Role of sialic acid-containing molecules and the $\alpha 4/\beta 1$ integrin receptor in the early steps of polyomavirus infection

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INTRODUCTION

Sialic acid (SA) residues are carbohydrate compounds abundantly and broadly expressed as terminal components of many glycoproteins and glycolipids on mammalian cells (Kelm & Schauer, 1997). They have also been demonstrated to be essential receptor components for initial attachment to host cells of many enveloped and non-enveloped viruses (Kelm & Schauer, 1997); however, SA–virus interaction seems to occur through a variety of modalities. For instance, SA is used as a co-receptor with integrin for cell binding of epidemic keratoconjunctivitis-associated adenoviruses (Arnberg et al., 2000a, b). In contrast, animal rotaviruses use SA and integrins as co-receptors in a two-step entry mechanism in which SA is necessary for initial cell binding, with integrins required for the secondary internalization step (Arias et al., 2002). Although SA has long been considered to be the sole cell receptor for influenza virus or reoviruses, recent reports have identified virus reassortants that are able to infect cells in a SA-independent manner, indicating the existence of alternative SA-independent cell receptors (Stray et al., 2000; Barton et al., 2001a).

Murine polyomavirus (MPyV) infection occurs through recognition of sialic acid (SA) residues present on the host cell membrane, but the nature of the molecules involved and the exact role of this interaction in virus cell entry still need to be clarified. In this work, mutations at residues R77 or H298 of the MPyV VP1 protein were shown to lead to a complete loss of virus infectivity, which, however, could be restored by lipofection of virus particles into the cytoplasm of the host cells. Using virus-like particles (VLPs), it was demonstrated that the non-infectivity of these mutants was due to impaired cell entry caused by total abrogation of SA-dependent cell binding. This indicates that SA residues are essential primary cell receptors for MPyV. As the $\alpha 4/\beta 1$ integrin has been identified recently as a cell receptor for MPyV, the relationship, if any, was investigated between SA-containing and $\alpha 4/\beta 1$ integrin receptors. The ability of mutants R77Q and H298Q and wt VLPs to bind to cells overexpressing the $\alpha 4/\beta 1$ integrin was studied in SA-positive (BALB/c 3T3 cells and Pro-5 cells) and SA-deficient (Pro5-derived Lec-2 cells) backgrounds. Overexpression of $\alpha 4/\beta 1$ integrin did not restore binding of mutant VLPs in any of these cell lines or, indeed, that of wt VLPs in a SA-deficient background. Moreover, evidence is provided that overexpression of the siaylated $\alpha 4/\beta 1$ integrin enhances wt VLP cell binding, suggesting that, in addition to its function at a post-attachment level, $\alpha 4/\beta 1$ integrin acts also as one of the SA-containing receptors for initial cell binding.
crystal structure of the major coat protein VP1 in complex with sialyllactose has been solved at 1·9 Å resolution (Stehle et al., 1994; Stehle & Harrison, 1996, 1997). As a result of these studies, the sialylated receptor has been predicted to bind to pre-formed pockets on the viral capsid surface. Pockets 1 and 2 accommodate the terminal SA and the penultimate α(2,3)-linked galactose, respectively, and pocket 3 accommodates a second α(2,6)-branched SA (Stehle & Harrison, 1996, 1997). Five residues (Y\textsuperscript{72}, R\textsuperscript{77}, G\textsuperscript{81}, N\textsuperscript{85} and H\textsuperscript{298}), mapped within the outfacing BC and HI loops of VP1, appear to be important for SA interactions, since they establish hydrogen bonds, salt bridges or hydrophobic interactions with the oligosaccharide (Stehle et al., 1994; Stehle & Harrison, 1996, 1997). On the basis of these structural data, Bauer et al. (1999) generated MPyV PTA mutant strains carrying single substitutions in each of these VP1 residues to abrogate such bonds. Transfection of mouse fibroblast cells with the mutated genomes, in contrast to transfection with the wt genome, did not result in any visible cytopathic effect even at 3 weeks post-transfection (p.t.), suggesting that each of these VP1 residues was visible cytopathic effect even at 3 weeks post-transfection (p.t.), suggesting that each of these VP1 residues was.

