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Homolog-Scanning Mutagenesis Reveals Poliovirus Receptor Residues Important for Virus Binding and Replication

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Poliovirus initiates infection of primate cells by binding to the poliovirus receptor, Pvr. Mouse cells do not bind poliovirus but express a Pvr homolog, Mph, that does not function as a poliovirus receptor. Previous work has shown that the first immunoglobulin-like domain of the Pvr protein contains the virus binding site. To further identify sequences of Pvr important for its interaction with poliovirus, stable cell lines expressing mutated Pvr molecules were examined for their abilities to bind virus and support virus replication. Substitution of the amino-terminal domain of Mph with that of Pvr yields a molecule that can function as a poliovirus receptor. Cells expressing this chimeric receptor have normal binding affinity for poliovirus, yet the kinetics of virus replication are delayed. Results of virus alteration assays indicate that this chimeric receptor is defective in converting native virus to 135S altered particles. This defect is not observed with cells expressing receptor recombinants that include Pvr domains 1 and 2. Because altered particles are believed to be an intermediate in poliovirus entry, these findings suggest that Pvr domains 2 and 3 participate in early stages of infection. Additional mutants were made by substituting variant Mph residues for the corresponding residues in Pvr. The results were interpreted by using a model of Pvr predicted from the known structures of other immunoglobulin-like V-type domains. Analysis of stable cell lines expressing the mutant proteins revealed that virus binding is influenced by mutations in the predicted C′-C″ loop, the C″ β-strand, the C″-D loop, and the D-E loop. Mutations in homologous regions of the immunoglobulin-like CD4 molecule alter its interaction with gp120 of human immunodeficiency virus type 1. Cells expressing Pvr mutations on the predicted C″ edge do not develop cytopathic effect during poliovirus infection, suggesting that poliovirus-induced cytopathic effect may be induced by the virus-receptor interaction.

Poliovirus is a member of the picornavirus family, a group of small, naked, icosahedral viruses that cause a wide range of human diseases. All three poliovirus serotypes utilize the poliovirus receptor, Pvr, to initiate infection of cells (32). Pvr is a member of the immunoglobulin (Ig) superfamily of proteins and is predicted to contain one V-like and two C2-like Ig domains.

The identification and molecular cloning of virus receptors enable the identification of features required for infection of cells. For virus receptors of the Ig superfamily, it is of particular interest to determine whether regions homologous to the complementarity-determining regions (CDRs) of antibody molecules constitute the virus binding site. CD4, a molecule involved in T-cell activation and recognition of antigens in the context of major histocompatibility complex class II molecules, is a receptor for the human immunodeficiency virus (HIV) (11, 24, 29). CD4 is an Ig gene family member with one N-terminal V-like and three C-like domains (57). The crystal structures of two truncated versions of CD4 containing only domains 1 and 2 have been determined (45, 56). The binding site for HIV type 1 (HIV-1) on CD4 is generally believed to be a sequence surrounding the region homologous to CDR2 of an Ig V domain (33, 49). However, regions homologous to CDR1 and CDR3 do not appear to participate in HIV-1 binding.

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Intercellular adhesion molecule 1 (ICAM-1) is an Ig family member receptor for rhinovirus, another member of the picornavirus family (17, 54, 55). ICAM-1 has five extracellular C-like domains; deletion experiments have shown that the amino-terminal domain 1 determines the specificity of rhinovirus binding, while the other domains contribute to quantitative rhinovirus binding (52, 53). Within domain 1, residues affecting rhinovirus binding have been mapped to several noncontiguous patches of residues (16, 31, 41). Because domain 1 is like C-like domains, residues affecting rhinovirus binding do not correspond to CDR-like regions.

