A Single-Amino-Acid Substitution in Polyomavirus VP1 Correlates with Plaque Size and Hemagglutination Behavior

ROBERT FREUND,¹ ROBERT L. GARCEA,² ROLAND SAHLI,³ AND THOMAS L. BENJAMIN^{1*}

Department of Pathology, Harvard Medical School,¹ and Division of Pediatric Oncology of the Dana-Farber Cancer Institute and the Children's Hospital,² Boston, Massachusetts 02115, and Institute of Microbiology, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland³

Received 3 July 1990/Accepted 11 October 1990

The plaque size and hemagglutination characteristics of five cloned wild-type strains of polyomavirus were determined. The strains fell into two groups, those with large or small plaques, each with distinctive hemagglutination behavior at different temperatures and pHs. The nucleotide sequence of VP1, the major capsid protein of the virus, was determined for each of the viral strains. The PTA (large-plaque) and RA (small-plaque) strains differed only at residue 92 of VP1, where there is a glutamic acid or glycine, respectively (R. Freund, A. Calderone, C. J. Dawe, and T. L. Benjamin, *J. Virol.* 65:335–341, 1991). The same amino acid difference in VP1 correlated with plaque size and hemagglutination properties of the other sequenced viruses. Mutagenesis converting amino acid 92 from glutamic acid to glycine converted the plaque size and hemagglutination behavior of the large-plaque PTA strain to that of a small-plaque strain. Furthermore, PTA and RA VP1 proteins produced in *Escherichia coli* behaved as their parental viruses did in hemagglutination assays. These results demonstrate that amino acid residue 92 of VP1 is involved in determining the plaque size and hemagglutination behavior of polyomavirus and strongly suggest that this region of the VP1 polypeptide interacts directly with cell receptors.

Investigations of the tumorigenic properties of different strains of wild-type polyomavirus have identified "high tumor" strains (inducing a high incidence of tumors) and "low tumor" strains (inducing a low incidence of tumors) (7). Characterization of the genomic regions responsible for the differences in tumorigenicity between the high tumor strain PTA and low tumor strain RA has indicated that a major determinant for induction of all tumors of epithelial origin resides in the coding regions of these virus strains (15). Comparison of the nucleic acid sequences of the PTA and RA strains revealed multiple single-base substitutions, three of which result in amino acid changes, one each in the large T antigen, in the middle T and small t antigens, and in VP1 (13a). Analysis of the tumor profiles induced by recombinant viruses containing various segments of the PTA and RA viral genome indicated that VP1 from PTA is the major determinant for the epithelial cell tropism of polyomavirus (13a). The difference between the VP1 genes of PTA and RA is a single amino acid, a glutamic acid or glycine at residue 92. This same determinant affects the ability of the virus to replicate and spread in the animal (9a). In the current study we show that this amino acid difference in VP1 also determines the plaque size and hemagglutination (HA) behavior of the viruses.

Plaque size variants of polyomavirus have been studied extensively for their differential properties in lytic infection, transformation, and HA (9, 10, 19, 27, 28). The most consistent difference found between large-plaque (LP) and small-plaque (SP) wild-type viruses is in their adsorption to cells, with SP viruses being more readily adsorbed than LP viruses. Other differences in virus output, efficiency of transformation, or selective advantage during long-term culture have been attributed to this apparent difference in adsorption. For example, the mutant Py235 (1), initially

isolated as temperature sensitive for growth and thought to be transformation defective, was characterized as having a defect in adsorption leading to an apparent decrease in transformation efficiency. HA by polyomavirus has been shown to be a reliable indicator of this adsorption property (6, 11). LP and SP variants have been distinguished by their HA properties under different conditions of pH and temperature (9, 12, 19). Although polyomavirus may recognize at least two classes of receptor proteins on the cell surface (2), adsorption differences between plaque variants appear to be based upon recognition of specific sialyloligosaccharide linkages (3, 4, 16). The linkages recognized by LP and SP variants appear distinct: both LP and SP viruses recognize the unbranched NeuAc α 2,3Gal β 1,3GalNAc structure equivalently; however, LP variants bind the branched structure NeuAc α 2,3Gal β 1,3(NeuAc α 2,6)GalNAc with a much lower affinity than SP variants do.

Here we report that the LP viruses contain glutamic acid and the SP viruses encode glycine at residue 92 of VP1. The results presented in this report, taken together with data on the tumor tropism and replication in mice, suggest that this region of VP1 interacts with cell surface receptors.

