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Conformational Changes of Murine Polyomavirus Capsid Proteins Induced by Sialic Acid Binding*

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Murine polyomavirus (Py) infection initiates by the recognition of cell membrane molecules containing terminal sialic acid (SA) residues through specific binding pockets formed at the major capsid protein VP1 surface. VP1 Pockets 1, 2, and 3 bind terminal SA, Gal, and second branched SA, respectively. The consequence of recognition on viral cell entry remains elusive. In this work, we show that preincubation of Py with soluble compounds within Pocket 1 (*N*-acetyl or *N*-glycolyl neuraminic acids) increases Py cell binding and infectivity in murine 3T6 fibroblasts. In contrast, Gal does not significantly alter Py binding nor infectivity, whereas sialyllactose, in Pockets 1 and 2, decreases cell binding and infectivity. Binding experiments with Py virus-like particles confirmed the direct involvement of VP1 in this effect. To determine whether such results could reflect VP1 conformational changes induced by SA binding, protease digestion assays were performed after pretreatment of Py or virus-like particles with soluble receptor fragments. Binding of SA with the VP1 Pocket 1, but not of compounds interacting with Pocket 2, was associated with a transition of this protein from a protease-sensitive to a protease-resistant state. This effect was transmitted to the minor capsid proteins VP2 and VP3 in virus particles. Attachment of Py to cell monolayers similarly led to a VP1 trypsin-resistant pattern. Taken together, these data present evidence that initial binding of Py to terminal SA induces conformational changes in the viral capsid, which may influence subsequent virus cell entry steps.

The first phase of viral infection is determined by the interaction of virus particles with specific receptors on host cell membranes followed by subsequent cell penetration. In the case of enveloped viruses such as retroviruses, paramyxoviruses, or coronaviruses, recognition and binding to cell receptors trigger a conformational change in the virus attachment protein, which in turn leads to the activation of the fusogenic properties of an associated viral capsid protein. This event results in the fusion of the viral envelope with the cell membrane (1–4). Cell entry of nonenveloped viruses is less well understood, but it has emerged from recent studies that this process can be induced by similar molecular mechanisms. For example, binding of poliovirus to its cell receptor promotes conformational changes of the major capsid protein, VP1, which are transmitted to the other capsid proteins. This results in the exposure at the viral capsid surface of hydrophobic sequences that may participate in membrane penetration (5). The interaction of reoviruses or papillomaviruses with their receptors has also been reported to promote conformational changes in the viral capsid, which are detected by changes in the sensitivity to proteases or in the antigenic properties of the viral protein (6, 7).

Polyomavirus (Py)[1] is a nonenveloped murine tumor virus with a T=7 icosahedral lattice capsid composed of 72 pentamers of the major capsid protein VP1 consisting of both pentavalent and hexavalent structures (8). The minor capsid proteins, VP2 or VP3, are positioned in the center of each pentamer, likely anchored to VP1 through their C terminus by hydrophobic interactions (9, 10). The Py cell attachment receptor contains *N*-acetyl neuraminic acid (a sialic acid (SA) residue) present on glycoproteins and glycolipids (11–15) that is specifically recognized by VP1 (8, 16, 17). Crystallography analysis of the virus complexed with an oligosaccharide receptor fragment has revealed the presence of four pockets on the VP1 surface. Pockets 1 and 2 accommodate the terminal SA and galactose moieties, respectively, whereas Pocket 3 can accommodate the (α-2,6)-linked SA of a branched chain receptor (8). So far, no specific binding function has been attributed to Pocket 4, which may interact with other as yet undefined sugar moieties (8). High resolution structure of a recombinant VP1 pentamer complexed with a receptor fragment has identified VP1 residues Tyr-72, Arg-77, Gly-78, His-298, and Asn-93 as essential components of Pockets 1 and 2 (16, 17). Single mutations in these residues result in the complete loss of infectivity (18). It has been demonstrated, at least for Arg-77 and His-298 mutants, that this effect is due to impaired cell entry caused by abrogation of SA-dependent cell binding (14).

The interaction of VP1 with terminal SA residues is the critical initial step of a complex multistage Py cell entry mechanism. We have recently demonstrated that the $\alpha_4\beta_1$ integrin is a cell receptor for Py post-attachment (13). Such an interaction, likely determined by the specific recognition of an LDV motif present on the VP1 DE loop by the $\alpha_4\beta_1$ integrin-ligand binding

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[1] The abbreviations used are: Py, polyomavirus; SA, sialic acid; VLP, virus-like particles; NANA, *N*-acetyl neuraminic acid; NGNA, *N*-glycolyl neuraminic acid; PBS, phosphate-buffered saline; SL, sialyllactose; WGA, wheat germ agglutinin; Ab, antibody; FACS, fluorescence-activated cell sorter.

site, only occurs subsequent to the initial VP1-SA interaction (13, 14). We also demonstrated that Py cell binding can be restored in neuraminidase-treated fibroblast cells if the virus is first preincubated with soluble SA (13). Taken together, these data led us to hypothesize that the initial interaction of Py with SA moieties may induce an alteration of the capsid conformation, which is necessary for the recognition of post-binding cell receptors and subsequent cell penetration. In this work, we have analyzed the effect of soluble receptor compounds on Py cell binding and infectivity. Potential conformational changes of the viral capsid induced by receptor binding have been investigated by testing the sensitivity of capsid proteins to protease digestion.