In the past, several attempts to isolate and identify candidate MPyV receptor molecules have been unsuccessful (Griffith & Consigli, 1986; Marriott et al., 1987a, b; Bauer et al., 1999). Recently, we demonstrated that the α4β1 integrin acts as a cell receptor for MPyV in fibroblast cells, probably by recognizing as a ligand the consensus LDV motif present in the DE loop of VP1. The interaction of MPyV with α4β1 integrin appears to be important for MPyV infectivity at a post-attachment level, since function-blocking antibodies directed against the ligand-binding site of the integrin block MPyV infectivity without affecting MPyV cell binding (Caruso et al., 2003).

The aim of the present work was to study further the function of SA residues as MPyV cell receptors and the potential relationship between SA-containing receptors and α4β1 integrin. For this purpose, two isogenic VP1 virus mutants of the MPyV A2 large plaque strain, designated MPyV R\textsuperscript{77}Q and H\textsuperscript{298}Q, were constructed and their infectious properties were analysed in murine fibroblast cells. Binding properties of the corresponding wt or mutant virus-like particles (VLPs) were also studied by FACS analysis in cells overexpressing or not expressing the α4β1 integrin in both SA-positive and SA-deficient backgrounds.

**METHODS**

**Plasmids.** The MPyV A2 large plaque wt genome (GenBank accession no. J02288.1) cloned into the EcoRI site of the plasmid vector pAT153 (Twigg & Sherratt, 1980) was used as the template for site-directed mutagenesis. The VP1-encoding sequence of wt MPyV is identical to the sequence under GenBank accession no. M34958, except for an amino acid substitution at position 91 (G→E), considering that VP1 numbering starts after the initial Met residue. Single amino acid substitutions were introduced using the Quick Change Mutagenesis kit (Stratagene) with the following pairs of complementary primers: for R\textsuperscript{77}Q, V61 (forward, 5′-tactatggtgg-agccgggattattggtcata-3′) and V62 (reverse, 5′-t-tacatggtgg-agccgggattattggtcata-3′); for H\textsuperscript{298}Q, V65 (forward, 5′-agaatcatgtagta-carcagtagagggctccc-3′) and V66 (reverse, 5′-agagagctccctcagttc-3′). The nucleotides underlined show the additional BrsI and BrGl restriction sites introduced as silent mutations in R\textsuperscript{77}Q and H\textsuperscript{298}Q, respectively, in order to specifically distinguish mutant from wt sequences. The presence of the desired mutations and integrity of the rest of the VP1-encoding sequence were controlled by DNA sequencing. A 1·8 kb Brf–BamII fragment (nt 2225–4053) and a 0·9 kb Asel fragment (nt 2925–3835) carrying the R\textsuperscript{77}Q and H\textsuperscript{298}Q mutations were then used to replace the corresponding wt sequence fragments in the plasmid pAT153-Py wt, respectively. The resulting MPyV mutants were designated MPyV R\textsuperscript{77}Q and H\textsuperscript{298}Q.

To generate VP1 pseudocapsids, a 1·6 kb EcoRV–Xhol fragment (nt 4110–2524) from the wt, R\textsuperscript{77}Q or H\textsuperscript{298}Q genomes of MPyV was introduced into the linearized Stul–Xhol pFASTBACI cloning vector (Life Technologies). The cloned VP1 genes were then transfected by transposition to the Bacmid vector, as recommended by the manufacturer.

**Cells.** Mouse fibroblast 3T6 and Swiss 3T3 cells were grown in DMEM supplemented with 10% FCS (Gibco) in a 5% CO\textsubscript{2} atmosphere at 37°C. Mock- or α4-transfected BALB/c 3T3 cells were grown in DMEM supplemented with 10% FCS in the presence of 1·3 μg puromycin ml\textsuperscript{-1}, as described previously (Caruso et al., 2003). Lec-2 cells, defective in SA expression, and the parental cell line Pro-5 (expressing SA), both originating from a Chinese ovary hamster (CHO) cell line, were grown as monolayers, as described previously (Stanley et al., 1975; Stanley & Siminovitch, 1977). Pro-5 and Lec-2 cells were transfected with the empty or recombinant pRK5 vector carrying the entire cDNA of the murine α4 integrin subunit (Rietzler et al., 1998) and the plasmid pBabe-Puro containing the puromycin-resistance gene as a selection marker (Morgenstern & Land, 1990) using the Lipofectamine Plus reagent (Invitrogen). At 2 days after transfection, cells were split 1:5 in DMEM supplemented with 5 μg puromycin ml\textsuperscript{-1}. Surviving colonies were pooled and amplified.