MATERIALS AND METHODS

Virus strains. Five wild-type virus strains were used in these experiments. The generation of virus stocks from the cloned strains PTA, RA, and A2 has been previously described (7, 14). The stocks of P16 and T1p were generated similarly. Lysates were obtained by infecting a 100-mm plate of primary baby mouse kidney cells with a single large plaque isolated from an uncloned stock of the Toronto SP strain (25, 37) obtained from R. Sheinin or with a small plaque from a P16 stock (9). These lysates were used to make viral DNA. The DNA was isolated by the method of Hirt (21) and purified by equilibrium centrifugation in CsCl and ethidium bromide. The viral genomes were molecularly

* Corresponding author.

FIG. 1. HA assay of cloned wild-type strains of polyomavirus. The HA titers of LP and SP viruses were determined at 4°C (panels A and B) and at 37°C (panels C and D) with respect to pH. The titers were normalized to the maximum titer of that virus stock at each temperature. Symbols: ▲, A2; ■, PTA; ●, Tlp; △, P16; □, RA; ○, PTA/RA-VP1.

cloned using standard recombinant DNA techniques (24). The cloned viral DNA was excised from the vector, ligated to promote self-closure, and used to transfect NIH 3T3 cells using the DEAE-dextran method (26). The reconstructed viruses were plaque purified on NIH 3T3 cells, and virus stocks were propagated on baby mouse kidney cells.

The mutant Py235, initially derived from an LP stock that had been mutagenized (1), was obtained from R. Consigli (Kansas State University) and plaque purified twice at 33°C, first on UC1B and then on NIH 3T3 cells. A single plaque (large and fuzzy [1]) was used to infect an NIH 3T3 cell monolayer to obtain a virus lysate.

Plaque assay. The method of plaque assays has been previously described (18). Briefly, 2×10^5 secondary mouse embryo fibroblasts, NIH 3T3 cells, or UC1B cells were seeded on 35-mm-diameter plastic petri dishes. Virus suspensions were diluted in phosphate-buffered saline solution containing 2% calf serum. The virus was allowed to adsorb for 2 h, and the infected monolayers were then overlaid with 3 ml of Dulbecco's modified Eagle's medium containing 5% calf serum and 0.9% agar. The cells were incubated at 37°C for 10 (UC1B and mouse embryo fibroblasts) or 7 (NIH 3T3) days. The plaques were photographed after the plates were

further overlaid with Dulbecco's modified Eagle medium containing 0.9% agar and 0.08% neutral red.

HA assay. HA was determined in TD saline (137 mM NaCl, 51 mM KCl, 0.38 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 24.8 mM Tris hydrochloride) by using a 0.2% (vol/vol) suspension of sheep erythrocytes. The TD saline was adjusted to the desired pH by titration. Serial twofold dilutions of 50 μl of virus or VP1 protein solution were made in V-bottom Linbro microtiter wells, and 50 μl of sheep erythrocytes was added subsequently. Recombinant VP1 protein initially at a concentration of 1 to 2 mg/ml in 1 M NaCl–0.1 mM EDTA–15 mM 2-mercaptoethanol–10 mM Tris hydrochloride (pH 7.2) was diluted into TD saline to a concentration of 1 to 2 $\mu\text{g}/\text{ml}$ immediately before assaying HA titer. The HA titer was the reciprocal of the highest dilution yielding detectable agglutination.

Nucleotide sequencing. The molecularly cloned viral DNAs from the strains PTA, RA, A2, P16, and Tlp were subcloned by standard recombinant techniques (24, 29). The large *Bam*HI-to-*Eco*RI fragment of the polyomavirus genome (nucleotide [nt] 1560 to 4632) containing the entire VP1 sequence was cloned into either M13mp19 (40) or mWB2311,

FIG. 2. (A) Amino acid sequence of VP1 from the strain RA. The nucleotide sequence of the VP1 gene from RA was determined and translated into the amino acid sequence. (B) Sequence differences between LP and SP viruses around amino acid 92 of VP1. From the translation of the nucleotide sequence, the amino acid sequence from residues 86 to 98 is shown for the indicated virus strains. Plaque size is indicated in parentheses.

a vector derived from mWB239 (34) with the M13mp11 polylinker.

Both strands of the VP1 gene from RA were sequenced. For one strand, overlapping DNA fragments were generated by the method of Hong (22) and sequenced by using the M13 universal primer (35). The opposite strand was sequenced by using Sequenase (United States Biochemical Corporation) with subcloned templates and appropriate oligonucleotide primers. One strand of the VP1 gene from the strains PTA, A2, P16, and Tlp was sequenced by the dideoxynucleotide sequencing method by using a set of oligonucleotide primers complementary to the plus strand of the VP1 gene (35). The viral DNA from Py235 was obtained by the method of Hirt (21), further purified by electrophoresis in 1% low-melt

agarose (Seaplaque) in Tris-acetate buffer, phenol extracted, and ethanol precipitated. The VP1 coding region was sequenced directly from the isolated viral DNA by using Sequenase and appropriate oligonucleotide primers.