EXPERIMENTAL PROCEDURES

Cells, Viruses, and Virus-like Particles (VLPs)—Mouse 3T6 fibroblast cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Invitrogen) in a 5% CO_2 atmosphere at 37 °C. Insect Sf9 cells were grown as monolayers in Grace's medium (Invitrogen) supplemented with 10% decomplemented fetal calf serum (Invitrogen) at 27 °C.

Py strain A2 was propagated at a low multiplicity of infection in 3T6 fibroblasts, and virus particles were purified by CsCl gradients, as described previously (13, 19). Production and CsCl gradient purification of VLPs were performed as described (14).

Reagents and Antibodies—*N*-acetyl neuraminic acid (NANA), *N*-glycolyl neuraminic acid (NGNA), and Gal, from Sigma, were dissolved in PBS (supplemented with 0.1 mM $CaCl_2$, 0.05 mM $MgCl_2$) to give a 320 mM (10%) stock solution. *N*-acetylneuraminyllactose (sialyllactose (SL)) was purchased from Sigma and dissolved in the same buffer to obtain a stock solution at 160 mM (5%). Aliquots of each stock solution were conserved at −20 °C. Wheat germ agglutinin (WGA) was purchased from Sigma.

Rabbit polyclonal anti-VP1 DE loop (designated 488) and mouse monoclonal anti-VP1 or -VP2/3 antibodies (Abs) were kindly provided by R. Garcea and J. Forstovà (20), respectively. Polyclonal large T antiserum was obtained from Brown Norway rats inoculated with syngeneic Py-transformed cells. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse immunoglobulin G (IgG) Abs were purchased from Bio-Rad, and R-phycoerythrin-conjugated streptavidin was purchased from BD PharMingen. Rat fluorescein isothiocyanate-conjugated IgG fraction Ab was obtained from Cappel.

FACS Analysis—Cell monolayers were detached by incubation in PBS, 5 mM EDTA and washed in buffer A (PBS supplemented with 0.1 mM $CaCl_2$, 0.05 mM $MgCl_2$, and 1% bovine serum albumin). Biotinylation of purified Py, Py VLPs, or WGA lectin was described previously (13). Biotinylated Py (0.8 μg), Py VLPs (0.5 μg), or WGA (75 ng) were pretreated for 1 h on ice with the sugar compound or buffer A (adjusted to pH 2.3 or 7.5) before being incubated with 4×10^5 cells for a subsequent hour on ice. Cells were washed and incubated with R-phycoerythrin-conjugated streptavidin in buffer A for 30 min on ice. After being washed twice, cells were fixed in paraformaldehyde, and their fluorescence intensity was analyzed by FACS using the CellQuest software.

Virus Infectivity Assays—2×10^5 3T6 cells, seeded for 3–4 h, were washed with Dulbecco's modified Eagle's medium just before infection. CsCl-purified viruses (1 μg corresponding to 1×10^7 plaque-forming unit) were preincubated with sugar compounds or buffer alone (PBS adjusted to pH 2.3 or 7.5) for 1 h on ice. Afterward, the virus volume was adjusted to 300 μl with Dulbecco's modified Eagle's medium, and the mixture was used to infect cells for 1.5 h at 37 °C. In some experiments, cells were pretreated with 50 milliunits of Neuraminidase (Sigma) for 1 h at 37 °C before viral infection. Unbound virus was removed, and Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, was added to the cells and left for 20 h at 37 °C. Cells were washed, fixed, and immunostained for virus (large T expression) and total nuclei (4′,6-diamidino-2-phenylindole) as described previously (13). Infectivity was measured as the number of large T-positive cells among total nuclei. A minimum of 1,000 nuclei was counted for each single plate, and each single experiment was performed in duplicate. 100% virus infectivity corresponded to an average of 10–20% large T-positive cells.

Protease Digestion Assays—Py or VLPs (1 μg) was incubated with the sugar compound or buffer alone (PBS adjusted to pH 2.3 or 7.5) for 1 h on ice. Dissociated VLPs were obtained by adding 3.3 mM dithiothreitol and 10 mM EDTA (final concentrations) to VLPs for 1 h. Samples were then mixed with 100 μg of trypsin (Invitrogen) or 25 μg of proteinase K (Sigma) and placed at room temperature. Aliquots from the digestion reactions were collected at various time points and lysed with Laemmli buffer. For assays performed on cell-bound viruses, 2×10^5 cells (monolayers) were precooled for 15 min at +4 °C, washed with ice-cold buffer, and then incubated with Py (2 μg) for a further hour on ice. Unbound viruses were removed, and trypsin (750 μg) was added to the monolayers at room temperature for 30 s, 2 min, or 20 min. Cells were washed three times with PBS containing protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin) and lysed with Laemmli buffer. After sample boiling, proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), stained with GelCode blue staining reagent (Pierce) or, alternatively, transferred to nitrocellulose filters (Schleicher & Schuell). Western blot analysis was carried out by using horseradish peroxidase-conjugated secondary Abs after blocking nonspecific reactivity with 4% nonfat dry milk in Tris-buffered saline, 0.05% Tween 20. Bands were detected by the enhanced chemiluminescence reaction (Pierce).

