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Poliovirus Variants Selected on Mutant Receptor-Expressing Cells Identify Capsid Residues That Expand Receptor Recognition

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Mutations in the predicted C -C -D edge of the first immunoglobulin-like domain of the poliovirus receptor were previously shown to eliminate poliovirus binding. To identify capsid residues that expand receptor recognition, 16 poliovirus suppressor mutants were selected that replicate in three different mutant receptor-expressing cell lines as well as in cells expressing the wild-type receptor. Sequence analysis of the mutant viruses revealed three capsid residues that enable poliovirus to utilize defective receptors. Two residues are in regions of the capsid that are known to regulate receptor binding and receptor-mediated conformational transitions. A third residue is located in a highly exposed loop on the virion surface that controls poliovirus host range in mice by influencing receptor recognition. One of the suppressor mutations enables the primate-restricted P1/Mahoney strain to paralyze mice by enabling the virus to recognize a receptor in the mouse central nervous system. Capsid mutations that suppress receptor defects may exert their effect at the binding site or may improve receptor binding by regulating structural transitions of the capsid.

All three serotypes of poliovirus initiate infection of a cell by binding to the poliovirus receptor (Pvr), which is a member of the immunoglobulin superfamily of proteins (18). Knowledge of the three-dimensional structure of the virus-receptor complex, coupled with genetic analysis of the interaction, will provide a detailed molecular description of the early events in infection. The study of poliovirus and Pvr mutants that are defective in the steps of cell entry has provided information on residues that control the virus-receptor interaction (for a review, see reference 25). The identification of viral mutations that suppress receptor defects and expand receptor recognition constitutes a different genetic approach to understanding virus-receptor interactions.

The poliovirion is composed of 60 protomers, each containing a single copy of the four capsid proteins VP1, VP2, VP3, and VP4 (12). The surface of the virion is characterized by prominent peaks at the fivefold and threefold axes of symmetry, and a surface depression, called the canyon, encircles each fivefold axis peak. Analysis of soluble receptor-resistant (*srr*) poliovirus mutants identified capsid residues that control interaction with the receptor (5). Surface capsid residues that regulate attachment to the receptor are located in the canyon, at an interface between protomer subunits. Internal capsid residues that influence receptor binding are located near a hydrocarbon-binding pocket of capsid protein VP1, which lies below the canyon at the interface between protomers.

Mutagenesis of Pvr domain 1 revealed receptor residues that are important for virus binding and replication (1, 3, 20). The effects of Pvr mutations on virus binding were interpreted in the context of a model of Pvr domain 1 predicted from the known structures of V-like domains of other immunoglobulin superfamily proteins. The results of these studies reveal that the C′-C″-D strands of Pvr domain 1 make up the major component of the virus binding site. Taken together, the study of *srr* mutants and Pvr mutants suggests that the C′-C″-D ridge of Pvr makes interactions with residues of the viral canyon floor along the protomer interface. Cryoelectron microscopy data reveal that ICAM-1, the receptor for the major group rhinoviruses, interacts with the canyon (23).

To understand the structural basis of expanded receptor recognition, we isolated viral variants that suppress the binding defect caused by mutations in Pvr. Sixteen poliovirus mutants were selected that replicate in cells expressing mutant forms of Pvr yet retain the ability to grow on cells expressing wild-type Pvr. Sequence analysis of the mutant Pvr-adapted viruses identified four amino acid changes at three residues in capsid proteins VP1 and VP2 that singly, and in certain combinations, confer the ability to replicate in cells expressing mutant Pvr. One mutation allows the primate-restricted P1/Mahoney strain to paralyze mice and is therefore a new host range determinant. Amino acid changes in adapted mutants are located in the VP1 B-C loop at the fivefold axis, at the interface between protomers near the hydrocarbon-binding pocket, and in the canyon. Capsid mutations in adapted viruses may suppress the effects of receptor mutations and restore the virus-receptor interaction by affecting receptor contact or by regulating structural transitions that the capsid undergoes during receptor interaction.

MATERIALS AND METHODS

Cells and viruses. HeLa S3 cells were grown in suspension cultures in Joklik minimal essential medium containing 5% horse serum and 10 μg of gentamicin per ml. For growth in monolayers, HeLa cells were plated in Dulbecco modified Eagle's medium containing 5% horse serum and 10 μg of gentamicin per ml. Stable L-cell transformants, expressing wild-type (20B-21) or mutated (d31-3, g28-11, and i212-12) Pvr cDNAs (20), were maintained in Dulbecco modified Eagle's medium containing 10% bovine calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 400 μg of geneticin sulfate (GIBCO) per ml for neomycin selection. Surface levels of Pvr, which were previously determined by fluorescence-activated cell sorting as a percentage of HeLa cell levels (20), are as follows: 20B-21, 15%; d31-3, 15%; g28-11, 20%; and i212-12, 23%.