Insect S9 cells were grown as monolayers in SF900II medium (Gibco) supplemented with 10% de-complemented FCS (Gibco) at 27°C.

**Viruses and VLPs.** To produce MPyV particles, fibroblast 3T6 cells were transfected with the re-ligated wt or mutant genomes, as described previously (García et al., 2000), using the DEAE/dextran procedure (McCutchan & Pagano, 1968). Cells were lysed 72 h.p.t. by repeated freeze-thawing and centrifuging for 15 min at 8000 g. The resulting supernatant was collected and virus particles were then concentrated by centrifugation through a 20% sucrose cushion in buffer B [150 mM NaCl, 10 mM Tris/HCl (pH 7·4) and 0·01 mM CaCl\textsubscript{2}]. For infection experiments, titration of non-infectious virus particles was performed by comparing their concentration in the supercoiled form I DNA molecules with that of wt MPyV particles titrated previously by plaque assay. For lipofection experiments, virus particles were purified by both CsCl and sucrose gradients, as described previously (Caruso et al., 2003), and Lipofectamine Plus reagent was used according to the manufacturer’s instructions.

To produce recombinant baculovirus particles, S9 cells were
transfected with recombinant Bacmid–VP1 plasmids using CellFectin (Gibco). Initial transfection lysates were used to subsequently infect cells in order to increase baculovirus titres. Production and purification of VLPs were by CaCl and sucrose gradients, as described previously (Forstová et al., 1995).

For cell-binding assays, VLPs or wheat germ agglutinin (WGA; Sigma) were bioinylated as follows: 15 μg protein was incubated with 1 mM biotin (Pierce) in a 50 mM Na2CO3 buffer (pH 8.5) for 30 min at room temperature and then dialysed extensively against buffer B. The efficiency of bioinylation was tested by Western blotting, as described previously (Caruso et al., 2003).

Negative staining. Virus, virus–lipofectamine complexes or VLP preparations were adsorbed to 400 mesh Formar carbon-coated copper grids and stained with 1 % uranyl acetate, pH 4.5. The preparations were observed under a Philips CM10 electron microscope operating at 80 kV.

Haemagglutination assays. A 5 % suspension of sheep red blood cells (Sclavo Diagnostics) was washed three times in PBS just before use and adjusted to a final concentration of 0.4 % in PBS. Cells were added (100 μl) to 100 μl serially diluted VLPs in 96-well, round-bottomed culture dishes (Falcon). Haemagglutination was read after an incubation time of at least 4 h at 4 °C.

Virus replication and gene expression analysis. For virus replication assays, total cellular RNA was obtained by cell lysis in 10 mM Tris/HCl (pH 8.0), 0.1 M EDTA, 0.5 % SDS and 200 μg proteinase K ml−1 and subsequent phenol/chloroform extraction. DNA was digested with EcoRI/MboI and restriction fragments were separated by electrophoresis in 1 % agarose gels and transferred to Gene Screen membranes (NEN Life Science Products), as recommended by the manufacturer. Filters were hybridized with the whole MPyV genome linearized by EcoRI, as described previously (Sambrook et al., 1989).

For viral gene expression assays, total cellular RNA was isolated from virus infected- or virus–lipofected cells by the method of Chomczynski & Sacchi (1987) and transferred to Gene Screen membranes. Filters were hybridized with the NdeI–EcoRI (nt 1562–2738) and Asel (nt 2925–3835) fragments specific for early and late genes, respectively, as well as with the β-actin-specific probe. The β-actin-specific probe was generated by PCR on 3T3 cellular DNA extracts using the following primers: V94 (forward, 5′-agtgaagctgacatgctgcc-3′) and V95 (reverse, 5′-ctattcagttgtctgctcag-3′). Autoradiography was carried out with Fuji photo films at −70 °C.