RESULTS

Effect of Different Sugar Compounds on Py and VLPs Cell Binding—Exposure of murine Py particles to soluble NANA effectively restores virus binding to cells pretreated with neuraminidase (13). To get better insight into the molecular mechanisms underlying this effect, we studied the effect of NANA on the ability of Py to bind to the permissive mouse 3T6 cell line. For this purpose, biotin-labeled purified Py was mixed for 1 h on ice with NANA at different concentrations (0, 32, 64, or 120 mM final concentration) and incubated with 3T6 cells for a further hour on ice. Cell-bound Py was detected by FACS using fluorochrome-labeled streptavidin. As shown in Fig. 1, preincubation of Py with NANA, but not with acidic buffer alone, increased cell binding in a dose-dependent manner. We further examined the effect of other sugar compounds on Py cell binding. Pretreatment of Py with NGNA, a larger compound than NANA, led to an increase of cell binding similar to that observed with NANA. The Gal compound, predicted to be accommodated within VP1 Pocket 2, did not modify Py cell binding. In contrast, preincubation of Py with SL (formed by NANA-α(2,3)-Gal-β(1,3)-GlcNAc), predicted to lie within Pockets 1 and 2 (16, 17), resulted in a dose-dependent decrease in cell binding (Fig. 1). Additionally, simultaneous pretreatment of Py with NANA and Gal resulted in increased cell binding, comparable with that seen with NANA alone (data not shown). The same experiments performed with Py VLPs, constituted by auto-assembled VP1 protein, showed effects similar to those observed with the viral particles (Fig. 1), indicating that the effects of sugars on cell binding were attributable solely to interactions involving the major capsid protein VP1. We ruled out that the increased cell binding observed with NANA-/NGNA-pretreated Py or VLPs could not be attributed to nonspecific binding of dissociated particles. Indeed, we ensured that integrity of Py or VLPs was perfectly maintained under acidic buffer conditions, by sedimentation of treated particles on CsCl gradients and direct electron microscopy observation (data not shown). In addition, we checked that the genomes packed into the viral particles were fully resistant to DNase I digestion (data not shown). The influence of these different compounds on the cell binding ability of the WGA lectin, which has been proposed to interact with *N*-linked α(2,3)-SA in a way similar to Py VP1 (17, 21), was also studied. We found that Gal did not influence WGA cell binding, similarly to Py VP1. In contrast, WGA binding was reduced in a dose-dependent manner in the presence of the other compounds used (Fig. 1). Taken together, these data indicate that the interaction of Py and VLPs, but not of WGA, with compounds accommodated within the VP1 binding Pocket 1, determines an increased cell binding ability.

Effect of Different Sugar Compounds on Py Infectivity—This topic was analyzed using the same 3T6 cell line after first determining that preincubation of Py with acidic or neutral buffer does not affect infectivity (data not shown). Our data