Results of deletion analysis of Pvr suggest that the first Ig-like domain, a V-type domain, contains the binding site for poliovirus (25, 50, 51). Our approach to studying regions of Pvr that are important for poliovirus infection is to analyze chimeric proteins of Pvr and its murine homolog, Mph, which has extensive sequence similarity to the extracellular domains of Pvr yet does not bind poliovirus (36). When the first Ig-like domain of Mph is substituted with that of domain 1 of Pvr, the chimeric protein can confer sensitivity to poliovirus infection to murine L cells (36). Here we show that cells expressing this chimeric protein do not produce poliovirus with wild-type kinetics and that they have a defect in viral alteration, suggesting that Pvr domains 2 and/or 3 are important for efficient receptor function. To identify Pvr residues critical for poliovirus binding and infection, residues within the first domain of Pvr were substituted with residues from the homologous Mph sequence. Analyses of poliovirus binding and infection in cell lines expressing mutant Pvr indicate that amino acid residues affecting poliovirus binding map to the predicted C′-C″-D edge

TABLE 1. Domain specificity of monoclonal antibodies

Cell line[a]	Antibody reactivity[b]		Receptor structure[c]
	711C	55D	
HeLa	+	−	
L	−	−	
20B	+	+	
E2	+	−	
h52neo10	+	+	

[a] Cell lines 20B, E2, and h52neo10 were created by transformation of L cells with expression plasmids previously described (36).

[b] The antibody reactivity of each cell line was determined by FACS analysis, as described in Materials and Methods. Whether the intensity of fluorescence staining with primary antibody did (+) or did not (−) exceed background staining obtained without primary antibody is indicated.

[c] Schematic drawings of the protein structures of each cell line, with stippled domains representing Pvr sequence and unstippled domains representing Mph sequence.

of Pvr domain 1, in a region similar to that of CD4 that is involved in HIV-1 binding. Cells expressing Pvr with mutations on the predicted C′-C″-D edge of Pvr replicated virus but did not develop cytopathic effect (CPE), suggesting a link between poliovirus receptor interaction and virus-induced cell death.

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 plaque assays, HeLa cells were plated in Dulbecco modified Eagle medium (DMEM) containing 10% horse serum and gentamicin. Mouse L cells were maintained in DMEM containing 10% calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Stable DNA transformants were maintained in the same medium with 100 mM hypoxanthine–0.4 mM aminopterin–16 mM thymidine (HAT) for thymidine kinase selection or with 400 μg of geneticin sulfate (GIBCO) per ml for neomycin selection. Hybridoma cell lines 711C and 55D were grown in basal Eagle medium with 10% fetal bovine serum.

Poliovirus strain P1/Mahoney was derived from the infectious cDNA clone (40). For binding assays, virus was labeled with [^{35}S]methionine (New England Nuclear), pelleted from cell lysates by centrifugation at 40,000 rpm and 10°C for 90 min in an SW41 rotor (Beckman), and centrifuged through a 7.5 to 45% sucrose gradient at 40,000 rpm and 10°C for 75 min in an SW41 rotor. The conversion factor of 9.4×10^{12} particles per optical density at 260 nm unit was used to determine virion concentration (44). The purified virus was brought to 5 mg of bovine serum albumin (BSA) (fraction V; Sigma) per ml and dialyzed against phosphate-buffered saline (PBS) containing 1 mg of BSA per ml and 20 mM $MgCl_2$.

Hybridoma cells and antibodies. Generation and properties of anti-Pvr monoclonal antibodies will be described in detail elsewhere. Briefly, BALB/c mice were immunized with a detergent extract of insect cells expressing Pvr (21). Fusions with the myeloma cell line X63Ag8.653 were performed as described previously (18), and hybridoma supernatants were screened by whole-cell enzyme-linked immunosorbent assay (ELISA) or by their ability to block virus-induced killing of cells (19). Antibody specificity was determined by performing an ELISA on a panel of cell lines expressing wild-type Pvr or several recombinants with Pvr and Mph domains (36). Specificity was confirmed by fluorescence-activated cell sorting (FACS) analysis of these cell lines with hybridoma supernatants (Table 1). Hybridoma 711C secretes an IgG2a antibody that reacts with Pvr domain 1, and hybridoma 55D secretes an IgG1 antibody which reacts with Pvr when both the first and second Ig-like domains are present but not when only the first domain is present. 711C antibody protects cells from poliovirus infection, and 55D antibody does not.