Poliovirus strains P1/Mahoney, P2/Lansing, and P3/Leon were derived by transfection of HeLa cell monolayers with RNA derived from cloned genomic cDNAs (26, 27, 32). Variants of these strains were also used (4, 5, 21). Poliovirus mutants generated as part of this work are described in detail below. Viral stocks were prepared in monolayers of HeLa cells or cells expressing mutant Pvr, with plaque-purified virus as an inoculum. Viral titers were determined by plaque assaying on HeLa cell monolayers as described elsewhere (28) or on monolayers of cells expressing mutant Pvr. For binding assays, virus was labeled with [^{35}S]me-

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thionine (New England Nuclear), pelleted from cell lysates by centrifugation at 40,000 rpm at 10°C for 90 min in an SW41 rotor, and centrifuged through a 7.5 to 45% sucrose gradient at 40,000 rpm at 10°C for 75 min in an SW41 rotor. Gradients were fractionated from the bottom, the radioactivity of each fraction was determined by liquid scintillation counting, and peak fractions were pooled and dialyzed against phosphate-buffered saline (PBS). The number of virion particles per ml was calculated from the A_{260}, assuming an absorbance of 1.0 for a virus concentration of 9.4×10^{12} particles per ml (30). Purified virus was adjusted to 5 mg of bovine serum albumin (BSA, fraction V; Sigma) per ml and stored in aliquots of 50 to 200 μl at −70°C.

Selection of mutant Pvr-adapted polioviruses. To select poliovirus mutants adapted to grow on d, g, and i cells, 1×10^8 to 5×10^9 PFU of wild-type P1/Mahoney was incubated with monolayers of cells expressing mutant Pvr for 45 min at 37°C. After adsorption, the monolayers were washed twice with PBS, overlaid with agar, and incubated at 37°C for 2 days. Viruses were isolated from plaques and subjected to two rounds of plaque purification on mutant Pvr-expressing cells. Stocks of mutant Pvr-adapted viruses were prepared by infecting monolayers of cells expressing mutant Pvr with the purified plaques.

Identification of mutations in mutant Pvr-adapted viruses. Stocks of d-, g-, and i-adapted viruses were used to infect confluent 15-cm plates of d, g, and i cells, respectively. Infected cells and medium were collected after 2 days at 37°C, virus was released by repeated cycles of freezing and thawing, and cellular debris was removed by centrifugation. Virus was pelleted from cell lysates by centrifugation at 45,000 rpm at 10°C for 45 min in a 60 Ti rotor and purified by CsCl density gradient centrifugation, and viral RNA was extracted as described previously (14) and used as a template for first-strand cDNA synthesis. The cDNA synthesis reaction was carried out for 30 min at 37°C in a total volume of 50 μl, which contained 50 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM dGTP, 1 mM dATP, 1 mM dTTP, 1 mM dCTP, 80 U of RNAsin (Promega Corp.), 400 U of SuperScript or SuperScript II reverse transcriptase (GIBCO), and 100 ng of oligo(dT). The first-strand cDNA product was then used as a template in a PCR with $Vent_R$ DNA Polymerase (New England Biolabs) that was performed according to the manufacturer's directions. The following two primers that flank the capsid-coding region of the poliovirus cDNA were used: a negative-sense primer annealed to bp 3410 to 3427 and a positive-sense primer annealed to bp 487 to 506. The 3.4-kb PCR product was treated with polynucleotide kinase (New England Biolabs), cloned into the vector pBR322 which had been digested with *Eco*RV (New England Biolabs), and treated with alkaline phosphatase (Boehringer Mannheim). Clones containing the desired insert were identified by restriction analysis.

The cloned capsid region was sequenced by the dideoxy method (31) to identify mutations. To confirm that a mutation identified by DNA sequencing was present in the virus, RNA was isolated as above for cDNA synthesis and sequenced with avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) as described previously (14).

Construction of P1/Mahoney cDNAs containing mutations in the capsid coding region. Nucleotide changes were introduced into the P1/Mahoney cDNA sequence by a strategy that used mutagenic and nonmutagenic primers in a series of PCRs (10). In PCR no. 1, full-length P1/Mahoney cDNA [linearized 3′ of the poly(A) tract in the poliovirus cDNA] was amplified by using a nonmutagenic, positive-sense primer that annealed 5′ of the target sequence and a negative-sense primer that contained a 1-bp mismatch with the target sequence. Different 5′ primers, depending on the target sequence, were used. A 5′ primer that annealed at nucleotides (nt) 2462 to 2482 and contained an *Nhe*I restriction site was used with mutagenic primers that annealed at nt 2753 to 2771 and nt 2948 to 2968. A second 5′ primer that annealed at nt 338 to 350 and contained an *Age*I restriction site was used with a mutagenic primer that annealed at nt 1364 to 1382. In PCR no. 2, linearized full-length P1/Mahoney cDNA was amplified with a nonmutagenic, negative-sense primer that annealed at nt 5595 to 5614 and contained a *Bgl*II restriction site and mutagenic, positive-sense primers that were complementary to the mutagenic negative-sense primers described above. Products from PCRs no. 1 and no. 2 overlapped in the region of complementarity between mutagenic primers and thus could be annealed and used as template in a third PCR with the appropriate flanking nonmutagenic primers. PCRs were performed with $Vent_R$ DNA Polymerase (New England Biolabs) according to the manufacturer's directions. The product of PCR no. 3 and pT7M (full-length P1/Mahoney cDNA cloned into pBR322) were digested with the appropriate restriction enzymes and ligated to regenerate full-length P1/Mahoney cDNA containing the desired mutation(s). Clones containing the full-length insert were identified by restriction analysis, and the infectivity of positive clones was determined by in vitro transcription and transfection of viral RNA as described below. Stocks of viruses with specific capsid mutation(s) were prepared, and viral RNA was sequenced directly to confirm that the intended mutation(s) was present in the genome.