FACS analysis. Cell monolayers were detached with PBS/5% FBS and washed with binding buffer BB (PBS, 0.1 mM CaCl2, 0.05 mM MgCl2 and 1 % BSA).

For integrin subunit cell surface expression, 4 × 10^5 cells were incubated with 0.5 μg monoclonal antibodies directed to the integrin subunits (CD49d, clone R1-2 for the α subunit, or CD29, clone 9E5/1 for the β1 subunit; BD Pharmingen) for 1 h on ice in buffer BB. Cells were washed twice in buffer BB and subsequently incubated with secondary R-phycocerythrin (PE)-conjugated anti-rat or anti-hamster antibodies (ICN) for 30 min on ice.

For cell-binding assays, 4 × 10^5 cells were incubated with biotin-labelled VLPs or WGA in buffer BB for 1 h on ice. Cells were washed twice in buffer BB and incubated with R-PE- or FITC-conjugated streptavidin (BD Pharmingen) for 30 min on ice.

After three final washes in buffer BB, cells were fixed in 4 % paraformaldehyde and their fluorescence intensity analysed on a FACS Calibur flow cytometer (Becton Dickinson) using the CELL QUEST software.

Western blotting. Cells were lysed for 1 h at 4 °C in 50 mM Tris/HCl (pH 7.4), 1 % Triton X-100, 150 mM NaCl, 2 mM CaCl2 and protease inhibitors (1 mM PMSF, 10 μg leupeptin ml−1 and 10 μg aprotinin ml−1). After centrifugation at 13 000 r.p.m. for 10 min at 4 °C, the protein content of each sample was quantified using the Bio-Rad Protein Assay reagent. A total of 300 μg protein was treated with or without 100 μU Clostridium perfringens neuraminidase (Sigma) for 3 h at 37 °C. Equal quantities of lysates were then run on 6 % SDS-polyacrylamide gels and transferred onto nitrocellulose filters (Schleicher & Schuell). Western blot analysis was carried out using anti-α4 antibodies (C-20) or anti-β1 antibodies (M-106) (Santa Cruz Biotechnology) after blocking non-specific reactivity with 2 % non-fat dried-milk in TBS/0.05 % Tween 20. Bands were detected with horseradish peroxidase (HRP)-conjugated anti-goat secondary antiserum (Santa Cruz Biotechnology) or anti-rabbit secondary antiserum (Bio-Rad), followed by the enhanced chemiluminescence reaction (Pierce).

RESULTS

SA-binding pocket 1 mutants are not infectious in fibroblast cells

To understand better the role of the SA–VP1 interaction in MPyV infectivity, two different MPyV mutant genomes, originating from MPyV strain A2, were generated. These two mutants carry single substitutions in the sequences encoding two residues of the VP1 protein (R297 and H298), predicted to be critical for correct accommodation of terminal SA within pocket 1 (Stehle et al., 1994; Stehle & Harrison, 1996, 1997; Bauer et al., 1999). To produce the mutant viruses MPyV R297Q and H298Q, murine fibroblast 3T6 cells were transfected with the mutant or wt viral genomes. The time-course of viral DNA replication was analysed by Southern blotting (Fig. 1A). Replication of mutant- and wt-transfected genomes occurred up to 48 h.p.t. After 96 h.p.t., mutant genome replication had greatly decreased, whereas, in contrast, wt DNA replication significantly increased, evidence of subsequent rounds of efficient infection. In accordance with results obtained by Bauer et al. (1999) with the MPyV PTA strain, our data suggest that mutation of the VP1 R297 or H298 residues leads to a complete loss of infectivity of MPyV strain A2.

First, the efficiency of genome packaging of mutant viruses MPyV R297Q and H298Q produced after transfection was controlled by a DNase I protection assay and analysed by Southern blotting. Mutant genomes contained in virus particles were mainly in the supercoiled state (form I) and appeared just as protected as wt genomes (Fig. 1B). When negatively stained and examined under electron microscopy, the morphology of mutant virus particles was similar to that of wt (Fig. 1C). Thus, the loss of infectivity of MPyV R297Q and H298Q was not related to an altered formation of virus particle.

