Canyon Rim Residues, Including Antigenic Determinants, Modulate Serotype-Specific Binding of Polioviruses to Mutants of the Poliovirus Receptor

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Several mouse cell lines expressing hybrid human poliovirus receptors (hPVRs) bearing mutations in the first immunoglobulin-like domain were previously characterized for their defective binding and replication of poliovirus type 1 Mahoney (G. Bernhardt, J. Harber, A. Zibert, M. DeCrombrugghe, and E. Wimmer, Virology, 203, 344–356, 1994). Here we report that these mutant hPVRs were utilized to explore differences in the binding behavior of the three serotypes of poliovirus. Type 3 polioviruses (both Sabin and the neurovirulent Leon strain) clearly bound to the hPVR mutant Q130G/GD, but were incapable of initiating infection. Also, binding at 25°C of poliovirus types 2 and 3 to cell lines expressing the hPVR mutants P84SYS/HPGA, L99GAE/AAAA, and D117F was greater than type 1 poliovirus. Further study of the serotype-specific interaction with mutant hPVRs was accomplished with antigenic hybrid viruses. Improved binding by antigenic hybrid viruses demonstrated that serotype-specific binding to mutant hPVRs is, in part, determined by the amino acid sequence of neutralization antigenic sites (NAgs) and the probable conformational rearrangement of amino acids adjacent to the NAg sites. Finally, site-directed mutants of poliovirus were utilized to determine the relative contributions, to hPVR interactions, of individual amino acids with solvent accessible side chains in the viral canyon. Of the 18 viable virus mutants produced, 3 (D1226A, I1089A, and VPEK1166HPGA) expressed impaired replication phenotypes on the mutant hPVR cell lines P84SYS/HYSA and D117F. A location at the rim of the poliovirus canyon was implicated for the interaction of the amino terminal domain of the poliovirus receptor with conserved and serotype-specific viral surface amino acids. The possible involvement of elements of neutralization antigenic sites in receptor binding may explain, in part, why poliovirus exists in only three serotypes.

INTRODUCTION

All wildtype (wt) polioviruses occur, surprisingly, in only three serotypes (discussed in Wimmer et al., 1993). They are closely related both genetically (Wimmer et al., 1993) and structurally (Filman et al., 1989; Yeates et al., 1991) and have protein sequences that are at least 85% homologous (Toyoda et al., 1984). Much of the sequence variation occurs in short segments of the three major viral capsid proteins (VP1, VP2, and VP3) which define major (NAgla, NAgII, and NAgIII) and minor (NAglb, NAglb, and NAglbIIb) neutralization antigenic sites on the surface of the virion (Hogle and Filman 1989; Murdin et al., 1992; Page et al., 1988). Through genetic manipulation, it is possible to exchange neutralization antigenic epitopes between viruses. The resulting antigenic hybrids carry either one (Burke et al., 1988; Evans et al., 1989; Jenkins et al., 1990; Martin et al., 1988; Minor et al., 1990; Murdin and Wimmer, 1989; Murray et al., 1988a,b) or two heterologous neutralization antigenic sequences (termed trivalent viruses; see Murdin et al., 1992). Antigenic hybrid viruses, however, usually express a small plaque phenotype, and they are impaired in replication. When infecting human and primate cells, all known polioviruses use only a single receptor entity, the poliovirus receptor (PVR) (Wimmer et al., 1994).

Polioviruses are members of the genus Enterovirus of the family Picornaviridae. Their nonenveloped capsids consist of 60 protomers that are composed of three surface proteins, VP1, VP2, and VP3, and the internal protein, VP4 (Hogle et al., 1985). The capsid surrounds a positively-stranded RNA molecule. The VP1, 2, and 3 capsid proteins fold as eight stranded antiparallel ß-barrels whereby the antigenic regions are hydrophilic ß-turns within these structures (Hogle and Filman, 1989; Rossmann and Johnson, 1989). The surface structure of the virion is convoluted (Fig. 1a). The fivefold axis of symmetry appears as a prominent star-shaped protrusion, while the threefold axis is located on a broad plateau. Surrounding each fivefold axis is a deep cleft, termed the canyon (Rossmann et al., 1985), that has been proposed to be the receptor binding site (Rossmann and Palmenberg 1988; Rossmann, 1989).