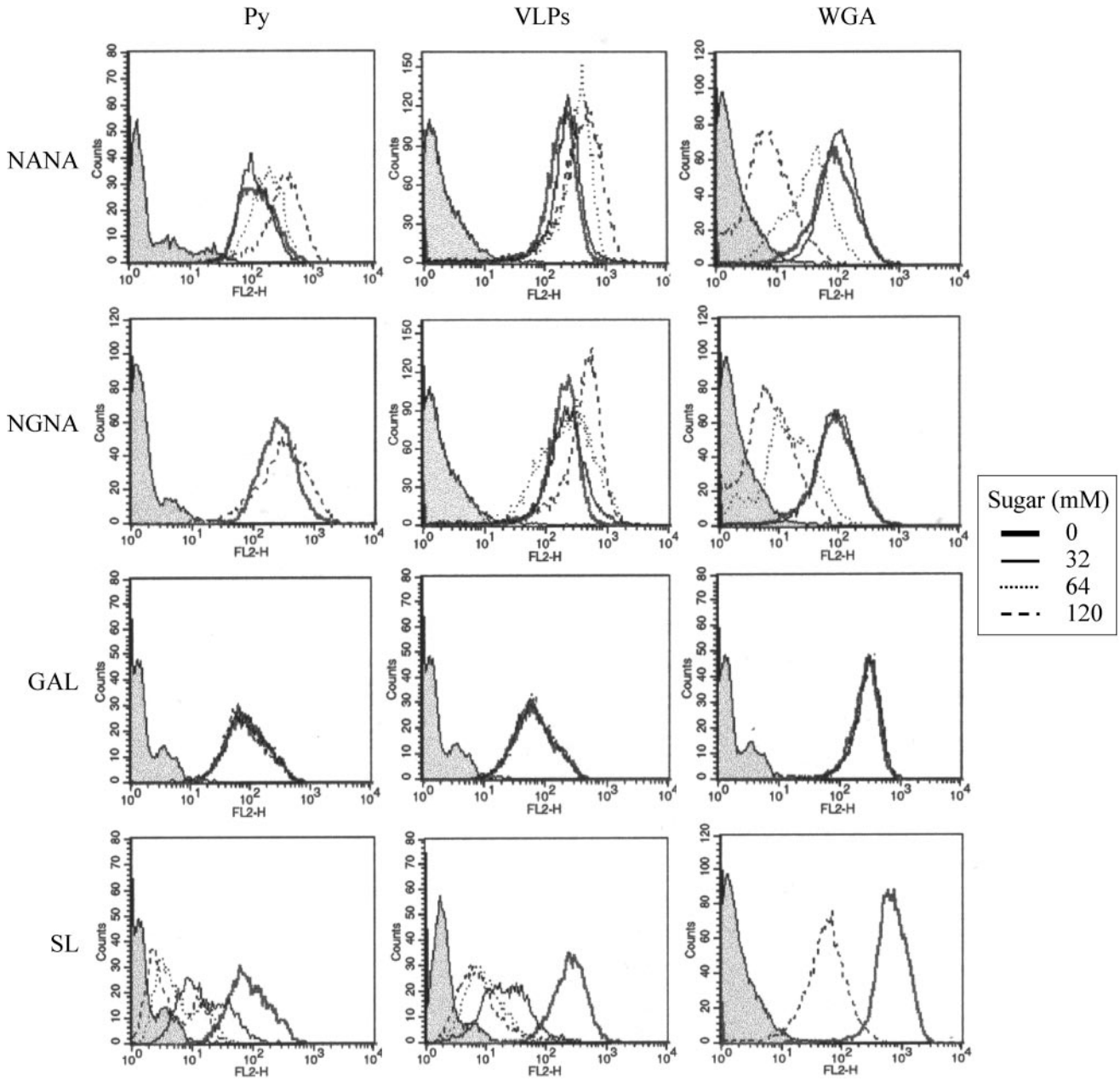

FIG. 1. **Effect of different sugar compounds on Py and VLPs binding to mouse 3T6 cells.** Biotinylated Py, VLPs, or WGA were incubated with buffer alone (0) or the respective sugar compound, as noted, at the concentrations indicated, and then added to 3T6 fibroblasts and incubated with R-phycoerythrin-conjugated streptavidin (see "Experimental Procedures"). Fixed cells were analyzed by FACS. Cells incubated with the binding buffer alone as a negative control are shown (*shaded area*). The results were reproducible in several independent experiments. Representative experiments are shown.

showed that SL reduced viral infectivity in a dose-dependent manner (36.5% ± 8.5% infectivity at 120 mM), whereas incubation with Gal only slightly reduced Py infectivity (81% ± 5.5% at 120 mM) (Fig. 2*A*). In contrast, infectivity was increased if Py was preincubated with NANA (139% ± 1% at 120 mM) or NGNA (data not shown). Taken together with cell binding assays, these data suggested a differential effect between VP1 Pockets 1 and 2 on Py infectivity.

Next, we asked whether the binding of Py to neuraminidase-treated cells, restored after exposure to NANA, results in a productive infection. Py, preincubated with or without 120 mM NANA, was used to infect cells that had been treated or not with the SA-removing enzyme. As shown in Fig. 2*B*, treatment of cells with neuraminidase reduced Py infectivity (to 38% ± 2%), but pretreatment of Py particles with NANA significantly restored infectivity (to 76% ± 8%). Thus, NANA binding to Py allows the recognition of SA-independent cell receptors for cell attachment and productive infection.

Preincubation of VLPs with NANA Partially Protects VP1 from Protease Digestion—To detect potential conformational changes of the Py VP1 protein due to soluble SA binding to Pocket 1, digestion protection assays were performed using trypsin protease. VLPs were preincubated with PBS buffer alone or 64 mM NANA, and trypsin was then added for various time periods. The resulting protease-resistant VP1 products were analyzed both by SDS-PAGE and by staining with the GelCode blue reagent (Fig. 3*A*) and by Western blot (Fig. 3*B*). A clearly different digestion profile was observed in samples treated or not with NANA. In NANA-VLPs samples, a large part of VP1 remained undigested, whereas digestion of PBS-VLPs, for as little as 2 min, resulted in complete disappearance of the intact VP1 and simultaneous appearance of smaller

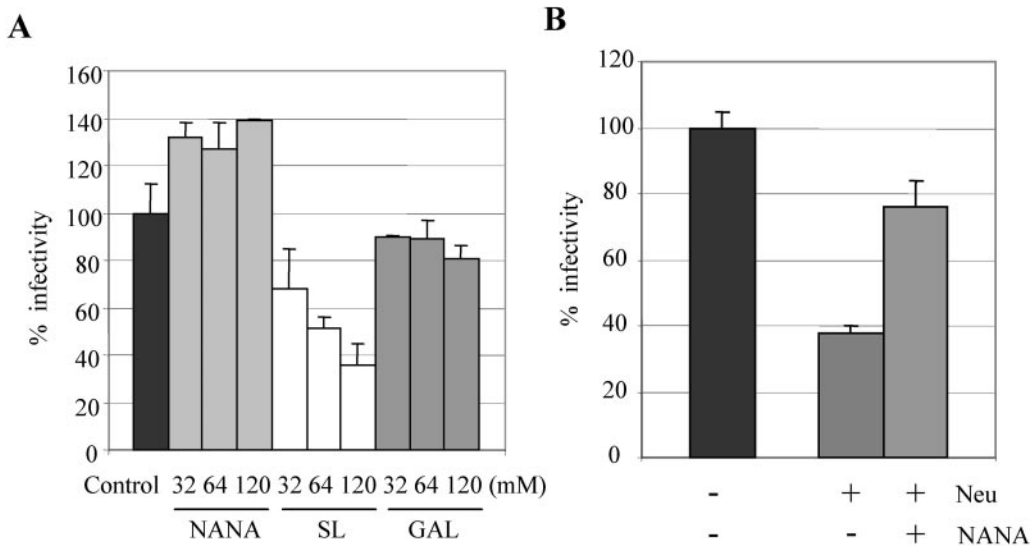

FIG. 2. **Effect of preincubation with sugar compounds on Py infectivity in 3T6 cells.** *A*, Py preincubated with different concentrations of NANA, SL, or GAL for 1 h at 4 °C, added to cells. *B*, Py preincubated with 120 mM NANA or buffer alone, added to cells pretreated with (+) or without (−) 50 milliunits of neuraminidase (*Neu*). Py infectivity was measured by indirect immunofluorescence. Results are presented as the percentage of Py infectivity obtained when cells were preincubated without sugar compounds (Control).