This strategy was used to construct a total of six site-directed P1/Mahoney mutants, each with one or two amino acid changes in the capsid. For every mutation that was introduced, two independent PCRs were performed at each PCR step to minimize the risk that a mutant phenotype was due to a mutation introduced by PCR. Therefore, for each site-directed mutation there are two independently constructed viruses.

Viral RNA synthesis and transfection. In vitro synthesis of RNA from cDNA templates was performed with T7 RNA polymerase (Pharmacia). The reaction mixtures contained 2 μg of full-length poliovirus cDNA, linearized 3′ of the poly(A) tract in the poliovirus cDNA, 1 mM each nucleoside triphosphates, 50 U of RNAsin (Promega), 0.5 μg of BSA (RNase and DNase free; Boehringer Mannheim) per ml, 5 mM dithiothreitol, 40 mM Tris-Cl (pH 8), 15 mM $MgCl_2$, and 35 U of T7 RNA polymerase (Pharmacia) in a total volume of 50 μl. After incubation at 37°C for 30 min, the reaction mixtures were used to transfect HeLa cell monolayers in 6-cm plates with DEAE-dextran as a facilitator (15).

Poliovirus binding assay. Cells were detached from tissue culture roller bottles by treatment with enzyme-free cell dissociation buffer (GIBCO). [^{35}S]methionine-labeled virus (5×10^9 particles) was incubated with 10^7 cells in 250 μl of Dulbecco modified Eagle's medium plus 10% bovine calf serum plus 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) for 4 h at room temperature with rotation. The number of virions added per cell was approximately equal to the number of virus binding sites per cell, because conditions of receptor or virus excess produce falsely high or low values for percent bound, respectively. For calculation of nonspecific binding, cells were detached from roller bottles, resuspended in undiluted anti-PVR hybridoma supernatant 711C, and incubated for 1 h at room temperature prior to the addition of virus. 711C is an immunoglobulin G2a antibody that reacts with domain 1 of the poliovirus receptor and protects cells from poliovirus infection by blocking poliovirus binding (20). Depending on the particular virus and cell line, nonspecific binding was 0.77 to 4.65% of total binding. After the binding period, unattached virus was separated from bound virus by centrifugation for 30 s in an Eppendorf microcentrifuge at 10,000 rpm, and supernatants were transferred to scintillation vials containing 1/10 volume of 1 N NaOH to cause cell lysis. The cell pellets were washed twice with 100 μl of PBS at room temperature, and each wash was added to the first supernatant. The cell pellet was then resuspended in a final volume of PBS equal to the total volume of the supernatants, and this was transferred to scintillation vials containing a 1/10 volume of 1 N NaOH. Radioactivity was quantitated by scintillation counting. Specific binding was expressed as a percentage of the total virus added. Binding assays were performed twice, and each datum point was determined in duplicate.

Neurovirulence assay in mice. Undiluted viral stocks (1.1×10^9 to 1×10^{10} PFU/ml) of P1/Mahoney, P2/Lansing, and the six site-directed mutants were inoculated intracerebrally (0.05 ml) into four male and four female CD1 mice. Infected animals were observed for 21 days for signs of paralysis as described previously (15).

Computer graphics. The atomic coordinates for the P1/Mahoney were provided by James M. Hogle. The capsid structure was visualized on a Silicon Graphics Personal IRIS 4D/35 by using Insight II, version 2.3.0 (Biosym Technologies, Inc., San Diego, Calif.).

RESULTS

Selection of poliovirus mutants adapted to grow on cells expressing mutant receptor. In previous work, Pvr mutants were constructed by replacing residues in domain 1 with the corresponding residues from Mph, the murine homolog of Pvr (19). Stable L-cell lines expressing three different Pvr mutants, d, g, or i, failed to bind or replicate poliovirus P1/Mahoney (20). The d, g, and i mutations are shown in Fig. 1 on a structural model of Pvr domain 1. To select mutant Pvr-adapted viruses, monolayers of d, g, and i receptor-expressing cells were inoculated with poliovirus P1/Mahoney. Wild-type virus stocks which typically yielded 1×10^9 PFU/ml on wild-type Pvr-expressing cells had titers of 1.8×10^2 to 4×10^3 PFU/ml on mutant Pvr-expressing cells (Table 1). Virus from plaques that formed on d, g, and i cells was isolated, plaque purified twice, and used to generate virus stocks.

To determine whether viruses that formed plaques on d, g, or i cells were viral variants with capsid mutations that suppressed the defects of the mutant receptors, stocks of these viruses were titrated on mutant and wild-type Pvr-expressing cells. Adapted viruses have titers on d, g and i cells that are 4.8 to 5.5 $\log_{10}$ higher than the titer of P1/Mahoney on mutant Pvr cells (Table 1). While adapted viruses gain the ability to grow on mutant Pvr cells, they retain the ability to grow on cells expressing wild-type Pvr. Adapted mutants are present in wild-type stocks at a frequency of approximately 1 in 10^6 to 10^7 PFU.