The human poliovirus receptor (hPVR) is a member of...
FIG. 1. Structure of the poliovirus and a model of the V-domain of the hPVR. (a) A complete capsid structure of PV1 (M) illustrated as a water-accessible molecular surface. One of the 12 pentameric subunits of the capsid and its five constituent triangular pseudoprotomeric subunits are illustrated. The 5\textsuperscript{1} and 3\textsuperscript{1} labels indicate the locations of the fivefold and the threefold axes of this pentamer. The twofold axes occur at the intersection of the three adjacent pentamers. The central pseudoprotomer illustrates the subunit geometry of VP1, VP2, and VP3(ii). The biologically relevant protomer (to viral assembly) is pear-shaped and consists of VP1, VP2, and VP3(i). The internal VP4 protein is not visible from the surface. The canyon’s north wall (A), south wall (C), and bottom (B) are indicated. The major poliovirus antigenic sites are labeled Ia, Ib, II, and III on an adjacent pseudoprotomer. (b) A three-dimensional model of the immunoglobulin-like V-domain of hPVR and the locations of mutations which compromise receptor function (Bernhardt et al., 1994b). The mutations are abbreviated in the texts as follows: 84, P84SYS/HYSA; 99, L99GAE/AAAA; 117, D117F; and 130, Q130G/GD. The orientation of the hPVR is such that the G* strand leads to the two C-domains and transmembrane region (not illustrated). The antibody-like variable loops are at the upper part of the molecule (CDR1, B–C loop; CDR2, C*–C9 loop; CDR3, F–G loop). Viral attachment has been determined to occur at the right side of the molecule, involving an area that includes the C*–C9 loop (not illustrated, see Bernhardt et al., 1994b).

the immunoglobulin superfamily with the domain structure V-C-C (Mendelsohn et al., 1989; Koike et al., 1990) and a molecular weight of greater than 80 kDa (Bernhardt et al., 1994a). Analysis of the alternate splicing products of the hPVR gene revealed that two membrane bound (hPVR\textsubscript{a} and hPVR\textsubscript{b}) and two secreted (hPVR\textsubscript{B} and hPVR\textsubscript{Y}) isoforms are potentially expressed by the cell (Koike et al., 1990). Expression of the two membrane forms of the hPVR is known to vary in tissue culture cells (Bernhardt et al., 1994a). The function of these hPVRs remains obscure (Freistadt, 1994; Freistadt et al., 1993). Several studies have demonstrated that the amino-terminal V-domain of the hPVR mediates binding of the virus to the cell (Koike et al., 1991; Selinka et al., 1991, 1992; Morrison and Racaniello, 1992). Amino acid substitutions were introduced into the V-domain of hPVR (Aoki et al., 1994; Bernhardt et al., 1994b; Morrison et al., 1994). Mutations influencing on PV1(M) binding were mapped to the putative C*–C9–D region, the D–E loop, the E–F loop, and the G* strand (see Fig. 1b). Interestingly, mutations in the largest amino terminal loop between \(\beta\)-strands B and C did not influence virus binding and replication. It was concluded that the PV binding region localizes to one side of the V-domain model (Bernhardt et al., 1994b). These studies also suggested that once virus binding has occurred, the virus is internalized and, hence, viral replication ensues (reviewed by Wimmer et al., 1994).

Thus far, mutations mapping to the V-domain of hPVR were studied primarily with the virulent poliovirus type 1, Mahoney [PV1(M)] (Aoki et al., 1994; Bernhardt et al.,
We therefore investigated binding and replication of all three serotypes of poliovirus with the mutant hPVR cell lines established by Bernhardt et al. (1994b) and were able to observe striking serotype-specific differences. Of particular interest is a receptor with a mutation located outside of the predicted binding site for PV1(M) (Q130G/GD). It was found to efficiently bind type 3 poliovirus, and not the other serotypes. The binding of type 3 viruses, however, did not progress to replication. Also, poliovirus types 2 and 3 displayed increased affinities [relative to PV1(M)] for three other hPVRs mutated in the predicted virus binding region (P84SYS/HYSA, L99GAE/AAAA, and D117F). Thus, affinity of the interaction of the three serotypes of poliovirus with hPVR can be distinguished by a simple panel of mutant hPVR cell lines. We were able to address the question of serotype specificity further by studying differences in binding of antigenic hybrid viruses with the mutant receptors. One result of this approach was that antigenic exchanges to PV1(M)-modulated binding to hPVRs. This suggests that antigenicity and receptor binding may be related to a greater extent than previously considered for poliovirus. A possible relationship of this phenomenon to evolution of poliovirus serotypes will be discussed.

In order to investigate the contribution of viral capsid residues to hPVR binding, a panel of 21 mutations in PV1(M) surface-accessible amino acids was constructed. Viable viruses were tested for binding and replication on mutant hPVRs in a manner consistent with assays of the serotype variants and hybrid polioviruses. Three of the mutants displayed a phenotype of defective binding to wildtype hPVR binding and defective replication on hPVR receptors P84SYS/HPGA and D117F. The location of these mutations and their influence on receptor binding will be discussed.

MATERIALS AND METHODS

Polioviruses

The wt polioviruses, PV1(M), PV2(MEF), and PV3(Leon) were obtained from the ATCC collection. The antigenic hybrids were constructed previously and are designated as follows: Site I/type 2 hybrid poliovirus (abbreviated I/2); site I/type 3 hybrid poliovirus (I/3) (Murray et al., 1988b); site II/type 2 hybrid (II/2) (Murdin and Wimmer, 1989); site II/type 3 hybrid (II/3) [Lu et al., manuscript in preparation]; sites I/3 + II/2 trivalent poliovirus and sites I/2 + II/3 trivalent poliovirus (Murdin et al., 1992).