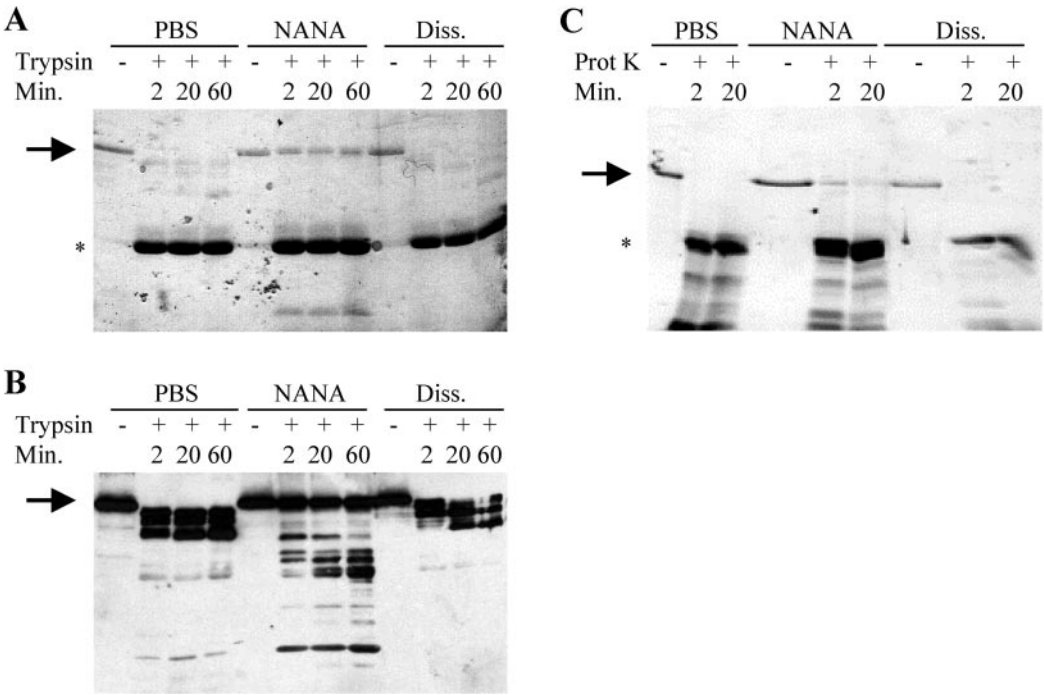

FIG. 3. **Effect of NANA on the sensitivity of VLPs to proteases digestion.** VLPs were pretreated with PBS or 64 mM NANA, or dissociated VLPs (*Diss.*), and then digested with trypsin for the indicated periods (in min) of time. Protein samples were analyzed by SDS-PAGE stained with GelCode blue staining reagent (*A*) and Western blots using anti-VP1 DE loop antibodies (*B*). *C*, VLPs were pretreated with PBS or 64 mM NANA, or dissociated VLPs, and then digested with proteinase K (*Prot K*) for the indicated periods (in min) of time. Protein samples were analyzed by SDS-PAGE stained with GelCode blue staining reagent. *Arrows* indicate the position of the undigested VP1. *Asterisks* show bands corresponding to the proteases.

polypeptides with 37, 39, and 40 kDa. In NANA-VLPs samples, the fraction of VP1 that was digested generated bands of 28–30 and 25–26 kDa, which were not detected in PBS-VLPs samples. Longer digestion times (up to 300 min) did not significantly change the two digestion profiles (data not shown). As a control, dissociated VLPs (treated with dithiothreitol and EDTA prior to protease digestion) appeared more digested than PBS-VLPs. Further experiments ruled out that low pH could have modified the cleavage specificity of the protease. That is, the trypsin digestion profile was shown to be identical whether VLPs had been pretreated with buffer adjusted to pH 2.3 or 7.5, and purified H1 histone, as alternative substrate, was similarly digested by trypsin if pretreated with PBS or 64 mM NANA (data not shown). Our data demonstrated that NANA binding to the VLPs converted VP1 from a trypsin-susceptible to a trypsin-resistant state. To determine whether such conformational changes were also detected with other proteinases, similar experiments were performed using proteinase K, which has broader cleavage specificity than trypsin. Protease-resistant VP1 fragments were analyzed by SDS-PAGE and stained with the GelCode blue reagent. As observed in Fig. 3*C*, NANA-treated VLPs were protected relative to untreated VLPs, whereas dissociated VLPs appeared almost completely digested even after 2 min. Taken together with the trypsin di-