Identification of mutations in adapted viruses and their locations in the capsid structure. To identify the mutation(s) in adapted viruses, the sequence of the capsid coding region was determined from cloned cDNA. After capsid mutations

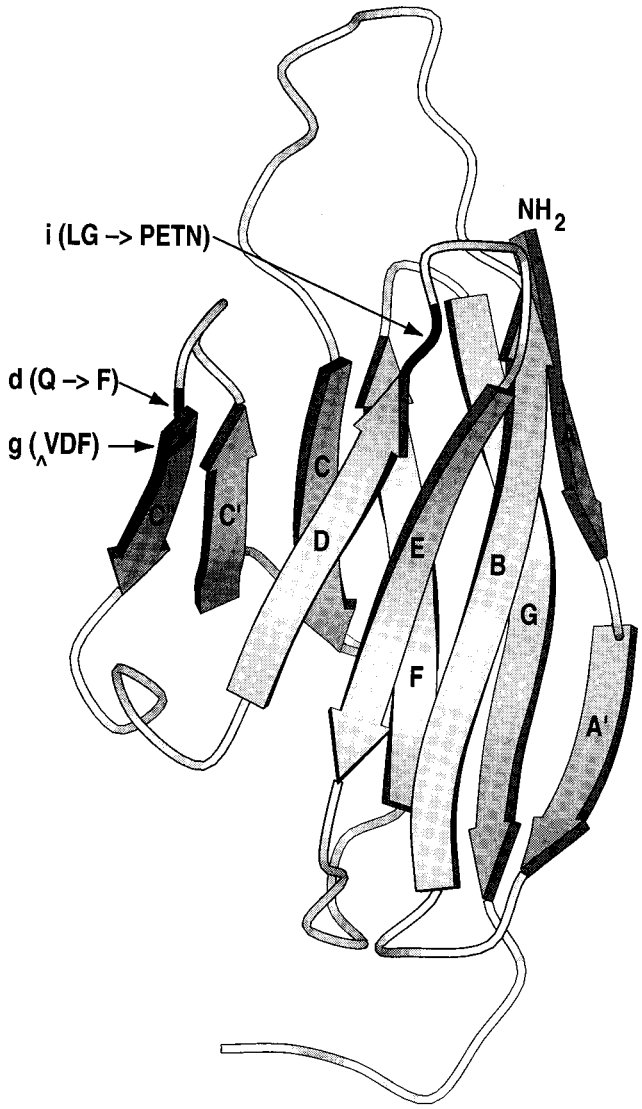

FIG. 1. Model of Pvr domain 1 showing the d, g, and i mutations. The structure of domain 1 was predicted as described previously (20). Capital letters, β strands; lowercase letters, the positions of the mutations. Amino acid changes are indicated.

were identified by DNA sequencing, specific regions of the viral RNA were sequenced directly to confirm that the mutations were present in the virus. The results of the sequence analysis are shown in Table 2. All of the adapted viruses have a Pro-to-Ser or Thr mutation at capsid residue 1095. Two d-adapted viruses and one g-adapted virus have two capsid mutations, P1095S and H2142Y. Six i-adapted viruses and one d-adapted virus have two changes in capsid protein VP1, P1095S and V1160I.

To begin to understand how mutations at positions 1095, 1160, and 2142 allow P1/Mahoney to grow on d, g, and i cells, their locations in the three-dimensional structure of P1/Mahoney were examined. Residue 1095 is located in the VP1 B-C loop, a highly exposed sequence on the virion surface near the fivefold axis of symmetry (Fig. 2a). Residue 1160 maps to the interface between fivefold related protomers and is near the hydrocarbon-binding pocket of VP1 (Fig. 2a and b). Residue 2142 is located in the canyon (Fig. 2a).

TABLE 1. Growth of wild-type and mutant poliovirus on cells expressing d, g, i, or wild-type receptor

Virus	Titer (PFU/ml)[a]			
	d cells	g cells	i cells	Wild-type cells
P1/Mahoney	4.0×10^3	2.2×10^3	1.8×10^2	1.6×10^9
P2/Lansing	3.1×10^2	1.4×10^8	<10	7.2×10^8
P2/P1 B-C loop chimera	5.4×10^3	2.7×10^3	<10	1.6×10^9
P3/Leon	5×10^8	4.7×10^3	<10	2.2×10^9
d-adapted virus[b]	2.5×10^8			1.2×10^8
g-adapted virus[c]		5.3×10^8		2.0×10^8
i-adapted virus[d]			6.3×10^7	2.8×10^8
vP1095S[e]	1.2×10^8	6.7×10^7	7.8×10^6	1.8×10^9
vP1095T[e]	4.6×10^8	6.5×10^7	9.6×10^6	5.5×10^8
vV1160I[e]	5.1×10^8	1.3×10^7	2.9×10^6	7.8×10^8
vH2142Y[e]	1.2×10^8	1.7×10^7	1.3×10^2	1.2×10^9
vP1095S + H2142Y[e]	9.7×10^8	6.2×10^7	6.5×10^6	6.6×10^8
vP1095S + V1160I[e]	1.7×10^8	3.0×10^8	8.4×10^7	8.3×10^8

[a] Determined by plaque assaying on cells expressing d, g, i, or wild-type receptor. The values are the averages of two experiments and, in the case of site-directed mutants, of two independent PCR isolates. Titers in independent experiments varied by a factor of 1.0 to 4.1 from the average value.
[b] Contains the mutations P1095S and H2142Y.
[c] Contains the mutation P1095T.
[d] Contains the mutations P1095S and V1160I.
[e] The lowercase v denotes that the virus was generated by site-directed mutagenesis, the second letter is the wild-type amino acid, the first digit indicates the capsid protein, the following three digits identify the amino acid position, and the last letter is the mutant amino acid.