Whole-cell ELISA for Pvr surface level detection. Control cells with known Pvr expression and test cells with unknown Pvr expression were grown in 96-well tissue culture plates (Corning) for 2 days, until fully confluent. The culture supernatant was aspirated from each well and replaced with 150 μl of hybridoma supernatant, and the entire plate was incubated at room temperature for 2 h. Hybridoma supernatant was aspirated from each well, and the cells were washed three times with 200 μl of wash buffer (PBS with 20 μg of $CaCl_2$ per ml, 20 μg of $MgCl_2$ per ml, and 3% BSA). Cells were incubated with 50 μl of a goat anti-mouse peroxidase-conjugated secondary antibody (Tago) diluted in wash buffer at room temperature for 1 h; secondary antibody was aspirated from each well, and the cells were washed two times with 300 μl of wash buffer each time and then washed once with PBS. The peroxidase reaction was developed with *O*-phenylene diamine hydrochloride substrate and quantified on an ELISA plate reader. Control cells included HeLa cells and an L-cell transformant expressing the *pvr* 20B cDNA (32) as well as several stable cell lines developed in this work expressing various forms of Pvr recombinants with Mph. Untransformed L cells and 2G7 cells, a derivative of the HeLa line that does not express Pvr (22), served as the negative-control lines.

DNA transformation. Ltk$^-$ Aprt$^-$ cells were seeded in plastic cell culture plates 1 day before use at 7.5×10^5 cells per 10-cm-diameter plate. The medium was changed 4 to 6 h before transformation. Plates were treated with the following DNA-calcium phosphate coprecipitates: for stable transformation with pSVL expression vectors, 1.0 ml of a mixture of 10 μg of linearized plasmid DNA and 3 μg of a plasmid containing the herpesvirus thymidine kinase gene; for stable transformation with pcDNA1neo expression vectors, 1.0 ml of a mixture of either 1 μg of linearized plasmid DNA and 9 μg of herring sperm DNA (allowing selection with geneticin sulfate) or 10 μg of linearized plasmid DNA and 3 μg of a plasmid containing the herpesvirus thymidine kinase gene (allowing HAT selection). After 18 h of incubation at 37°C, the medium was replaced, and incubation continued for an additional 24 h. For isolation of stable transformants with HAT selection, cells were grown in HAT medium for 2 weeks, and HAT-resistant colonies were subcultured. For isolation of stable transformants with Geneticin, cells were grown in medium with 400 μg of geneticin sulfate (GIBCO) per ml for 9 to 14 days, and Geneticin-resistant colonies were subcultured. After initial selection and characterization, Geneticin-resistant cell lines were maintained in medium containing 200 μg of Geneticin per ml.

FACS staining and analysis. Cells were detached from tissue culture plastic by treatment with 50 mM EDTA or enzyme-free dissociation buffer (GIBCO). All staining and washing steps were performed at 4°C unless otherwise indicated. For analytical determination of Pvr surface levels, aliquots of approximately 7×10^5 cells in 25 μl were incubated with 25-μl portions of 711C or 55D hybridoma supernatant or with 25-μl portions of staining medium (SM; PBS with 3% newborn calf serum) as a negative control, on ice for 15 min. The cells were washed three times with 150 μl of SM, resuspended in 25 μl of SM containing a 1:50 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse IgG and IgM antibody (Tago), and incubated on ice for 15 min. The cells were washed three times as described above and resus-

pended in 500 μl of SM containing 0.1 μg of propidium iodide per ml. Samples prepared in this way were subjected to FACS analysis on a Becton-Dickinson FACStar machine with the laser excitation wavelength set at 488 nm. Propidium iodide was used to allow exclusion of dead cells without interfering with the green emission of fluorescein-conjugated secondary antibody (12). The FACS machine was calibrated with fluorescent beads (Polysciences, Warrington, Pa.) before each analysis.