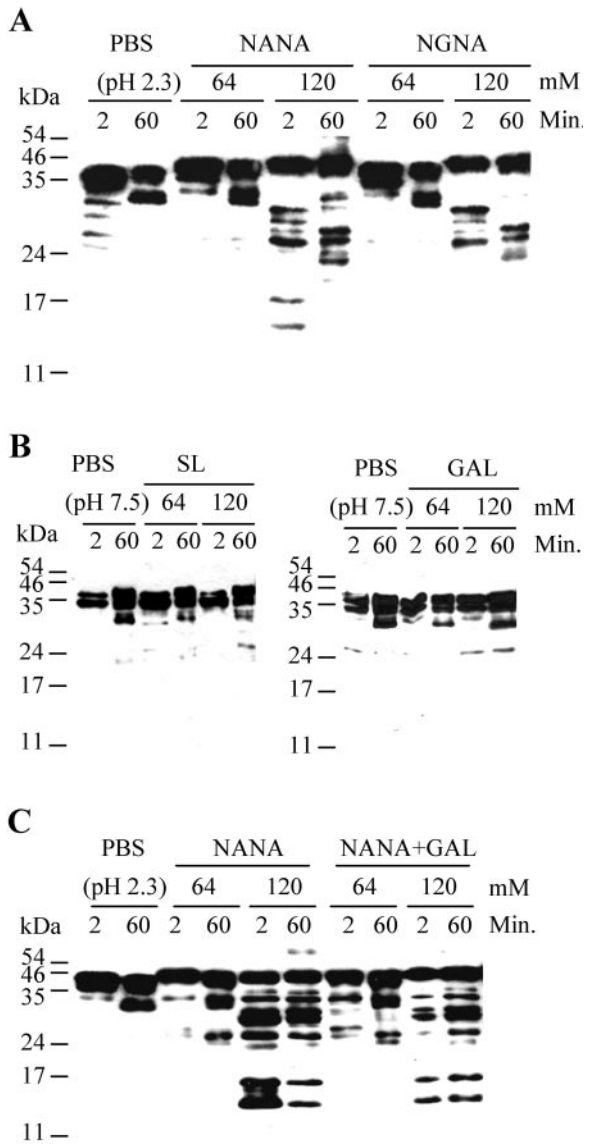

FIG. 4. **Effect of the sugar compound on the sensitivity of VLPs to trypsin digestion.** VLPs were pretreated with NANA or NGNA (*A*), SL or GAL (*B*), or NANA + GAL (*C*) at 64 or 120 mM and then digested with trypsin, as indicated. Protein samples were analyzed by Western blots using anti-VP1 DE loop antibodies.

gestion assays, these data indicated that the interaction of NANA with VP1 changes the accessibility of the VP1 cleavage sites to proteases, and therefore, suggested that binding of the soluble receptor to the VP1 Pocket 1 may alter the conformation of the VLPs. Since the digestion profiles obtained by proteinase K treatment were more complex to analyze, further studies were performed with the trypsin protease.

Effect of Sugar Compounds on the Sensitivity of VLPs to Trypsin Digestion—Sugar compounds (NANA, NGNA, Gal, SL, or NANA + Gal) were assessed with regard to susceptibility of VLPs to trypsin digestion. VLPs were incubated with the sugar compounds at two different concentrations, and then trypsin digestion was carried out. The resulting protease-resistant VP1 fragments were analyzed by Western blots (Fig. 4). It was found that the protection effect of NANA on VLPs was dose-dependent; that is, a higher proportion of VP1 was trypsin-resistant at 120 mM than at 64 mM (Fig. 4*A*). Incubation of VLPs with NGNA resulted in a digestion pattern similar to that observed with NANA (Fig. 4*A*). In contrast, preincubation of VLPs with either SL or Gal (at any concentration) gave trypsin digestion patterns comparable with those of PBS-VLPs (Fig. 4*B*). Finally, simultaneous incubation of VLPs with NANA and Gal conferred a digestion profile similar to that observed for NANA-VLPs (Fig. 4*C*). These data are consistent with the notion that a conformational change of VP1 is induced by the compounds that exclusively bind to Pocket 1.

Effect of NANA-induced Conformational Changes of VP1 on the Minor Capsid Proteins—We investigated whether the conformational change observed on VLPs upon SA binding also occurs in the viral capsid context, which includes the two minor capsid proteins VP2 and VP3 and the packed minichromosome. To this end, Py was incubated with SA-containing compounds (NANA or SL) at different concentrations or with PBS buffer alone before trypsin digestion. As shown in Fig. 5*A*, the incubation of viruses with NANA, but not with PBS or SL, was associated with VP1 resistance to trypsin digestion in a dose-dependent manner, similar to VLPs. The same samples were analyzed by Western blot using monoclonal Abs that recognize both the VP2 and the VP3 proteins. In PBS-Py samples, as early as 2 min after digestion, both minor capsid proteins had been totally digested. A similar result was observed in SL-Py samples at any concentration. In contrast, in NANA-Py samples, a different digestion profile could be observed depending on the concentration. In fact, at 32 mM, some VP2/3 products were still detected and, at 64 and 120 mM, minor proteins were almost fully protected from trypsin digestion. These data show that the change of VP1 conformation induced by NANA binding also occurs in the virus and modifies the accessibility of minor coat proteins to proteases.

Effect of Py Binding to Host Cells on VP1 Sensitivity to Trypsin—To determine whether the VP1 resistance to trypsin observed after incubation of Py or VLPs with soluble NANA also occurs at the host cell surface, Py was allowed to bind to 3T6 fibroblast monolayers, and trypsin was added to cells for the times indicated in Fig. 5*B*. The amount of trypsin used for this experiment was increased by 7.5-fold with respect to that used for the soluble virus and was determined not to be a limiting factor in the digestion assay (data not shown). As detected by Western blots, the VP1 of cell-bound viruses was totally trypsin-resistant, whereas that from unbound viruses appeared sensitive to trypsin (Fig. 5*B*). The possibility that the apparent resistance of cell-associated VP1 to trypsin resulted from viral cell entry was ruled out by measuring the virus internalization in these experimental conditions. Biotin-labeled purified Py (0.8 μg) was added to 2.10^5 3T6 cells for 1 h on ice. Unbound particles were washed away, and cells were incubated at room temperature for the indicated times. Then, cell-bound Py was detected by FACS analysis as described (see "Experimental Procedures"). The percentage of internalization (*% Py Int.*) was calculated as the difference of fluorescence intensity of cells before and after the temperature switch. *0% Py Int.* corresponds to the fluorescence observed after 1 h of Py binding at +4 °C. As indicated in Fig. 5*B*, only 8% of total cell-bound Py had become internalized within 30 s, and the percentage of Py internalization only reached 23% after 20 min. Therefore, as seen with the soluble receptor, the binding of Py to the cell membrane triggers conformational changes in the major capsid protein VP1.