Next, the infectious properties of the mutant viruses produced after transfection were tested by infecting a permissive fibroblast Swiss 3T3 cell line, with equal quantities of mutant or wt virions (m.o.i. of 10). The presence of early transcripts was analysed by Northern blotting. As shown in
Fig. 1(D), early transcripts were detected in wt MPyV-infected cells after 18 h post-infection (p.i.), with an increase in signal at 24 h p.i. In contrast, no signal was ever observed in MPyV R77Q- or H298Q-infected cells, indicating that the mutant virus particles were not able to initiate early transcription, therefore confirming that MPyV R77Q and H298Q were not infectious.

Lipofection of mutant virions into fibroblast cells restores viral gene expression

To assess whether the lack of infectivity of the MPyV R77Q and H298Q mutants was due to a deficiency in cell entry, 1 or 2 μg purified mutant or wt virus particles (corresponding to 10⁷ and 2 x 10⁷ p.f.u., respectively) were delivered directly into the cytoplasm of Swiss 3T3 cells by lipofection. The equivalent m.o.i. was estimated to be of 10 and 20, respectively.

Total RNA was extracted after 24 h and viral gene expression was analysed by Northern blotting (Fig. 2). Early and late gene expression was enhanced in fibroblasts lipofected with wt virus particles as compared to cells inoculated with wt particles alone (without Lipofectamin Plus reagent). In addition, we found that lipofection of Swiss 3T3 cells with mutant virions could restore early and late gene expression efficiently. The positive signals obtained did not result from contaminating free DNA present in the virus preparation, as pre-treatment of viruses with DNase I prior to lipofection gave similar results (data not shown). Moreover, electron microscopy observation of virus–lipofectamine complexes showed that the addition of lipofectamine to the virus did not affect either the morphology or the stability of virions (data not shown). Furthermore, we confirmed that the late messengers detected by Northern blot analysis in R77Q- or H298Q-lipofected cells corresponded to the mutant sequences (data not shown). This control was carried out as follows: total RNA from the samples was reverse-transcribed and the VP1-encoding region amplified specifically by PCR. Amplified fragments were then digested with BstNI or BsrGI to discriminate between the two mutants and wt sequences. Therefore, these data indicated that bypassing the entry step could restore mutant virus infectivity. Consequently, we concluded that mutant virus particles were altered at the cell entry stage.

Cell binding of mutant VLPs is totally abolished

To analyse the cell-binding properties of MPyV R77Q and H298Q, and in order not to be limited by the amount of virus particles available (obtainable only after transfection), we decided to transpose our analysis to the corresponding mutant VLPs. Mutant and wt VLPs were first produced and purified from insect cells.
stained R77Q and H298Q VLPs had an appearance similar to that of wt VLPs (Fig. 3A). First, the haemagglutination properties of mutant VLPs were tested on sheep red blood cells. As shown in Fig. 3(B), in contrast to wt VLPs, mutant VLPs did not show ability to agglutinate erythrocytes, suggesting that the interaction of the mutants with SA was altered.

The ability of mutant and wt VLPs to bind to the Swiss 3T3 cells was then tested using biotin-labelled VLPs, after having controlled the efficiency of labelling by Western blotting with HRP-conjugated streptavidin (Fig. 3C). Cell-bound biotin-labelled VLPs were detected by FACS using fluorochrome-labelled streptavidin. Whereas wt VLPs showed efficient cell binding, which was dose-dependent (data not shown), no significant binding of R77Q or H298Q VLPs was observed on these cells (Fig. 4A). The binding properties of mutant and wt VLPs were also compared in the CHO-derived cell lines Pro-5 and Lec-2. The Lec-2 cell line is a Pro-5-subline reported to express 10% of the total amount of SA on the cell membrane with respect to the parental cell line (Stanley et al., 1975; Stanley & Siminovitch, 1977). First of all, we controlled by FACS the expression level of SA on both cell lines using biotinylated WGA, a lectin that interacts with N-linked α(2,3) and α(2,6) SA residues in a manner similar to that of VP1 (Stehle & Harrison, 1997). As expected, 99% of Pro-5 cells were fluorescent with respect to 11% of Lec-2 cells (Fig. 4B, upper panel). Then, cell binding of biotinylated mutant or wt VLPs was analysed by FACS (Fig. 4B, lower panel). wt VLPs bound to Pro-5 cells in a dose-dependent manner but did not show any binding signal on the SA-deficient Lec-2 cells. In addition, neither R77Q nor H298Q VLPs demonstrated the ability to bind to these two cell lines. Taken together, these data indicate that the R77Q and H298Q mutations totally abolish cell binding by specifically altering the interaction of VP1 with SA.