Pvr surface levels were calculated by comparing the median fluorescence intensity (MFI) of a given transformant cell line with the MFI of HeLa cells stained with the same antibody (Ab) in the same assay, according to the following formula: percent HeLa surface level = (linear MFI of cells with Ab − linear MFI of cells with SM)/(linear MFI of HeLa cells with Ab − linear MFI of HeLa cells with SM) × 100 (23, 27, 39). Because the fluorescence intensity of the cells varies from day to day, final estimates of Pvr surface level were achieved by averaging the percentages of HeLa surface levels from five to eight independent FACS analyses with a given antibody performed over a period of 3 to 4 months. The amount and source of surface level variation will be discussed below.

Virus infections. For analysis of virus replication in stable L-cell transformants, replicate 25-cm^2 tissue culture flasks were seeded with 10^6 cells. Two days later, the confluent monolayers were infected with poliovirus strain P1/Mahoney at the indicated multiplicity of infection (MOI). After a 45-min adsorption period at room temperature, all flasks were washed with PBS to remove unbound virus (four washes for high-MOI infections, two washes for low-MOI infections), medium was replaced, and infection was allowed to proceed at 37°C. Duplicate flasks were transferred to a −70°C freezer at the indicated intervals after infection. All flasks were freeze-thawed three times to release total virus, and the resulting lysates were titrated by plaque assay on HeLa cell monolayers.

Construction of domain recombinants. Recombinants of the human poliovirus receptor cDNA clone in plasmid pMEM.20BKpn and the murine *pvr* homolog cDNA clone in pMEM.brmp18 were constructed as described previously (36). Schematic drawings of the expected recombinant polypeptides appear in Table 1. The final constructs are as follows: pMEM.m1-1 contains pSVL with *pvr* domain 1 sequences up to the *Kpn*I site introduced by mutagenesis, followed by murine sequences 3′ of the *Kpn*I site. Cells carrying this construct are designated E2 cells. pMEM.h5neo7 contains pcDNA1neo with *pvr* domain 1 and 2 sequences up to the naturally occurring *Kpn*I site between domains 2 and 3, followed by murine sequences for domain 3, the putative transmembrane-spanning region, and the cytoplasmic tail; this construct contains the *Kpn*I site introduced between domains 1 and 2 by mutagenesis of the human receptor cDNA. Cells carrying this construct are designated h52neo10 cells.

Establishment of stable cell lines. Murine L cells, which do not have the human *pvr* gene, were transformed with CsCl-purified plasmids as described above, and the resulting colonies were screened by two methods. Initially, individual colonies were expanded into 24-well plates and seeded in duplicate 96-well plates for Pvr surface level detection by ELISA with monoclonal antibodies 711C and 55D. Alternatively, transformation plates were expanded and stained for FACS analysis in bulk by using monoclonal antibodies 711C and/or 55D. This approach allowed simultaneous selection for higher surface levels and immediate elimination of nonexpressing clones. Those single cells with the highest surface expression of Pvr epitopes were sorted on the FACS machine under sterile conditions into 96-well tissue culture plates and grown until ready for expansion to 24-well plates (approximately 2×10^5 cells), at which time they were restained for FACS analysis to confirm their Pvr-expressing status and the general level of Pvr on their surfaces. The highest expressors were expanded for use in further experiments. Cell lines are designated by a letter representing the Pvr mutation, followed by numbers enabling us to trace the origin of each clone.

Oligonucleotide-directed mutagenesis. Mutagenesis was performed on human poliovirus receptor cDNAs subcloned in bacteriophage M13 and grown in *Escherichia coli* CJ236 as previously described (1). The enzymes used were T4 polynucleotide kinase and Klenow enzyme (both from Boehringer Mannheim Biochemicals). The coding regions of all mutated templates were sequenced in their entirety by the dideoxy method (47), either manually or by automated sequencing (ABI, Inc.), to confirm that they did not contain unintended amino acid changes. The mutagenized coding regions were cloned into the *Xba*I site in the eukaryotic expression vector pcDNA1neo (Invitrogen) or into the *Sma*I site in expression vector pSVL (Pharmacia). These constructs are designated pMEM.d1a through pMEM.d1j (numbers follow the lowercase letters representing the Pvr mutation).