Specific capsid mutations expand the receptor recognition of P1/Mahoney. Six P1/Mahoney variants were constructed by site-directed mutagenesis to determine the effect of single and double mutations on the ability of P1/Mahoney to replicate in cells expressing d, g, i, or wild-type Pvr. With one exception, the titers of these site-directed variants and the adapted viruses on d, g, and i cells are comparable and are higher than the titer of wild-type P1/Mahoney on d, g, and i cells by a factor of 3.8 to 5.7 $\log_{10}$ (Table 1). The site-directed variants grow as well as wild-type P1/Mahoney on cells expressing wild-type Pvr (Table 1).

Comparison of the titers of site-directed variants on mutant Pvr cells to the titer of wild-type P1/Mahoney on wild-type Pvr cells indicates the extent to which different capsid mutations

TABLE 2. Capsid mutations in mutant Pvr-adapted viruses

Virus	No. of mutants	Mutation at the following capsid positions[a]:		
		1095	1160	2142
d-adapted viruses	3	Ser		
	1	Ser	Ile	
	2	Ser		Tyr
g-adapted viruses	2	Ser		
	1	Thr		
	1	Ser		Tyr
i-adapted viruses	6	Ser	Ile	
P1/Mahoney		Pro	Val	His
P2/Lansing		Asp	Ile	Phe
P3/Leon		Glu	Ile	Tyr

[a] The first digit in each capsid position indicates the capsid protein, and the following three digits identify the amino acid position. Wild-type P1/Mahoney sequence is from Racaniello and Baltimore (28).

FIG. 2. Locations of adapting mutations in the capsid structure. (a) An alpha-carbon trace of a single poliovirus protomer viewed from the side (sixty protomers form an entire capsid). Note the peaks at the 5× and 3× axes of symmetry, as well as the canyon which separates them. Adapting mutations are indicated by yellow dots, and residue numbers are indicated. The VP1 B-C loop is shown in green; the GH loop of VP1, in which the majority of soluble receptor-resistant mutants map, is highlighted in white; and the light-blue molecule represents the occupant (possibly sphingosine) of the hydrocarbon-binding pocket of VP1. (b) Two protomers viewed from outside the virion, showing the interface between protomers. The color coding and labeling are the same as for part a.

confer mutant receptor adaptation to P1/Mahoney. All six site-directed variants have titers on d cells that are within 1 $\log_{10}$ PFU of the titer of P1/Mahoney on wild-type Pvr cells, demonstrating that the capsid mutations largely compensate for the effect of the d mutation on the virus-receptor interaction (Table 1). All six site-directed mutants also replicate to high titers in g cells; however, with the exception of vP1095S + V1160I, the titers are 25- to 125-fold lower than the titer of P1/Mahoney on wild-type Pvr cells (Table 1). These findings suggest that only the double mutation P1095S + V1160I fully overcomes the block in the virus-receptor interaction caused by the g mutation. Of the six site-directed mutants, vP1095S + V1160I has the highest titer on i cells, although its titer is 20-fold lower than the titer of P1/Mahoney on wild-type Pvr cells (Table 1). Mutant vH2142Y grows as poorly as P1/Mahoney on i cells, and the remaining four site-directed mutants have titers on i cells that are 170- to 550-fold lower than the titer of P1/Mahoney on wild-type Pvr cells (Table 1). These results suggest that the double mutation, P1095S + V1160I, best suppresses the growth defect of P1/Mahoney on i cells, while other mutations compensate less well or not at all for the effect of the i mutation on the virus-receptor interaction.

To determine whether other capsid mutations confer mutant receptor adaptation to P1/Mahoney, the replication of six additional P1/Mahoney variants in d, g, and i cells was examined. These viruses included two *srr* mutants, each containing a single amino acid change in the canyon (L1228F and G1225D) that reduces binding to wild-type Pvr-expressing cells (5); two viruses with single mutations in the amino terminus of VP1 (E1040G and P1054S) that overcome the host range restriction of P1/Mahoney in mice (21); a P1/Mahoney recombinant containing VP2 from P1/Sabin (4); and a mutant with an Ala-to-Ser substitution at position 65 of VP4 (4). These viruses grow as poorly as P1/Mahoney on d, g, and i cells but replicate to high titers on wild-type Pvr-expressing cells (data not shown). These results are consistent with the idea that specific capsid mutations selected during replication in d, g, or i cells allow P1/Mahoney to recognize mutant receptors.