Fig. 2. Northern blot analysis of viral gene expression of MPyV R77Q-, H298Q- or wt-lipofected Swiss 3T3 cells. Cells were incubated with 1 or 2 μg purified MPyV R77Q, H298Q or wt virus particles (corresponding to 10⁷ and 2×10⁷ pfu, respectively), either with (+) or without (–) Lipofectamine Plus. Total RNA (10 μg) was analysed for the presence of early and late transcripts and then re-hybridized with the β-actin-specific probe as control.

Fig. 3. Characterization of VLPs. (a) Electron microscopy analysis of purified VLPs. (b) Agglutination properties of wt and mutant VLPs on sheep red blood cells. (c) SDS-PAGE of purified VLPs stained with GelCode Blue staining reagent and Western blot analysis of biotinylated VLPs carried out using HRP-conjugated streptavidin.
Overexpression of the α4β1 integrin does not complement the lack of cell binding due to an altered VP1–SA interaction

We have demonstrated recently that α4β1 integrin acts as one of the cell receptors for MPyV in fibroblast cells, probably required at a post-attachment level (Caruso et al., 2003). To investigate whether SA-containing receptors and α4β1 integrin are alternative receptors or are co-receptors involved in the same multistep entry process, binding experiments were performed on Pro-5 and Lec-2 cells that

Fig. 4. FACS analysis of wt and mutant VLP binding in cells expressing or not expressing the α4β1 integrin in a SA-positive (BALB/c 3T3 and Pro-5 cells) or SA-deficient (Lec-2 cells) background. Cells incubated with the binding buffer alone are shown as the negative control (shaded areas). (a) Binding of biotinylated wt or mutant VLPs (1 μg) to Swiss 3T3 fibroblasts. (b) Binding of biotinylated WGA (100 ng) or VLPs (3 μg, unless stated otherwise) to SA-expressing (Pro-5) and SA-deficient (Lec-2) cells. (c) Binding of biotinylated mutant or wt VLPs (0.5 μg) to α4β1 integrin-deficient (α4−) or α4β1 integrin-expressing (α4+) cells.
had been mock- or α4-stably transfected (Table 1). As shown in Fig. 4(C), R77Q and H298Q VLPs were not able to bind to any CHO-derived cell lines, whereas wt VLPs bound to SA-containing cells only, demonstrating that VLPs cannot bind to cells overexpressing the α4β1 integrin if the VP1–SA interaction is compromised. Similarly, high levels of α4β1 integrin expression in the α4-transfected BALB/c 3T3 (α4+) cells did not rescue the lack of cell binding of mutant R77Q and H298Q VLPs (Fig. 4C). These results indicate that SA-containing molecules are the only receptors used by MPyV strain A2 to bind to these cells and that α4β1 integrin cannot be used as an alternative SA-independent receptor for the initial attachment step.