DISCUSSION

The present work provides for the first time biological and biochemical evidence that the initial binding of Py to terminal SA alters the conformation in both major and minor capsid Py proteins, which may be necessary for subsequent virus entry into cells. The use of soluble receptor compounds is an approach that has previously been adopted to analyze the receptor specificity of Adenovirus 37 on Chang cells. In this case, NANA, NGNA, and

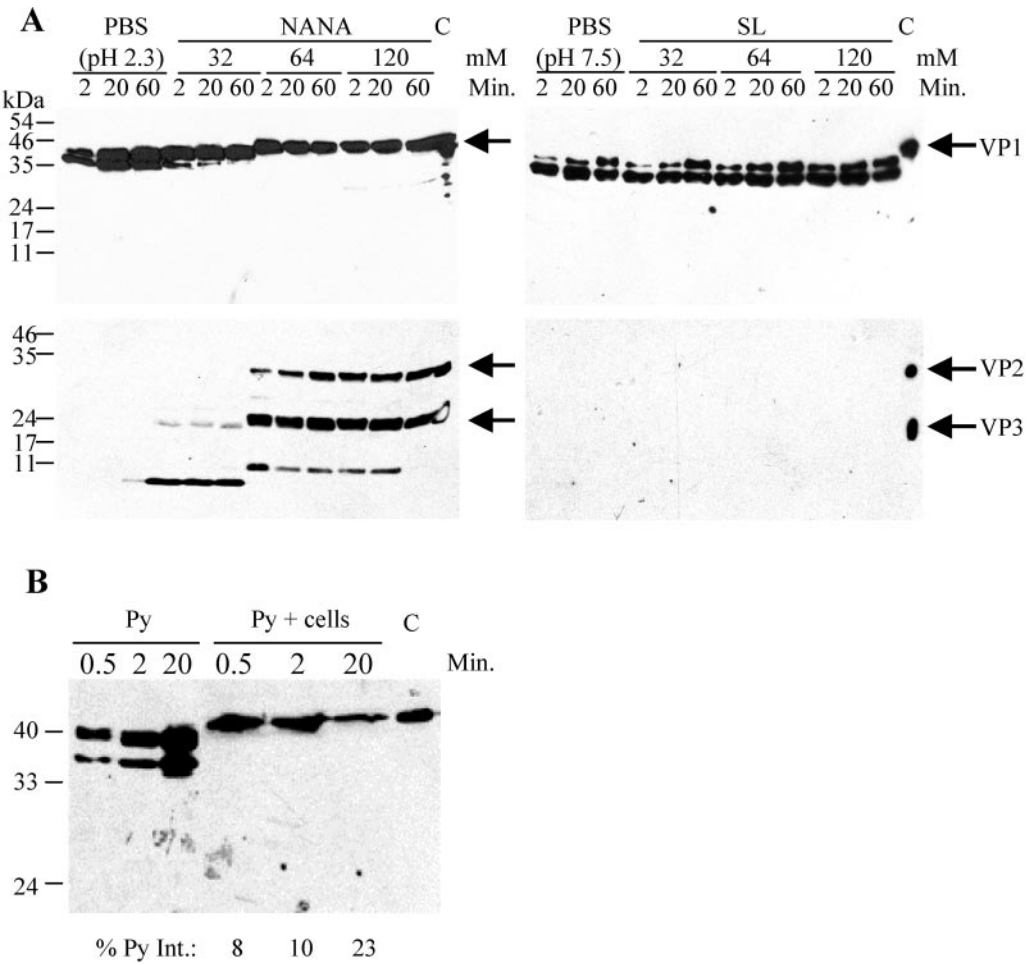

FIG. 5. **Effect of binding of Py to soluble or cell membrane-associated receptor on capsid proteins.** *A*, sensitivity of Py plus soluble receptors to trypsin digestion. Py was pretreated with PBS or with the indicated concentrations of NANA or SL and then digested with trypsin for times, as noted. Protein samples were analyzed by Western blots using anti-VP1 DE loop (*upper panels*) and anti-VP2/3 (*lower panels*) antibodies. *B*, the effect of Py binding to 3T6 cells on VP1 sensitivity to trypsin. Unbound Py and Py prebound to 3T6 cells (Py + cells) were digested with trypsin for the indicated times. Protein samples were analyzed by Western blots using anti-VP1 DE loop antibodies. For Py + cells samples, the total amount of protein lysed from each single plate was loaded onto the SDS-PAGE gel. Control (*C*) corresponds to undigested Py particles. The percentage of Py internalization (*% Py Int.*) was calculated as the decrease of fluorescence intensity of cell surface-bound labeled Py after the temperature switch at room temperature. 0% *Py Int.* corresponds to the fluorescence observed after 1 h of Py binding at +4 °C, before the temperature switch.