The capsid sequences of poliovirus serotypes 1, 2, and 3 differ significantly (24), and therefore it was of interest to determine whether P2/Lansing and P3/Leon were adapted to d, g, and i cells. The titers of P2/Lansing and a g-adapted virus on g cells are similar (Table 1), indicating that P2/Lansing can recognize the g receptor. Similarly, the titers of P3/Leon and a d-adapted virus on d cells are comparable (Table 1), demonstrating that P3/Leon is adapted to the d receptor. A P1/Mahoney chimera containing the VP1 B-C loop of P2/Lansing does not plaque efficiently on d, g, or i cells but grows well on cells expressing wild-type Pvr, suggesting that this sequence is not sufficient for g adaptation of P2/Lansing.

Capsid mutations in adapted viruses improve binding of P1/Mahoney to mutant Pvr and do not reduce binding to wild-type Pvr. Binding assays were performed to test the hypothesis that a mechanism of action of adapting mutations is

TABLE 3. Binding of wild-type and mutant poliovirus to cells expressing d, g, i, or wild-type receptor

Virus[a]	% Of total virus bound[b]			
	d cells	g cells	i cells	Wild-type cells
P1/Mahoney	14.5	5.5	1.3	90.6
vP1095S	84.4	22.6	7.7	92.6
vP1095T	84.8	38.9	14.1	87.0
vV1160I	60.3	22.5	6.0	83.8
vH2142Y	79.6	32.9	2.4	90.6
vP1095S + H2142Y	80.2	40.7	14.5	74.8
vP1095S + V1160I	87.8	41.9	24.6	82.7

[a] The lowercase v denotes that the virus was generated by site-directed mutagenesis, the second letter is the wild-type amino acid, the first digit indicates the capsid protein, the following three digits identify the amino acid position, and the last letter indicates the mutant amino acid.

[b] Values are the averages of two experiments. Values in independent experiments varied by 0.2 to 5.9 percentage points from the average.

that they improve growth on d, g, and i cells by improving binding of P1/Mahoney to mutant receptors. The site-directed mutants bind significantly better to d cells than does wild-type P1/Mahoney (Table 3). With the exception of vV1160I, the site-directed variants bind as well to d cells as P1/Mahoney binds to wild-type cells (Table 3). The site-directed mutants also have improved binding to g cells. The levels of binding of vP1095T, vP1095S + V1160I, and vP1095S + H2142Y to g cells are higher than those of other site-directed variants, although not as high as the level of binding of P1/Mahoney to wild-type cells (Table 3). Of the site-directed mutants, vP1095S + V1160I has the highest level of binding to i cells, which is lower than the level of binding of P1/Mahoney to wild-type cells (Table 3).

These results suggest that the capsid mutations in adapted viruses overcome the binding defect of the d receptor but do not completely compensate for the binding defects of the g and i receptors, although they are sufficient to overcome the growth defect on g and i cells. All of the site-directed P1/Mahoney variants display high levels of binding to wild-type Pvr-expressing cells (Table 3), which is consistent with the finding that these mutants replicate well in wild-type Pvr cells (Table 1).

A specific capsid mutation in adapted viruses allows P1/Mahoney to paralyze mice. The host range of P1/Mahoney, which is restricted to primates, can be expanded by certain mutations that allow the virus to interact with a receptor in the mouse central nervous system (6, 7, 17, 21, 22). To determine whether mutations in adapted viruses enable poliovirus to recognize a mouse receptor, the ability of site-directed mutants to cause paralysis in mice was examined. Intracerebral inoculation of mice with high levels of P2/Lansing resulted in paralysis of all eight mice inoculated, while similar levels of P1/Mahoney had no effect, as expected. Of the six site-directed mutants tested, only vV1160I was neurovirulent, paralyzing three of eight mice inoculated. These results indicate that the mutation at VP1-160 is a new host range determinant in mice.

DISCUSSION

Poliovirus variants that are adapted to grow on cells expressing mutant Pvr retain the ability to grow on cells expressing wild-type Pvr. The capsid mutations that confer mutant Pvr adaptation to P1/Mahoney, therefore, expand rather than change receptor specificity. Lambda phage mutants selected for the ability to grow on bacteria expressing mutated lambda phage receptor also retain the ability to grow on wild-type bacteria and have an expanded host range (2, 11).

Mechanism of action of adapting mutations. Previously described mutations in Pvr that block poliovirus binding are believed to define parts of the molecule that contact virus (1, 3, 20). Viruses selected for the ability to replicate in cells expressing mutant receptors d, g, and i contain specific adapting mutations. An attractive explanation for the mechanism of action of adapting mutations is that they act at regions of the capsid that contact the mutant receptor. Residue 2142 is located in the canyon, an area of the capsid that is believed to be involved in receptor contact, on the basis of genetic and structural studies (5, 23). The change from His to Tyr at this position may restore contact with d and g receptor residues.