Construction of poliovirus capsid mutants

A fragment (nt 496 to 3625) of the poliovirus cDNA containing the capsid-encoding region was excised from a full-length cDNA by PflMI digestion, and it was inserted into plasmid pGEM9zf(−) (Promega) and denoted PC-ΔE. Single-stranded DNA was generated from this construct (denoted PC-ΔE) for oligonucleotide-directed mutagenesis. The oligonucleotides for mutagenesis (Table 1) were designed with the map/silent option of the GCG Wisconsin DNA program (Devereux et al., 1984), and they were used to create both the capsid amino acid mutations of interest and a diagnostic silent restriction enzyme site. Site-directed mutagenesis reactions were carried out as previously described (Kunkel et al., 1987; Zoller and Smith 1984).

RNA was transcribed from the T7 promoter of the cDNA clone with T7 RNA polymerase, and transfected into mouse L-cells (van der Werf et al., 1986). When CPE was observed, the cells were frozen and thawed three times to release the virus. Viruses were then characterized by plaque assays. Absence of progeny virus at this stage was retested by transfection of six 10-fold dilutions of the RNA from the transcription cocktail onto HeLa cell monolayers. The HeLa monolayers were overlaid with DMEM containing 2% calf serum and 1% noble agar and observed for the formation of plaques. RNAs that again failed to yield progeny virus were further analyzed for integrity of the open reading frame by in vitro translation (Molla et al., 1991).

Viable viruses from L cell lysates were amplified once on a single 10-cm plate of HeLa cells; infected cells were frozen and thawed three times, and the lysates were then spun at 5000 g to remove aggregates. All viruses with point mutations were passaged no more than three times in HeLa cells. Viral RNA sequencing using a standard protocol (Boehringer Mannheim Biochemicals) confirmed the presence of the mutations in third-passage virus.

Mutant receptor cell lines

Mouse cell lines expressing mutant poliovirus receptors were established as previously described (Bernhardt et al., 1994b). To simplify the nomenclature of cell lines bearing the mutated hPVR, the following designations are made: 82/92, Q82A/L92P; 84, P84SYS/HYSA; 99, L99GAE/AAAA; 117, D117F; and 130, Q130G/GD. Three cell lines (84, 99, and 117) have reduced binding and replicative capacities for PV1(M). The binding and replication of PV1(M) in cell line 99 was consistently marginal relative to cell lines 84 and 117. The cell line 130 does not replicate PV1(M). Expressions levels of the receptor were monitored during each third passage of the cell lines with a fluorescence-activated cell sorter (Becton Dickinson and Coulter Instruments). The anti-hPVR monoclonal antibodies p242, p216, and p437 used to monitor receptor expression were generous gifts of A. Nomoto. All mutant hPVR cell lines retained an uncompromised level of D171 binding relative to the wt hPVR cell line. The absence of the p216 antibody epitope and diminished p242 antibody binding is diagnostic for cell line 117.
TABLE 1

Oligonucleotides Used in Single Strand Mutagenesis Reactions to Generate Mutant Poliovirus DNA Clones

<table>
<thead>
<tr>
<th>Mutation(s)</th>
<th>Site +/-</th>
<th>Oligo sequence 5' to 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>I1089A</td>
<td>SalI</td>
<td>GCTGGGTTGGCGGTACGCTGATGCTG</td>
</tr>
<tr>
<td>T1091K</td>
<td>SalI</td>
<td>GCTGGGTTGGCGGTACGCTGATGCTG</td>
</tr>
<tr>
<td>K1109R</td>
<td>SnaBI</td>
<td>GACAGATCTTTATACCTCCACACCTG</td>
</tr>
<tr>
<td>D1114E</td>
<td>XmnI</td>
<td>GCGTAAGGTAATCCTTTAATAAGTCTG</td>
</tr>
<tr>
<td>D1114T/AA</td>
<td>PvuII</td>
<td>GCGTAAGGTAATCCTTTAATAAGTCTG</td>
</tr>
<tr>
<td>P1162G</td>
<td>SmaI</td>
<td>GGAGCGCCCAGGTAGTACGAC</td>
</tr>
<tr>
<td>P1162Q</td>
<td>Bsu36I</td>
<td>GGAGCGCCCAGGTAGTACGAC</td>
</tr>
<tr>
<td>T1143ET/AAA</td>
<td>PvuII</td>
<td>GCATGGCCATTGTTAGCAGCTGCGAAATTTGACG</td>
</tr>
<tr>
<td>DD1171/2TS</td>
<td>PmII</td>
<td>GTAGTCGTCCCAGGCTCCCGGGTGGAGCGCC</td>
</tr>
<tr>
<td>K1214R</td>
<td>NaeI</td>
<td>CTTAGTCCCTAGGGACCTCAGTATCAGTACG</td>
</tr>
<tr>
<td>D1226E</td>
<td>Avai</td>
<td>GAATGAGGTTTGCCACGTGTAGGAGGTCTTTTTCGGGCAC</td>
</tr>
<tr>
<td>D1226A</td>
<td>Avai</td>
<td>GAATGAGGTTTGCCACGTGTAGGAGGTCTTTTTCGGGCAC</td>
</tr>
<tr>
<td>D1236H</td>
<td>XmnI</td>
<td>GCGTAAGGTAATCCTTTAATAAGTCTG</td>
</tr>
<tr>
<td>D1247E</td>
<td>BclI</td>
<td>CTTAGTCCCTAGGGACCTCAGTATCAGTACG</td>
</tr>
<tr>
<td>K1256T</td>
<td>BstEI</td>
<td>GTAGTCGTCCCAGGCTCCCGGGTGGAGCGCC</td>
</tr>
<tr>
<td>KD1287AA</td>
<td>PvuII</td>
<td>GCATGGCCATTGTTAGCAGCTGCGAAATTTGACG</td>
</tr>
<tr>
<td>R2172A</td>
<td>PvuII</td>
<td>GCATGGCCATTGTTAGCAGCTGCGAAATTTGACG</td>
</tr>
<tr>
<td>ID 3180.MV</td>
<td>SalI</td>
<td>GCTCTATATGGTACGCTGACCATGGTACG</td>
</tr>
<tr>
<td>T3229V</td>
<td>AclI</td>
<td>GCTCTATATGGTACGCTGACCATGGTACG</td>
</tr>
</tbody>
</table>