Poliovirus binding assay. Cells were detached from tissue culture dishes as described above and then incubated in medium at 4°C for at least 4 h. Eppendorf tubes to be used in binding experiments were first filled with growth medium and incubated overnight to block nonspecific adsorption of virus to the tube walls. Cells and virus prepared as described above were incubated in medium at 4°C in preblocked Eppendorf tubes on a rotator for a minimum of 22 h. After this incubation, tubes were spun in a 4°C microcentrifuge (Eppendorf Inc.) at 5,000 rpm to pellet the cells, and supernatants were removed to scintillation vials containing 1/10 volume of 10× cell lysis buffer (1× lysis buffer is 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 20 mM $MgCl_2$, and 0.5% Nonidet P-40 in PBS). The cells were resuspended in an additional 100 μl of ice-cold medium and repelleted to wash off unattached virus, and this 100-μl wash was added to the first supernatant. The cell pellet was then resuspended in a final volume of medium identical to the total volume of the supernatants, and the cells were transferred to scintillation vials with lysis buffer. After incubation overnight at room temperature to allow cell lysis, 6 ml of Aquasol (DuPont) was added to each vial, and the vials were left in the dark for 1 h to reduce chemiluminescence before scintillation counting.

All binding assay points were determined in duplicate tubes. Nonspecific binding of transformant cell lines was determined from control tubes containing L cells, which do not express Pvr, and nonspecific binding of HeLa cells was determined from control tubes containing 711C hybridoma supernatant in addition to HeLa cells and virus. HeLa cells for nonspecific binding estimates were pelleted and resuspended in undiluted 711C supernatant prior to the binding period. Virus concentrations from 1.0 pM to 1.3 nM were used for each cell line.

Pilot experiments were performed to determine the appropriate incubation time and cell concentration for each cell line. To permit single-variable analysis of binding data, cell concentrations were adjusted to give less than 10% specific binding of input virus at the calculated dissociation constant (K_d) (7). K_d values were determined from saturation binding experiments by nonlinear regression analysis with Inplot (GraphPad, San Diego, Calif.). Because poliovirus binding is virtually irreversible under the conditions used, only an apparent K_d can be calculated.

Poliovirus alteration assay. Radiolabeled poliovirus strain P1/Mahoney was prebound to cells at room temperature for

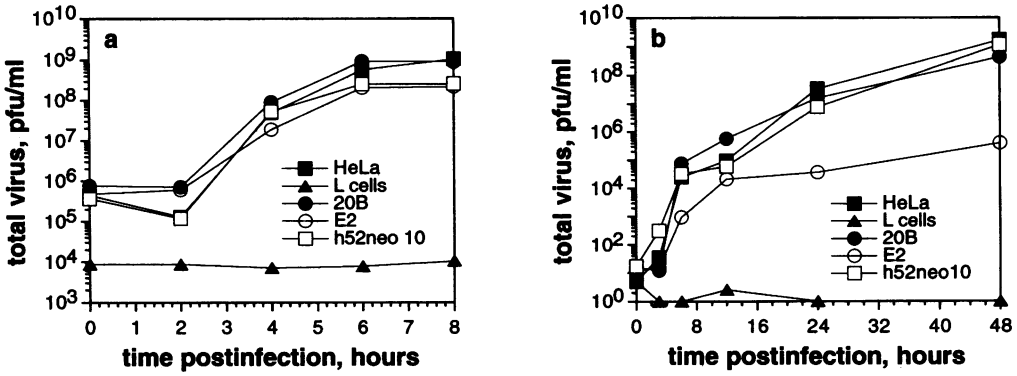

FIG. 1. Poliovirus growth curves in whole-domain recombinant and control cell lines. Confluent monolayers of the indicated cell lines were infected with poliovirus strain P1/Mahoney at a MOI of 20 (a) or 0.001 (b), and the total virus titer was determined by plaque assay at different times postinfection.

