrison,S.C. (1994) Structure of murine polyomavirus complexed with an oligosaccharide receptor fragment. *Nature*, **369**, 160–163.
- Tsai,B. and Rapoport,T.A. (2002) Unfolded cholera toxin is transferred to the ER membrane and released from protein disulfide isomerase upon oxidation by Ero1. *J. Cell Biol.*, **159**, 207–216.
- Tsai,B., Rodighiero,C., Lencer,W.I. and Rapoport,T.A. (2001) Protein disulfide isomerase acts as a redox-dependent chaperone to unfold cholera toxin. *Cell*, **104**, 937–948.
- van den Akker,R., Sarfaty,S., Twiddy,E.M., Connell,T.D., Holmes,R.K. and Hol,W. (1996) Crystal structure of a new heat-labile enterotoxin, LTIIb. *Structure*, **4**, 665–678.
- Wolf,A.A., Fujinaga,Y. and Lencer,W.I. (2002) Uncoupling of the cholera toxin-G(M1) ganglioside receptor complex from endocytosis, retrograde Golgi trafficking, and downstream signal transduction by depletion of membrane cholesterol. *J. Biol. Chem.*, **277**, 16249–16256.

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