while cell line 84 has partially lost the p242 epitope. Cell line 99 retained all antibody epitopes. Cell lines 82/92 and 130, which did not bind PV1(M), can be distinguished in that 82/92 has lost the p242 epitope, whereas 130 displayed mildly diminished p437 binding.

Virus binding and replication assays

Cells expressing the appropriate mutant hPVR were grown to 30–50% confluence in 60-mm dishes (1 × 10⁶ cells). Cell density was monitored to provide a minimal variance between samples. A typical assay included 10⁷ PFU of unlabeled, or 10⁴ cpm of ³⁵S-labeled virus sample per 60-mm dish of cells. Virus was incubated at 4 or 25°C for 1 hr with the monolayers. For the determination of growth parameters, the plates were carefully washed four times at the incubation temperature with medium (DME at 4 or 25°C), and overlaid with 2 ml of DME (without serum). "Zero time points" corresponded to cells that were frozen immediately following washing; for subsequent time points, the cells were placed in a 5% CO₂ incubator. All cells were prepared for titering by freezing– thawing three times. Viral titers were determined by plaque assays on HeLa monolayers in 6-well dishes. For binding assays involving radiolabeled virions, the cell monolayers were solubilized with a solution of 1% SDS and 0.2 N NaOH, and radioactivity was determined by standard procedures.

Purification of radiolabeled poliovirus

Virus at an m.o.i. of 10 was applied to a 10-cm HeLa cell monolayer (typically 5 × 10⁷ PFU) for 1 hr at room temperature. Without washing, the cells were overlaid with 4 ml of warm (37°C) DME, and placed in an incubator. Without washing, the cells were overlaid with 4 ml of warm (37°C) DME, and placed in an incubator. Virus at an m.o.i. of 10 was applied to a 10-cm HeLa cell monolayer (typically 5 × 10⁷ PFU) for 1 hr at room temperature. Without washing, the cells were overlaid with 4 ml of warm (37°C) DME, and placed in an incubator. Without washing, the cells were overlaid with 4 ml of warm (37°C) DME, and placed in an incubator.
tion with cell monolayers at 4°C, the sample dishes were placed on ice and washed five times with ice-cold DMEM. The samples were then lysed and counted. Growth properties were determined as described above. Heat-lability was measured by incubating virus (1 × 10^5 PFU) for 1 hr at 37 or 45°C. The reduction in titer was determined as the ratio of titer at 45°C/37°C.

Molecular graphics

Figures 1a and 5a–5c were generated from atomic coordinates obtained from the Protein Data Bank at Brookhaven (Bernstein, 1977) for PV1(M) [entry number 2PLV (Hogle et al., 1985)], and for the domains 1 and 2 of the CD4 molecule [entry number 1CDH (Ryu et al., 1994)]. All molecular surfaces were calculated with the program GRASP (Nicholls et al., 1991). Domains 1 and 2 of the CD4 molecule served as a model for the corresponding hPVR domains, and the model was docked into the canyon on a Silicon Graphics Crimson computer with the program MIDAS-PLUS (Ferrin 1988).

The ribbon diagram of the molecular model of the hPVR (Fig. 1b) was generated on a Silicon Graphics IRIS 4D with the programs molscript (Kraulis, 1991) and Insight II (Biosym Technologies, San Diego, CA) as previously described (Bernhardt et al., 1994b).