Site-directed mutagenesis. A glutamic acid-to-glycine mutation was introduced at position 92 of VP1 from PTA by oligonucleotide mutagenesis. The mutagenesis was performed as previously described (5) by using the large *Bam*HI-to-*Eco*RI fragment (nt 1560 to 4632) of PTA cloned into M13mp19 and the 23-mer GTGTATTATTCCTGGG GAATCC, which is complementary to sequence nt 3792 to 3812 on the plus strand with mismatches (underlined) at nt 3802 and 3803. The construction and propagation of the resulting virus, designated PTA/RA-VP1, was previously described (13a).

Recombinant VP1 protein. The large *Eco*RI-to-*Bam*HI fragment (nt 1560 to 4632) of PTA was subcloned into pUC13. The RA VP1 expression vector, pALVP1, has been previously described (23). In order to express PTA VP1, the *Hind*III-to-*Pvu*II fragment (nt 3918 to 2032) from the PTA subclone, which contains the VP1 coding sequence from amino acid 52 to the carboxy terminus, was ligated into the same restriction sites of the expression vector pALVP1tac NCO (17). The *Nco*I deletion present in the original plasmid was restored upon introduction of the PTA fragment. This construction replaced the RA VP1 coding sequence with that of PTA from amino acid 52 to the carboxy terminus of VP1. Recombinant RA and PTA VP1 proteins expressed in *Escherichia coli* RB791 from these vectors were purified as previously described (23).

Nucleotide sequence accession number. The GenBank accession number for the nucleotide sequence data of the VP1 gene is M34958.

RESULTS

Plaque size and HA behavior of viral strains. The viral DNAs from the strains PTA, RA, A2, P16, and Tlp were molecularly cloned and transfected into NIH 3T3 cells to generate virus stocks. The plaque size of each of these wild-type stocks was determined by plaque assay by using NIH 3T3 cells, UC1B cells, or secondary mouse embryo cultures as indicator cells. The strains PTA, A2, and Tlp

FIG. 3. Plaque size of the site-directed mutant, PTA/RA-VP1. Plaque assay was performed on NIH 3T3 cells by using the virus stocks RA (A), PTA (B), PTA/RA-VP1 (C), and mock (D).

FIG. 4. HA assay on purified recombinant VP1 protein. HA titers of purified VP1 protein produced in *E. coli* were determined at 4°C (panel A) and 37°C (panel B) with respect to pH. The assays were performed with equivalent amounts of RA and PTA protein and normalized to maximum titers at each temperature. Symbols: ■, PTA protein; □, RA protein.

gave predominantly large plaques, while RA and P16 gave small plaques (data not shown).

The same virus stocks were used to measure agglutination of sheep erythrocytes at different pHs and at both 4 and 37°C. The results obtained were similar to those previously described for SP and LP strains (9). Figure 1 shows the HA behavior of the LP (PTA, A2, and Tlp) and SP (RA and P16) strains. At both 4 and 37°C, the SP strains RA and P16 hemagglutinated most efficiently at a higher pH (7.5), whereas the LP strains PTA, A2, and Tlp hemagglutinated better at a lower pH (6.5). A comparison of the absolute HA titers at each temperature revealed that the LP strains were more sensitive to temperature than the SP strains. For example, at pH 7.0, the titer of PTA is 32-fold lower at 37°C than it is at 4°C, while the titer of RA is only 4-fold lower at 37°C than it is at 4°C. The HA behavior of the mutant Py235 was similar to that of the LP strains, as originally reported (1).

Nucleotide sequence of VP1. It has been shown previously by marker rescue experiments (13, 30) that plaque size and HA properties of polyomavirus map to the major coat protein gene, VP1. To correlate these parameters with the amino acid sequence of VP1, the nucleotide sequence of the VP1 gene from each strain was determined. VP1 of all five strains contains 384 amino acids, the same number as previously reported for VP1 of other polyomavirus strains (8, 33, 36), but the primary sequences differ in certain regions. Figure 2A shows the inferred amino acid sequence of VP1 from the RA strain. A comparison of the amino acid sequences of the different strains revealed few differences. The PTA and RA VP1 differed only a residue 92; PTA encoded a glutamic acid at that position, while RA contained a glycine. Both the LP strain Tlp and the SP strain P16 contain aspartic acid at position 88 and isoleucine at position 291, while the LP strains PTA and A2 and the SP strain RA encode a glutamic acid and valine at these positions, respectively. Since LP and SP viruses have the same amino acid at positions 88 and 291, these differences do not affect plaque size and HA behavior strongly. Figure 2B shows the amino acid sequence around position 92 for the strains PTA, RA, P16, A2, Tlp, and the mutant Py235. All the LP strains studied contain glutamic acid at position 92, and all the SP