time periods ranging from 2 h to overnight. Cells and bound virus were pelleted and washed as described above for the binding assays. Pellets with bound virus were incubated at 37°C for 15 min, and then 150 μl of prewarmed binding medium was added to each pellet. Alteration was allowed to occur during a 2-h incubation with rotation at 37°C. Cells were lysed by the addition of 1/10 volume of cell lysis buffer containing Nonidet P-40, followed by a 10-min incubation on ice, and cell debris was removed by centrifugation at maximum speed for 5 min. Lysis and debris removal were repeated. The combined supernatants were centrifuged through 15 to 30% sucrose gradients at 40,000 rpm and 10°C for 75 min in an SW41 rotor. Fractions (11 drops) were collected from the bottom of the tubes; 6 ml of Aquasol was added to each fraction, and the vials were left in the dark for 1 h before scintillation counting. Virus sedimentation markers were prepared by mixing unheated sucrose gradient-purified P1/Mahoney (160S) with purified P1/Mahoney that had been heated at 56°C for 10 min (80S).

Molecular modeling and computer graphics. Domain 1 of Pvr was modeled using the Homology module of the Insight II program (Biosym Technologies, San Diego, Calif.) on a Silicon Graphics Iris R4000-50 VGX workstation. Members of the Ig superfamily for which high-resolution structures are known were screened for their sequence similarity with Pvr, and redundant sequences were eliminated. Eight fragments with known structures were initially chosen, the maximum allowed by the program. These models were the light-chain variable domains of pdb2rhe (15), pdb1rei (13), pdb1mcp (48), pdb2fb4 (30), pdb3fab (38) and the heavy-chain variable domains of pdb1mcp, pdb3fab, and domain 1 of pdb1cd8 (26). With the exception of the CD8 coordinates which were obtained directly from W. Hendrickson, all coordinates were obtained from the Brookhaven Protein Data Bank (2). The Pvr and CD4 sequences were aligned with the rest of the sequences, but CD4 was not used as one of the modeling structures because of its low sequence similarity with Pvr.

The sequences of Pvr and the models were initially aligned by the method of Rossmann and Argos (43). This alignment was further refined by using structural information, with priority given to the alignment of loop and β sheet regions and gaps and insertions being placed in loop regions. After the alignment, the backbones of the models were superimposed to the best possible least-squares fit, taking particular care to superimpose the most highly conserved parts of the structures.

Next, the segments of the Pvr sequence believed to lie within structurally conserved regions (mostly the β sheets of the β barrel) were assigned the coordinates of the corresponding sequence in the model with the highest sequence similarity. In this manner, a somewhat generic β barrel was constructed.

Loop regions were built by searching a data base of loops from the Brookhaven Protein Data Bank (2). The algorithm for loop selection considers the alpha carbon distance between the anchor residues and the number of residues in the loop, with the program providing the 10 best loops in the data base on the basis of these criteria. The potential loops were screened for their plausibility, and generally, extended loops were chosen to prevent unfavorable Van der Waals contacts with other parts of the structure. Once all the coordinates were assigned, the loop and anchor regions were refined by using the conjugate gradient minimization option of the Discover module of the Insight II program (Biosym Technologies).

RESULTS

Pvr domain 1 is not sufficient for wild-type Pvr function. To identify Pvr domains important for poliovirus infection, we previously constructed Pvr-Mph chimeras (36). Replacement of the first Ig-like domain of Mph with the domain from Pvr was sufficient to convert Mph into an active poliovirus receptor in a transient expression system. When stable cell line E2, expressing the domain 1 chimera, was infected with poliovirus at a low MOI, the virus yield throughout the growth cycle was significantly lower than in cells expressing wild-type Pvr, such as HeLa or 20B (Fig. 1b). Viral replication was normal in h52neo10 cells, which express a two-domain recombinant. At a high MOI, the defect in viral growth in E2 cells was not evident (Fig. 1a). These results indicate that Pvr domain 1 alone is sufficient to convert Mph into a functional poliovirus receptor, but cells expressing the chimeric receptor do not support replication of poliovirus to levels observed in cells expressing Pvr.