RESULTS

Binding of poliovirus serotypes to mutant hPVR cell lines

We have previously tested several cell lines expressing mutants of hPVR for their ability to bind PV1(M) (Bernhardt et al., 1994b). We have now extended these studies to the other viral serotypes whose surface properties differ somewhat from PV1(M), particularly with respect to the neutralization antigenic sites. Whereas the binding to wt hPVR is similar for all serotypes (Bibb et al., 1994; and Figs. 2a and 2b), an unexpected variation of binding was observed when different hPVR mutant cell lines were tested. This is particularly striking in the case of cell line 130 (hPVR cell line Q130G/GD; see Materials and Methods for nomenclature) which fails to bind type 1 and type 2 viruses, but binds efficiently type 3 poliovirus (Figs. 2a and 2b; column 130). Attachment, however, did not lead to observable replication at 37°C. Figure 2c shows the results of an assay in which virus was bound at room temperature, and incubated further at 37°C for 24 hr to allow replication. The wt hPVR cell line displayed a typical 0 to 24 hr replication profile for all viruses tested, including the neurovirulent PV2(MEF) and PV3(Leon) strains (data not shown). In contrast, the type 3 Sabin (Fig. 2c) and Leon (data not shown) virions attached to cell line 130, but the virus titer did not increase. Because the titer did not change significantly during the 24-hr incubation, we consider it likely that the process of uncoating is defective for type 3 virions on cell line 130 (see below).

The binding and replication phenotypes of the three serotypes to other mutant hPVR cell lines was intermediate to that of wt or 130. However, distinct differences in binding between the three serotypes were apparent. Whereas at room temperature PV1(Sabin) bound to cell lines 84, 99, and 117 at a level corresponding to no more than 15% of that bound to wt hPVR, Sabin 2 and Sabin 3 viruses bound at high levels to the mutant receptors, particularly to cell line 99 (Fig. 2a). In general, binding to receptor cell lines increased when the assays were performed at 25°C instead of 4°C (Bernhardt et al., 1994b; Fig. 2b). One notable exception is the binding of PV3(Leon) to cell line 130 (Fig. 2b). The reason for the apparent decrease is not known.

The binding data shown here demonstrate that serotype-specific interactions occur with the hPVR. However, they are apparent only with hPVR mutants. A possible explanation of this phenomenon is that subsets of interacting amino acids in the virus/receptor mutants are not the same for the three serotypes of the virus. Therefore, we investigated the possibility that neutralization antigenic determinants of virions of the different serotypes influenced the interaction with the receptor.

Neutralization antigenic (NAg) sequence determinants modulate binding of hybrid PV1(M) viruses to mutant hPVR cell lines 84, 99, and 117

Sequences specifying NAgIa and NAgIIa of PV1(M) have been exchanged with the corresponding PV2(Lansing) and PV3(Leon) sequences in a series of six antigenic hybrid viruses designated as follows: PV1(M) site I/type 2 virus (I/2) (Martin et al., 1988; Murray et al., 1988a); PV1(M) site I/type 3 virus (I/3) (Burke et al., 1988; Murray et al., 1988b); PV1(M) site II/type 2 virus (II/2) (Murdin et al., 1992); and PV1(M) site II/type 3 (II/3) (Lu et al., manuscript in preparation). Two trivalent antigenic hybrid viruses were utilized as well. These consisted of combinations of site Ia and IIa exchanges: the PV1(M) I/2 + II/3 trivalent hybrid and the PV1(M) I/3 + II/2 trivalent hybrid (Murdin et al., 1992). In binding assays, all antigenic hybrids retained the character of the PV1(M) virus from which they are derived in that at 4°C binding to any of the mutant hPVR cell lines showed the PV1(M) profile (data not shown). However, when the binding assay was performed at 25°C, half of the hybrid viruses were capable of significant binding to the mutant hPVR cell lines well above the binding levels of PV1(M) (Fig. 3), resulting in viral replication (data not shown). The increased binding of the mouse neurovirulent hybrid I/2 to the mutant receptor-expressing cell lines suggests that a component of the receptor binding site of type 2 virus which mediates 25°C binding with the mutant receptors 84, 99, and 117 resides in the structural region of NAgI of type 2 viruses.
The site II/3 virus also displayed elevated levels of cell binding, virus at 25°C on cell lines 84 and 99, but none of the chimeric carrying type 3 antigenic determinants resembled the type 3 pattern seen in Fig. 2. Instead, they retained two characteristics of PV1(M): a level of binding to cell line 117 and the absence of measurable binding over background to cell line 130. A trivalent hybrid virus containing both exchanges (site I/type 2 + site II/type 3) had a 25°C binding level which was intermediate of its constituent hybrid virus counterparts. The binding of the reciprocal hybrid viruses (site I/type 3; site II/type 2 and the trivalent carrying both) showed no influence of temperature (Fig. 3). In fact, the I/3 virus and the trivalent hybrid carrying this exchange both showed reduced binding to the mutant cell lines 84, 99, and 117.

PV1(M) canyon mutants with reduced binding to wt hPVR are replication-defective in hPVRs mutant cell lines 84 and 117.