strains contain glycine at that position; thus, the amino acid at position 92 in VP1 determines the plaque size and HA properties of the viral strains. The Py235 virus contains a glutamic acid at residue 92 of VP1, consistent with its LP phenotype. The Py235 VP1 gene also contains two nonconservative amino acid differences, a proline-to-leucine change at position 44 and an aspartic acid-to-asparagine change at position 89, and a two-amino-acid deletion at residue 147. These additional differences may contribute to the temperature-sensitive growth defect of the mutant virus.

Site-directed mutation of the VP1 gene of PTA. The data presented above suggest that the amino acid at position 92 of VP1 is responsible for the differences in plaque size and HA behavior between the LP and SP strains. As a further test, the mutant strain PTA/RA-VP1 constructed from the LP PTA strain by changing the glutamic acid at position 92 to glycine (13a) was examined. The plaque size and HA behavior of the PTA/RA-VP1 virus was compared with those of PTA and RA. The plaque size of PTA/RA-VP1 was essentially identical to that of the SP strain RA (Fig. 3). In addition, PTA/RA-VP1 agglutinates erythrocytes in a manner identical to that of RA at all temperatures and pHs tested (Fig. 1). Thus, a single-amino-acid change at residue 92 in VP1 of PTA is sufficient to change the plaque size and HA behavior to those of an SP strain.

HA properties of recombinant VP1 protein. To show whether the viral protein VP1 by itself is responsible for the agglutination of sheep erythrocytes by the virus strains, HA assays were performed with purified recombinant VP1 from PTA and RA. The DNA sequences encoding VP1 from PTA and RA were cloned into an *E. coli* expression vector (23). The viral VP1 proteins expressed in *E. coli* were purified and tested in HA assays at different pHs and at both 4 and 37°C. Figure 4 shows that the recombinant VP1 proteins from the SP virus RA and the LP virus PTA behaved similarly to their parental virus with respect to HA at the different pHs and temperatures. Thus, these data demonstrate that the major capsid protein VP1 determines the HA properties of the virus and that the determinant within VP1 is amino acid residue 92; glutamic acid conveys LP characteristics, and glycine conveys SP characteristics.

DISCUSSION

In this report we demonstrate that the region of the VP1 protein containing amino acid 92 determines the plaque size and HA properties of polyomavirus. To prove the importance of this residue in virus-cell adsorption, a single-site mutation was introduced into the LP strain PTA by site-directed mutagenesis, which changed the glutamic acid at position 92 of VP1 to glycine. This virus, PTA/RA-VP1, displayed an SP phenotype. Furthermore, by using purified recombinant VP1 protein from PTA and RA in HA assays, it has been shown that VP1 alone is responsible for agglutinating erythrocytes and that amino acid 92 determines, at least in these cases, the agglutinating properties of the viruses.

Since the viruses used in this study are indistinguishable in their overall growth properties *in vitro*, we conclude that this region of substitution (amino acid 92 of VP1) does not have a long-range effect on overall protein structure but is localized within the specific sialic acid-binding domain responsible for HA and virus-cell adsorption in fibroblasts. This is supported by X-ray diffraction studies of simian virus 40 and polyomavirus, which indicate that the region of VP1 containing amino acid 92 is in a disordered loop structure on the outside of the virus capsid (19a). Furthermore, this region of VP1 is a major determinant in tumor tropism (13a) and viral spread (9a) in mice. These data, taken together, strongly suggest that the domain surrounding amino acid residue 92 in VP1 interacts directly with the cellular receptors for polyomavirus.

HA and recognition of specific sialyloligosaccharide linkages by polyomavirus are similar in several respects to those properties of influenza virus and paramyxoviruses (3, 20). Studies of the escape and horse serum-resistant mutants of influenza virus have demonstrated that single-amino-acid substitutions can lead to changes in sialic acid recognition associated with changes in virus tropism (31, 32, 39). The recent solution of the structure of the influenza virus HA molecule cocrystallized with sialyllactose (38) has provided the first insight into the specific chemical interactions between proteins and sialoglycoproteins. These interactions may be fundamental to a number of important biologic questions of cell-cell recognition and communication as well as those concerning viral pathogenesis. The crystal structure of polyomavirus and characterization of the sialic acid binding domain of polyomavirus may provide another example of how glycoprotein recognition is achieved.

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