Overexpression of the sialylated α4β1 integrin enhances wt VLPs cell binding

The α4 and β1 integrin subunits contain 13 and 12 potential N-glycosylation sites, respectively. The presence of potential terminal SA residues on these subunits was first confirmed by Western blotting on BALB/c 3T3 (α4+) cell lysates pre-treated with or without C. perfringens sialidase. Both the sialylated and hyposialylated species of the α4 and β1 subunits were detected in the untreated cell extracts, whereas neuraminidase-treated lysates contained only the fastest electrophoretic mobility species, i.e. the desialylated form (Fig. 5A). In accordance with the fact that α4β1 integrin was a sialylated molecule, we observed that overexpression of the α4 subunit in BALB/c 3T3 cells resulted in a higher cell surface SA content with respect to the mock-transfected ones (Fig. 5B, left panel). We then questioned whether the α4β1 integrin plays a second role in MPyV infectivity as a SA-containing receptor for cell binding. As shown in Fig. 5(B, right panel), analysis of the binding of wt VLPs on mock- and α4-transfected BALB/c 3T3 cell lines indicated a higher (twofold) binding efficiency that was both consistent and reproducible in α4-expressing cells. Therefore, these data suggest that α4β1 integrin, through its SA residues, is one of the SA-containing molecules used by MPyV to bind to host cells.

### DISCUSSION

SA have been identified as essential cell receptors for many viruses (Kelm & Schauer, 1997). For polyomaviruses, enzymatic or biochemical removal of SA from the host cell...
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surface correlates with reduced cell binding and infectivity (Chen & Benjamin, 1997; Herrmann et al., 1997). Moreover, single substitutions in the VP1 R77 or H298 residues located in the SA-binding pocket 1 of MPyV strain PTA destroy the ability of the virus to promote cytopathic effects in cell culture (Bauer et al., 1999). In the present work, we intended to investigate the reason of the non-viability of such mutants and to determine whether the SA-containing receptors and α4β1 integrin are alternative or co-receptors involved in the same multistep entry process. Our results showed that MPyV strain A2 cell infection depends solely on SA for initial attachment to host cells, suggesting that α4β1 integrin can act by itself as a SA-containing receptor.

In accordance with data reported by Bauer et al. (1999), our MPyV strain A2 mutants MPyV R77Q and H298Q were unable to replicate in fibroblast cells. We showed further that MPyV mutant virus particles were formed and perfectly encapsidated, ruling out the possibility that the total loss of infectivity of MPyV mutants was due to structural alterations in the viral capsid. We also demonstrated that direct delivery of mutant viruses into the host cell cytoplasm allowed viral gene expression. The post-entry events, including uncoating, studied on adenovirus and liposomes–adenovirus complexes (Fasbender et al., 1997), have demonstrated that virus lipofection bypasses natural cell binding and uptake and that the subsequent steps necessary for efficient infection are adenovirus-dependent. The fact that lipofection of wt viruses enhances, rather than decreases, early gene expression with respect to natural infection may indicate that, as in adenoviruses, liposome-mediated MPyV cell entry does not specifically affect the process of virus uncoating and genome expression.

The hypothesis that the virus cycle of MPyV mutants is impaired at the cell entry level, as suggested by lipofection experiments, was confirmed by FACS analysis. Binding of mutant R77Q or H298Q VLPs appeared to be totally abrogated on both SA-expressing (BALB/c 3T3 and Pro-5) and SA-deficient (Lec-2) cells, whereas wt VLP binding occurred in a SA-positive background only. Therefore, abrogation of the VP1–SA interaction (due to the VP1 R77Q and H298Q mutations or to the absence of SA residues on host cells) results in a total loss of virus cell binding. To our understanding, this represents the first biological demonstration that MPyV cell binding depends solely on SA-containing molecules, irrespective of cell type. In this regard, the critical role of SA in MPyV cell binding notably differs from that reported for other viruses. For example, binding of reoviruses to SA enhances virus infection by accelerating adsorption but does not represent an essential event for the virus life cycle (Barton et al., 2001b).

Despite the fact that cell binding for several viruses, such as reoviruses, influenza virus and rotaviruses, was identified initially as being SA-dependent, recent reports have indicated that cell binding/entry may also occur through recognition of other receptor molecules (Barton et al., 2001a, b; Stray et al., 2000; Arias et al., 2002). For instance, rotavirus strain RRV uses SA as a primary cell receptor and subsequently interacts with the α2β1 integrin at a post-attachment step to infect host cells, while its neuraminidase-resistant (SA-independent) variant nar3 recognizes this integrin as its primary cell attachment site (Arias et al., 2002). The identification of α4β1 integrin as one of the cell receptors involved at a post-attachment level for MPyV (Caruso et al., 2003) prompted us to investigate whether a lack of SA-mediated cell binding could be rescued by cell surface overexpression of the α4β1 integrin receptor. FACS analysis showed that high expression levels of the post-attachment receptor could not restore either mutant VLP binding to SA-containing and SA-deficient cells or wt VLP binding to SA-deficient cells. These results indicate that, as with rotavirus strain RRV, SA residues and integrins are not alternative receptors for MPyV and confirm the two-step entry mechanism hypothesized previously, in which initial cell binding occurs strictly through VP1–SA recognition; such an interaction may, in turn, trigger subsequent recognition of the α4β1 integrin ligand-binding site at post-attachment level (Caruso et al., 2003).