The binding and replicative properties of poliovirus were further investigated with a panel of PV1(M) variants generated by site-directed mutagenesis. Using maps of the surface of the virus (A. Palmenberg and J.-Y. Sgro, unpublished results), and amino acid alignments of the three serotypes (A. Palmenberg, personal communication), 21 virus mutants with changes in amino acid sites in the region of the viral canyon and rim were constructed (Table 2). Site-directed mutagenesis yielded 18 viable derivatives of PV1(M). Three viral RNA constructs (DD1171TS, KD1287AA, and R2172A) were incapable of producing infectious virions by transfection, despite confirmation of the integrity of the poliovirus polyprotein and proteolytic processing by in vitro translation using a HeLa cell-free extract (Molla et al., 1991; data not shown). Three viruses (I1089A, VPEK1166HPGA, and D1226A) were greatly diminished in their ability to bind to a wild-type hPVR cell line at 4°C, and they failed to replicate on mutant hPVR cell lines 84 and 117 (Table 2) (cell line 99 was not included in the replication screen because of very low levels of PV1(M) replication). The one-step growth patterns of these viruses on HeLa cells is shown in Fig. 4. The D1226A mutation did not display a delay in eclipse and was not heat sensitive, an observation indicating that its defect may be correlated with binding to the hPVR. In contrast, the I1089A virus was markedly delayed in eclipse and both the I1089A and VPEK1166HPGA viruses were slower in producing newly synthesized infectious particles. In addition, P1162G, a small plaque virus, was also found to display a delay in eclipse (data...
not shown) but exhibited no binding deficiencies on the
wt hPVR cell line or replication deficiencies (in 0/24 hr
assays) on wt, 84, and 117 hPVR cell lines. A further
characterization of the mutant viruses revealed that two
of the viruses (11089A and VPEK1166HPGA) were tem-
porary sensitive. Analyses of other viruses showed that
the DT1114AA variant was also temperature sensitive
(Table 2).

DISCUSSION

In view of the genetic plasticity of viral RNA genomes,
the restriction of poliovirus to only three serotypes is a
perplexing phenomenon (Wimmer et al., 1993). If neutral-
ization antigenic regions surrounding the canyon had no
other function than to assist in hiding the receptor bind-
ing site, we would expect to find far more than three
poliovirus serotypes because new antigenic determi-
nants would evolve as the virus attempted to evade the
immune system. If, however, neutralization antigenic
sites contribute to the docking process of virions to the
hPVR, then the serotype restriction may be in part ex-
plained. Indeed, evidence has been presented suggest-
ing that some RNA viruses have diverged antgeni-
cally independent of immune selection (Domingo et al.,
1993). Our approach of testing the binding and replication
of wt poliovirus, or of engineered viral variants, with cells
expressing mutated hPVR molecules provides a new
strategy for defining the relationship between the three
poliovirus serotypes and their interaction with their re-
ceptor.

By conventional binding assays with intact cells, we
have been unable to observe serotype-specific differ-
ences in the interaction between polioviruses and the wt
hPVR (Bibb et al., 1994; this study). This suggests that
the binding energy between the virus and wt receptor
appears to be roughly the same within the contact do-
main amongst the three serotypes. In this study, however,
striking differences in serotype-specific receptor interac-
tions were revealed when receptor molecules with alter-
ations in the V-domain were used in binding experiments.
We interpret this to mean that both conserved and variant
amino acids in the region of the viral binding site (canyon
and elements of the antigenic sites) are responsible for
serotype-specific binding to hPVR. This phenomenon
may also serve to explain preferred inhibition of PV2
docking to HeLa cells by mcAbs to CD44. CD44 may
form a complex with hPVR or may alter the conformation
of hPVR through the cytoskeleton or signal transduction
pathways (Shepley, 1988; Shepley and Racaniello, 1994).

The phenomenon of serotype-specific polymorphism
in V-domain binding is most pronounced for mutant re-
ceptor 130 which carries two amino acid replacements
(Q130G and G131D) in the region located between the
proposed G and G′ β-strands (Fig. 1b; Bernhardt et al.,
1994b). This region is located adjacent to the C2 domain
of the receptor. At 25°, the complete inhibition of binding
of poliovirus serotypes 1 and 2 to this receptor derivative
was surprising, given the efficient binding of type 3 poli-
virus to the 130 cells. At present, we have no explanation
for the phenomenon of serotype-specific polymorphism
in V-domain binding. The mutant receptor domain ex-
pressed by 130 cells may be unable to convert the type
3 virions to A-particles, a process thought to be the first
step in poliovirus uncoating (Wimmer et al., 1994). Cell
line 130-bound type 3 viruses (Sabin or Leon), when incu-
bated for 24 hr at 37°, did not go through a phase of
typical eclipse but showed a slight loss of input titer.
Indeed, numerous attempts have failed to detect replica-
tion of PV3 in 130 cells even if the virus was administered
at high m.o.i. (unpublished observations). The G – G′ re-
region of the V-domain may therefore be involved in medi-
at ing conformational changes that unlock the virion struc-
ture and lead to the release of VP4. The fact that the
binding of PV3 to 130 cells does not progress to produc-
tive infection appears to be a unique case in which bind-
ing and uncoating are separated. This property makes
the complex PV3/130 receptor a desirable candidate for
structural analysis by crystallography since destabilizing
changes which accompany the formation of a picornavi-
The structural location of mutations using the strand assignments of Hogle et al. (1985).