In every adapted mutant, Pro-1095 in the VP1 B-C loop is substituted with Ser or Thr. One explanation for the mechanism of action of mutation P1095S/T is that it alters the conformation of the VP1 B-C loop, thereby affecting the ability of the capsid to accommodate mutant receptors. Comparison of the structures of P1/Mahoney, P3/Sabin, and a Lansing/Mahoney VP1 B-C loop chimera support the idea that mutation of residue 1095 changes the conformation of the B-C loop. The distinctive B-C loop conformations of P3/Sabin and the Lansing/Mahoney B-C loop chimera are due in part to hydrogen bonding between residues 1095 and 1102, which is absent in P1/Mahoney (33). Replacement of Pro-1095 with Ser/Thr in adapted viruses may introduce a hydrogen bond between B-C loop residues 1095 and 1102, altering the structure of the B-C loop. This change in the B-C loop structure may influence the conformation of the adjacent E-F loop. The E-F loop forms part of the interface between protomers, and regulation of the stability of this interface is important in controlling assembly and disassembly of the virus (5, 8). By influencing the E-F loop, a mutation in the B-C loop might affect the flexibility of the capsid, allowing recognition of a wider range of receptor structures.

Alternatively, the VP1 B-C loop may be part of the contact site for Pvr. In the structurally similar HRV14, residues of the VP1 B-C loop lie within the footprint of the receptor, ICAM-1 (23). However, the VP1 B-C loop lies outside the canyon, which is the region of the poliovirus capsid believed to be involved in receptor contact (5). Furthermore, the VP1 B-C loop is dispensable for poliovirus replication in primate cells (21). Thus, it is not clear whether the poliovirus VP1 B-C loop contacts Pvr.

Mutation at residue 1160 may expand receptor recognition by acting at regions of the capsid that contact the receptor and/or by regulating the flexibility of the capsid. Residue 1160 is located above the hydrocarbon-binding pocket of VP1, and the amino acid change at this residue may alter the position of the occupant of the pocket (possibly sphingosine), changing the conformation of the overlying G-H loop, which is likely to be involved in receptor contact (5). Replacement of sphingosine by certain antiviral drugs does not cause major changes in the native capsid structure but is believed to prevent structural changes associated with receptor binding and cell entry (9). Because of its proximity to the pocket, the mutation at 1160 may facilitate the structural changes necessary to recognize mutant receptors. Residue 1160 is within the E-F loop, which is located at the protomer interface, and therefore mutation at this position might also directly influence the stability of the interface.

The effects of the capsid mutations on receptor recognition may not be solely determined by their influence on virus-receptor contact. For example, capsid mutations in adapted viruses do not completely compensate for the binding defects

of the g and i receptors, although the mutations are sufficient to overcome the growth defect on g and i cells. These observations suggest that the capsid mutations also suppress the growth defect by acting at postbinding steps, such as alteration and uncoating.

Mutation of residue 1095 is sufficient to confer adaptation to d and g receptors, and several isolates contain a single mutation at this position. Interestingly, mutations V1160I and H2142Y did not occur in the absence of mutation P1095S in viruses selected for adaptation to mutant receptors. However, vV1160I grows well on d, g, and i cells, and vH2142Y replicates to high titers on d and g cells (Table 1). Single mutants may not have been detected, because only a small number of mutants were selected. Single mutants did not replicate as well as the double mutant vP1095S + V1160I on i cells, suggesting why single mutants were not isolated from i cells.

Wild-type poliovirus types 2 and 3 are adapted to d and g receptors. It is interesting that P1/Mahoney does not replicate in cells expressing d, g, or i receptors, while P2/Lansing and P3/Leon replicate to high titers on g and d cells, respectively. P2/Lansing recognizes a receptor in the mouse central nervous system, and this expanded receptor recognition can be conferred to P1/Mahoney by substitution of the VP1 B-C loop (17, 22). However, the VP1 B-C loop of P2/Lansing is not sufficient to confer g adaptation to P1/Mahoney (Table 1). Growth of P2/Lansing on g cells may be due to Ile at position 1160, which allows P1/Mahoney to recognize the g receptor (Tables 1 and 2). Growth of P3/Leon on d cells may be explained by the presence of Ile at 1160 and Tyr at 2142, which allow P1/Mahoney to infect d cells (Tables 1 and 2).

A comparison of the structures of different VP1 B-C loops also suggests that a specific conformation is required for expanded receptor recognition of P2/Lansing and P3/Leon. In P3/Sabin, which has the same VP1 B-C loop sequence as P3/Leon, and a Lansing-Mahoney VP1 B-C loop chimera, residues 1095 and 1102 form a hydrogen bond, which is not present in the VP1 B-C loop of P1/Mahoney (33). The conformation of the VP1 B-C loop may therefore contribute to the expanded receptor recognition of P2/Lansing and P3/Leon and may contribute to the restricted receptor recognition of P1/Mahoney.

The adapting mutation V1160I is a poliovirus host range determinant. The host restriction of poliovirus P1/Mahoney in mice is controlled at the level of the virus-receptor interaction, because transgenic mice expressing the human Pvr gene can be infected with this strain (13, 29). The host range of P1/Mahoney can also be expanded to mice by substitution of the VP1 B-C loop with the sequence from P2/Lansing, which is a mouse-adapted strain of poliovirus (17, 22). The B-C loop is also the site of mutation P1095S/T, which enables P1/Mahoney to recognize d, g, and i receptors. Virus containing the mutation V1160I, which permits replication on d, g, and i cells, is also able to paralyze mice. Mutations at either position in the capsid therefore allow poliovirus to recognize mutant human receptors and a receptor in the mouse central nervous system. These results imply that adaptation to d, g, i, and mouse receptors have similar mechanistic bases. Either directly or by their effect on neighboring residues, VP1 B-C loop residues and residue 1160 might modulate contact with the receptors or structural flexibility of the capsid. Other mutations that enable P1/Mahoney to utilize a mouse receptor are located in the interior of the capsid and are believed to expand receptor recognition by affecting capsid transitions during cell entry (6, 7, 21).