SA residues used for MPyV cell binding are present on molecules the nature of which has not yet been identified. In the present work, we present evidence that the α4β1 integrin is one of these molecules. Indeed, expression of the sialylated α4β1 integrin in BALB/c 3T3 cells correlated with an increased cell surface SA content and enhanced the cell-binding ability of wt VLPs by twofold. We have reported recently that transfection of BALB/c 3T3 cells with the α4 subunit enhances MPyV infectivity and that function-blocking antibodies directed against the ligand-binding site of the α4β1 integrin reduce MPyV infectivity in α4-transfected BALB/c 3T3 cells without affecting virus cell binding (Caruso et al., 2003). These data led us to propose a role for the α4β1 integrin, probably through its ligand-binding site, in MPyV infectivity at a post-attachment level. In a similar manner to MPyV particles, wt VLP cell binding is not reduced by pre-incubation of α4-transfected cells with anti-α4 antibodies (data not shown). Taken together with our present results, this suggests that the α4β1 integrin plays a dual function in MPyV infectivity, being involved both initially at a cell-attachment level as a SA-containing receptor and then at a post-attachment level through its ligand-binding site. These two different functions probably involve separate parts of the receptor molecule, as the potential N-glycosylation sites of the α4 and β1 subunit chains do not map to the ligand-binding site (Shih et al., 1993; Irie et al., 1995, 1997). To date, the contribution of N-glycosylation to modulation of the α4β1 integrin function, in the presence of terminal SA residues, is not understood fully. Recent reports suggest that hypo-sialylation of integrins enhances binding of the integrin to fibronectin by exposing the active ligand-binding site to the ligand (Pretzlaff et al., 2000; Semel et al., 2002). Further studies will, therefore, be needed to answer the question of whether MPyV binding to the SA residues of α4β1 integrin induces modulation of the integrin activity by promoting...
exposure of the active binding site for interaction with the VP1 LDV motif.

The molecular mechanisms that determine MPyV tropism, pathogenicity and tumourigenicity are complex. They rely both on post-cell entry events (regulated by the enhancer and promoter elements of the viral genome) and on the ability of MPyV to recognize branched or straight SA chains and the tightness of the VP1-SA interaction (Amati, 1985; Maione et al., 1985; Caruso et al., 1990; Freund et al., 1991a, b). We showed previously that α4β1 integrin enhances cellular permissivity to MPyV at a post-attachment level (Caruso et al., 2003) and, in this study, we present evidence that this molecule is also used as a SA-containing cell attachment receptor. These observations suggest that the nature of the proteinaceous part of the SA-containing glycoprotein receptor is a major determinant of tissue tropism. In addition, we have proposed previously that more than one molecule is involved in this process (Caruso et al., 2003). The characterization of other as yet unidentified MPyV cell receptors is therefore necessary to understand MPyV pathogenicity better. Moreover, according to this hypothesis, branched SA-containing molecules, also designated by Bauer et al. (1999) as pseudoreceptors, could represent glycoproteins that allow MPyV cell attachment but not secondary post-attachment interactions with the protein moiety required for virus internalization. Further investigation in this direction is required to develop tissue-restricted MPyV VLP-based vectors for gene therapy (Forstová et al., 1995; Soeda et al., 1998; Krauzewicz & Griffin, 2000).

ACKNOWLEDGEMENTS

We wish to thank R. Maione for useful suggestions and critical discussions and N. Falcone for technical assistance. This work was supported by MIUR and ASI (004143) funds. M. Caruso is funded by a fellowship from the Adriano Buzzati-Traverso Foundation.

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