<table>
<thead>
<tr>
<th>PV1 (M) surface mutation(s)</th>
<th>Three serotypes conserved</th>
<th>Viable virus</th>
<th>Plaque size</th>
<th>Receptor footprint</th>
<th>Temperature sensitivity of the viruses</th>
<th>Defective binding on wt PVR cell line</th>
<th>Replication impaired on mutant PVRs</th>
<th>Structural location</th>
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<tbody>
<tr>
<td>I1089A</td>
<td>Yes</td>
<td>+</td>
<td>S</td>
<td>No</td>
<td>+</td>
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<td>T1091K</td>
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<td>+</td>
<td>M</td>
<td>No</td>
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</table>

a Nomenclature of the poliovirus type 1 Mahoney coordinates deposited to the Protein Data Bank (Hogle et al., 1985).
b Derived from picornavirus alignments (courtesy of Dr. Ann Palmenberg).
c Production of virus was monitored by RNA transfection (see Materials and Methods).
d Following 2 days incubation on HeLa cells, S, <1 mm; M, 1 –2 mm; L, >2 mm.
e The hypothetical footprint of the hPVR on the poliovirus capsid assumes that the interaction resembles the structure of HRV16 complexed with a two domain fragment of its receptor, ICAM-1 (Chapman and Rossman, 1993).
f A score of + indicates that reduction of titer of >1 log over the PV1(M) value of infective virus occurred in the heat activation assay (see Materials and Methods).
g Viruses displaying 10% or less of the PV1(M) value in the 4° binding assay (see Materials and Methods).
h The replication of a designated virus on the mutant cell lines (84 and 117) in a single-step growth curve displaying less than a 5% increase in plaques of the wt value.
i The structural location of mutations using the strand assignments of Hogle et al. (1985).

rus-receptor complex are mitigated. Analyses of the kinetic parameters of PV3/130 cell binding, A-particle formation in the presence of 130 cells, and attempts by blind passage to find PV3 mutants able to replicate on 130 cells are in progress.

The hPVR mutant 82/92 (carrying the double mutation Q82A/L92P) that completely suppressed PV1 binding and replication (Bernhardt et al., 1994b) did not show any variation in serotype-specific binding. In contrast, we have observed pronounced binding differences of the poliovirus serotypes with cell lines 84 (P845YS/HYS5A), 99 (L99GAE/AAAAA), and 117 (D117F). Cell line 99 stands out in that at 25° it bound both serotypes 2 and 3 to wt levels.

Binding of PV1 to wt hPVR or its derivatives is higher at 25° than at 4° (Bernhardt et al., 1994b). This is also the case for PV3 (Fig. 2b) and PV2 (data not shown). Surprisingly, whereas PV3(Leon) binds at wt levels to cell line 99 at 25°, its binding is greatly impaired at 4°. Such large temperature-dependent differences of binding have not been observed with the 130 cell line (Fig. 3C), and PV1(Sabin) has been found to bind to the 130 line at 37° at 87% of the level of binding to the wt receptor (data not shown). The crystal structure of poliovirus (Hogle et al., 1985) provides a fixed view of the capsid whereby specific segments of the amino acid chains of VP1 and all of VP4 are clearly an internal feature. In solution, at 25° or physiological temperatures, however, such “internal” segments may be exposed to the outside solvent since they become accessible, in part, to antibody binding (Li et al., 1994). This strongly suggests that certain regions of the poliovirus are subject to spontaneous structural (reversible) rearrangements when incubated in appropriate buffers at physiological temperature. Whether structural alterations or kinetic parameters, or both, account for the serotype-specific difference in receptor binding remains to be determined.

It is highly likely that the viral canyon is a major contact point for the poliovirus receptor activity.
FIG. 4. Single-step growth curves of three virus mutants and PV1(M). HeLa cell monolayers were infected at similar m.o.i. with PV1(M) and the mutants and incubated for the indicated times (given in hours). The titer of each virus was determined by plaque assay.

Area with hPVR (see below), as was predicted by Chapman and Rossmann (1993). However, the binding differences discussed above could be the result of interactions involving not only the canyon but also neutralization antigenic sites lining the canyon. These sites, NAg1 (VP1 B-C loop) and NAg2 (VP2 E-F loop), are highlighted (magenta shading) in Fig. 5. Indeed, results with antigenic hybrid viruses reported here suggest that NAg1 may influence binding to the receptor variants as the binding phenotype to cell lines 84 and 99 clearly covaries with the presence or absence of the type 2 NAg1 loop. A contribution of the type 3 NAg1 loop to binding of PV1 was also observed, but none of the hybrid viruses acquired the phenotype of binding to the 130 cell line.

As noted above, the involvement of neutralization antigenic determinants in receptor binding could be, in part, responsible for the observation that poliovirus occurs in only three serotypes (Wimmer et al., 1993). Divergence of the neutralizing determinants may shift receptor specificity of the virion which could lead to the emergence of a new “species” of enteroviruses with altered tissue tropism. For example, a phylogenetic tree derived from amino acid similarities in capsid proteins of picornaviruses suggests that the coxsackie viruses A 21 and A 24 (CAV21 and CAV24) are enteroviruses that are very closely related to polioviruses (L. Kinnunen, T. Pöyry, and T. Hovi, personal communication). Whereas polioviruses can cause poliomyelitis, CAV21 and CAV24 cause respiratory disease. Perhaps, CAV21 and CAV24 were at one time polioviruses until divergence of the antigenic sites drove them apart by allowing them to accept distinct cellular receptor moieties.