Although the VP1 B-C loop of P2/Lansing can expand the host range of P1/Mahoney, the mutations P1095S/T, which are located in the VP1 B-C loop, are not sufficient to allow P1/Mahoney to infect mice. This result is not surprising, because previous work has shown that changing residue 1095 to Asp, the residue in P2/Lansing, is not sufficient to confer mouse adaptation to P1/Mahoney; replacement of at least two residues is required (16). Interestingly, P1095S suppresses the expanded host range conferred to P1/Mahoney by V1160I. P1095S may therefore functionally override the effect of the mutation at residue 1160 on receptor recognition.

Variants of P1/Mahoney selected to persist in neuroblastoma cells contain the mutations P1095S and H2142Y (6), which are identical to those observed in mutant receptor adapted viruses. Because these mutations enable poliovirus to recognize mutant receptors in L cells, it is possible that their selection in persistently infected neuroblastoma cells also reflects adaptation to suboptimal receptors. Whether persistence in neuroblastoma cells is related to receptor interactions is unknown. It was reported that a P1/Mahoney mutant containing the H2142Y mutation is virulent in mice (6), in contrast to the data reported here. This difference may reflect variations in the mouse or virus strains used in the two laboratories.

The analysis of viral variants presented here reveals specific capsid residues that allow wild-type P1/Mahoney to replicate in cells expressing mutant receptors and to paralyze mice. A complete understanding of how specific mutations expand poliovirus receptor recognition awaits resolution of the atomic structure of the virus-receptor complex.

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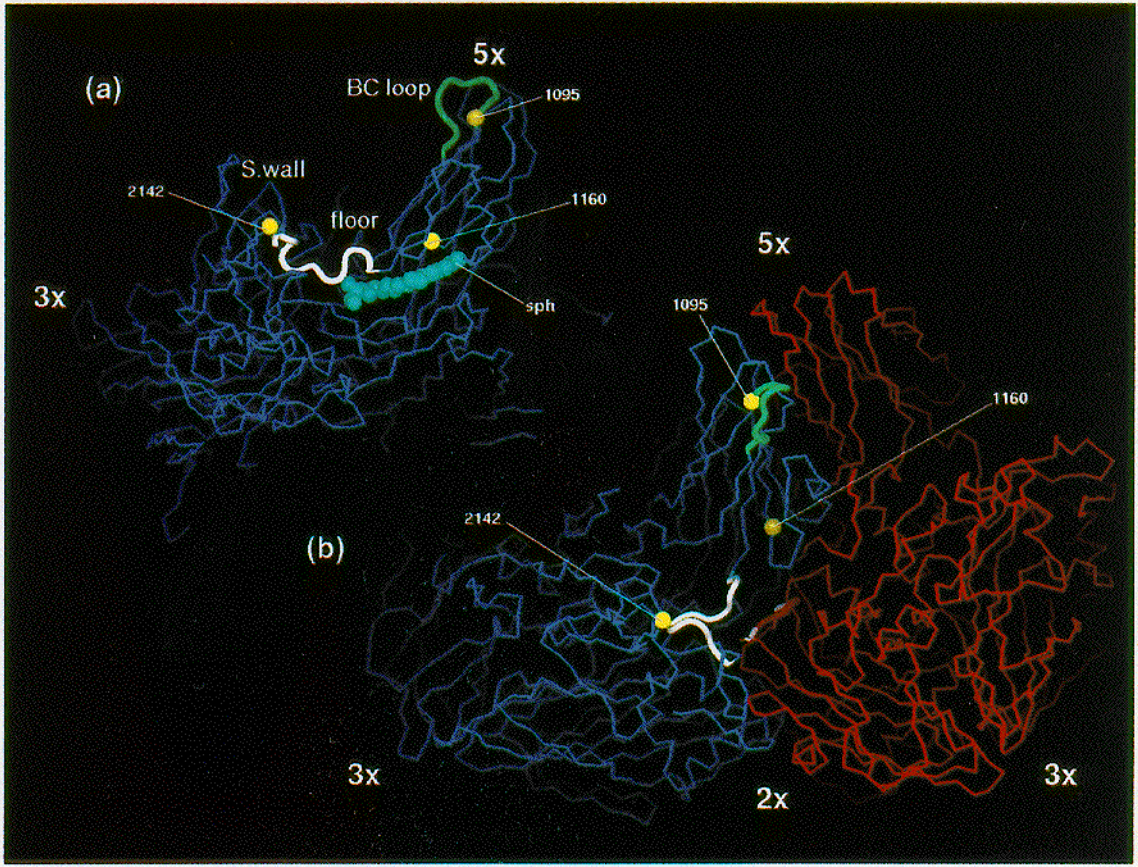
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