SL used at concentrations of 160 mM were all shown to block 80–90% of Ad37 cell attachment (22). In our experiments, a similar blocking effect was observed on WGA cell binding. In contrast, we observed a dose-dependent increase of Py cell binding in the presence of compounds only accommodated within Pocket 1 of VP1. Such a result was not specific of the cell line since it was detected in other murine permissive cell lines (data not shown). The effect of NANA on Py cell binding was not due to non-specific pH effect since control experiments, performed at acidic pH, did not alter the host cell membrane nor influence Py cell attachment. Since increased cell attachment upon SA binding was also detected with both Py virions and VLPs, the observed effect must be dependent on the major capsid protein VP1. For Py viral particles, increased cell binding resulted in increased infectivity. A similar phenomenon has been reported for human immunodeficiency virus (HIV) or coronaviruses, whose incubation with their soluble cell receptors activate viral infectivity due to a conformational change of the envelope protein (1, 4). For Py, soluble NANA binding to VP1 was accompanied by a change in VP1 sensitivity to proteases, the data indicating that the interaction of terminal SA with VP1 Pocket 1 triggers a conformational change in the protein that results in favored cell entry of particles and infection.

The NGNA is an SA variant that is expressed on the surface of murine cells in various tissues (16). Structural analysis suggested that its additional hydroxyl group (with regard to NANA) could possibly hydrogen-bond with the side chains of Tyr-72 and Asp-85 of the clockwise VP1 neighbor in the viral pentamer. In accordance with this hypothesis, we found that NGNA has effects on Py cell binding, infectivity, and protease digestion that are similar to NANA. The data raise the interesting question whether NGNA may also be a functional cell receptor for Py on murine cells.

Intriguing data were obtained with regard to the VP1 interaction with Gal. Naturally occurring terminal SA are linked to Gal moieties (23), and crystallographic evidence has indicated that Gal is accommodated within VP1 Pocket 2 through hydrogen bonding with Asn-93. Hydrogen bonding of Gly-78 with Gal and terminal SA contributes (together with Arg-77) to form a ridge with both sugar moieties separated onto either side of the ridge (17). Mutation of any of these sites in VP1 leads to nonviability of the Py strains (18). In our experiments, Gal did not modify Py cell binding, nor did it significantly affect viral infectivity, and no particular conformational change of VP1 was detected in the protease digestion assays. Further, 3′-sialyllactose had a blocking effect on Py cell binding and infectivity, similar to that seen for the WGA lectin, and no associated conformational change of VP1, in line with previous

crystallographic studies performed with this particular compound (17). In contrast, simultaneous binding of unlinked NANA and Gal moieties resulted in VP1 conformational changes. Taken together, these data suggest a critical role of the SA-Gal linkage conformation as a determinant for productive interaction between Py and its receptor. The conformation of the SA-Gal linkage, determined by the presence of internal hydrogen bonds in each SA and Gal and between both moieties, may depend on the nature of the oligosaccharide receptor. *In vivo*, it is conceivable that Py infection will be productive if the interaction of VP1 only occurs with sialylated receptors carrying a SA-Gal linkage conformation that is compatible with conformational changes that occur in the capsid protein. Incompatibility may lead to "pseudoreceptors," a hypothesis already proposed for VP1 binding to second branched SA through Pocket 3 (18). In support of this model is the observation that, among large plaque strains, mutation of the Val-296 to Ala (PTA and LID strains, respectively) confers weaker binding to Pocket 1 due to abrogation of a hydrophobic interaction with terminal SA and results in a higher virulent phenotype (18). Exploration of the conformational changes induced by SA binding in the VP1 proteins of PTA and LID strains may help define this possibility.

Conformational change of viral attachment proteins upon receptor binding is associated with structural alterations of the other capsid proteins for several viruses (2, 24, 25). For Py, we found that NANA binding to VP1, but not SL, promotes a transition of VP2 and VP3 from a protease-sensitive to a protease-resistant state, indicating that the interaction of VP1 with SA also confers changes to the conformation of VP2 and VP3. Consistent with our data, several reports support a role for the minor capsid proteins during the early stages of Py infection (26, 27). In the case of VP2, this function seems to depend primarily on the amino acid composition rather than the presence of the myristyl moiety at the N terminus (26), although this modification has been demonstrated to play a central role in cell penetration for other viruses (28, 29). Determination of the crystal structure of the C-terminal segment of VP2/3 complexed with a VP1 pentamer at 2.2-Å resolution shows that this portion of the minor capsid proteins is strongly anchored to VP1 through specific hydrophobic interactions (10). Although the mechanism by which VP1 confers conformational changes on the minor proteins remains to be further defined, one may hypothesize that this could be mediated by the region of hydrophobic interactions taking place between the major and the minor proteins. The fact that VLPs, which are only constituted from VP1, behave similarly to viral particles after SA binding indicates that there must be VP1 sequences, similar to those of VP2/3, that become exposed upon SA binding to interact with host cell membranes and further promote viral entry.

In conclusion, we have demonstrated that the recognition of SA moieties on host cell membranes provokes initial conformational changes in the Py capsid, which appear crucial for productive infection. In this regard, Py resembles other viruses in which a primary interaction with SA-containing receptors also leads to conformational alterations of capsid proteins (3, 6). This addresses the question of whether the recognition of SA moieties as primary cell receptors can be a mechanism to induce conformational changes in viral capsids necessary for viral entry, common to other viruses such as polyomavirus species associated with human diseases (30).

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Conformational Changes of Murine Polyomavirus Capsid Proteins Induced by Sialic Acid Binding

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