Making use of surface maps of poliovirus (A. Palmenberg and J.-Y. Sgro, unpublished results), we have entertained the possibility of uncovering contact points between the V-domain and the virion by generating viral mutants and analyzing their receptor binding phenotypes. The three virus variants that showed clear phenotypes with respect to binding to wt receptor carry mutations mapping either to (1) the “north wall” of the canyon (I1089A; located near the fivefold axis in a border region between VP1 subunits), (2) a region adjacent to NAg1 (V1166PEK/HPGA), or (3) the south rim of the canyon (D1226A). It is possible that all these regions are involved in the various stages of receptor binding. Interestingly, all three mutant viruses were impaired in replication on cell lines 84 and 117. The srr mutants selected by incubation of virus with soluble receptor (Kaplan et al., 1990) have yielded amino acid changes in regions identical to those described here (Colston and Racaniello, 1994). Most recently, these authors have reported differences of growth properties of poliovirus serotypes in cell lines expressing hPVR mutants (Colston and Racaniello, 1995). Although no binding studies were carried out, this observation may support our hypothesis of serotype-specific interaction of polioviruses with hPVR variants.

The D1226A mutation is located in the G-H loop of VP1 (canyon rim) where the wt aspartic acid is part of an amino acid triplet (G-D-S) that is conserved in all serotypes of poliovirus. This sequence aligns with the conserved R-G-D triplet of the VP1 G-H loop of foot-and-mouth disease viruses (FMDV) that has been shown to participate in FMDV binding to host cells (Fox et al., 1989; Mason et al., 1994; Berinstein et al., 1995). Residue D1226 of the poliovirus GDS triplet has been suggested to form a salt bridge to R2172 of the E-F loop of VP2 (Hogle et al., 1985). Whereas the mutation D1226A did not abrogate viral replication, an R2172A change was found to be lethal (Table 2). The significance of this observation is not apparent. Our hypothesis that the poliovirus G-D-S adhesion motif plays a role in receptor interaction may be supported by the observation that several of the srr mutants selected by Colston and Racaniello (1994) were mutated at D1226. The implication of the
FIG. 5. Locations of hPVR binding mutations on the poliovirus capsid and a virus-receptor model. (a) Stereo view showing details of the fivefold depression, referred to as the canyon. The axes of icosahedral symmetry are labeled around a single representative of the 60 triangular pseudoprotomeric facets. The view is seen along the icosahedral twofold axes of symmetry looking down upon the canyon area. Residues exchanged in the antigenic hybrids (NAgI and NAgII) are represented in magenta color, while the amino acid substitutions resulting from site-directed mutagenesis are colored in cyan. The sphingosine molecule occupying the hydrophobic pocket of the VP1 protein is shown in yellow. (b) Stereo view of the same area as that shown in (a) except that the view is perpendicular to the axes of the icosahedral five- and twofold symmetry. (c) A poliovirus receptor modeled after the CD4 molecule is docked into the canyon. Orientation is the same as that in (b). Domain 1, an immunoglobulin V-like domain (gold) enters the canyon. The smaller domain 2, an immunoglobulin C-like domain, is colored green and sits above the surface of the virion. It is possible that domain 1 contacts residues of the north wall (nearest NAgI) and south wall (the NAgII face) simultaneously based on spatial considerations alone. Also, binding of the receptor to the canyon rim regions does not necessarily involve contacts of the receptor to the bottom of the canyon.

involvement of the G–H loop in receptor interaction may lead to the elucidation of possible evolutionary mechanisms that alter picornavirus receptor specificity and tissue tropism.

We had expected to find mutations of the canyon floor which greatly impaired replication on the mutant receptor cell lines, but this was not the case. Although we mutated residues in positions analogous to those previously reported for defective rhinoviruses (Colonno et al., 1988), the canyon floor mutations yielded only viruses with ts and plaque size variants in our assays. Of interest was canyon floor mutation DT1114AA of the VP1 C strand that lies adjacent to the hydrophobic pocket and confers a ts phenotype. It will be interesting to see whether this vari-
ant displays a difference in thermal denaturation patterns in the presence or absence of drugs such as the WIN compounds (Rombaut et al., 1991).

A model of the binding of the V and C2 domains of the hPVR to the poliovirus is shown in Fig. 5. Although many aspects of this model are hypothetical, the illustration serves to depict the size relationship and a possible orientation of the receptor toward the canyon. This model is based on the assumption that the C1–C2 region of the V-domain and the canyon (rim G–H loop) are likely to interact in the docking event. The orientation of the virus-bound hPVR illustrated here accounts for our observation that the antibody-like loops CDR2 and CDR3 may interact with the PV1(M) virion during docking, but the prominent CDR1 region does not.

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REFERENCES


Colston, E., and Racaniello, V. R. (1994). Soluble receptor-resistant poliovirus mutants identify surface and internal capsid residues that control interaction with the cell receptor. EMBO J. 13, 5855–5862.


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