To determine whether the defect in poliovirus replication in E2 cells was caused by reduced binding affinity of the chimeric receptor, saturation virus binding experiments were conducted. The apparent binding affinities of poliovirus for cells expressing the E2 and h52 chimeric receptors are identical to those for control 20B cells (Table 2) and HeLa cells (10). The defect in virus growth in E2 cells is therefore not due to a deficiency in virus binding; it may reflect an inefficiency of the E2 cell receptor in mediating subsequent steps in cell entry and uncoating.

The Pvr domain 1 recombinant is defective in viral alter-

TABLE 2. Saturation binding properties of selected cell lines

Cell line	K_d[a] (pM)
20B	83 ± 31 (n = 4)
20B-4[b]	56
E2	92
h52neo10	75
a22-10	44
h21-11	36

[a] Cells were incubated with radiolabeled P1/Mahoney virus as described in Materials and Methods. Apparent K_d values were calculated by nonlinear regression analysis performed with Inplot. All K_d values fall within the 95% confidence interval of the range of K_ds for the 20B cell line (control).

[b] 20B-4 cells were included as a control in this experiment because, under the cell concentration conditions needed to produce adequate virus binding to the a and h mutant lines, the 20B line bound too high a fraction of virus to enable a proper K_d determination.

ation. To test the hypothesis that the E2 cell chimeric receptor is defective in steps of the virus life cycle after binding, we performed virus alteration assays with cell lines expressing domain recombinants. Under conditions that allow full alteration of virus bound to control HeLa or 20B cells expressing wild-type Pvr (Fig. 2a), the E2 cells exhibit an alteration defect (Fig. 2b). In contrast, h52neo10 cells, which express the two-domain recombinant, have no alteration defect (Fig. 2c). Pvr domain 2 is therefore involved in the process of virus alteration.

Isolation of cell lines expressing mutant Pvr. To identify Pvr sequences within the first Ig-like domain critical for poliovirus binding and infection, single or multiple amino acids in Pvr were substituted with amino acids from the Mph sequence. Amino acid alignments between Pvr, Mph, human CD4, and murine CD4 were used to identify potential loops and β-strands that differ between Mph and Pvr, and 10 different Pvr mutants were constructed (Fig. 3).

The mutated Pvr cDNAs were used to establish stable L-cell lines as described in Materials and Methods. Expressing cell lines were obtained for all mutated Pvr cDNAs except that carrying the b mutation, despite three independent transformations with six different b mutant plasmids.

Quantitation of Pvr surface level by FACS. To permit meaningful comparison of the effects of Pvr mutations on virus binding, it was necessary to determine the relative levels of Pvr protein on the cell surface. FACS analysis was used to determine Pvr surface levels, using two different anti-Pvr monoclonal antibodies. In general, mutant Pvr transformants did not express as much Pvr on their surfaces as HeLa cells did (see Fig. 5). There was significant variation in Pvr cell surface expression on all of the cells used in this study, as much as 50% from day-to-day variation. This variability was not due to variable staining of the cells with the hybridoma supernatants, as the supernatant/cell ratios used for FACS staining were shown to be capable of completely blocking the binding of radiolabeled poliovirus to the cells (35). The source of the variation is not known, but the apparent inaccuracy of any single surface level measurement was reduced by performing multiple FACS determinations for each cell line.

For most of the wild-type or mutant Pvr proteins, the ratio of the absolute values of FACS signals with 711C to those with 55D was close to 2.8:1. For f mutant-expressing cells, however, this ratio was almost reversed: 55D consistently gave brighter staining than 711C. The f mutation may change the accessibility of one or both epitopes, and therefore estimates of f mutant surface levels may not be accurate.

Poliovirus replication in Pvr mutant cell lines. To determine

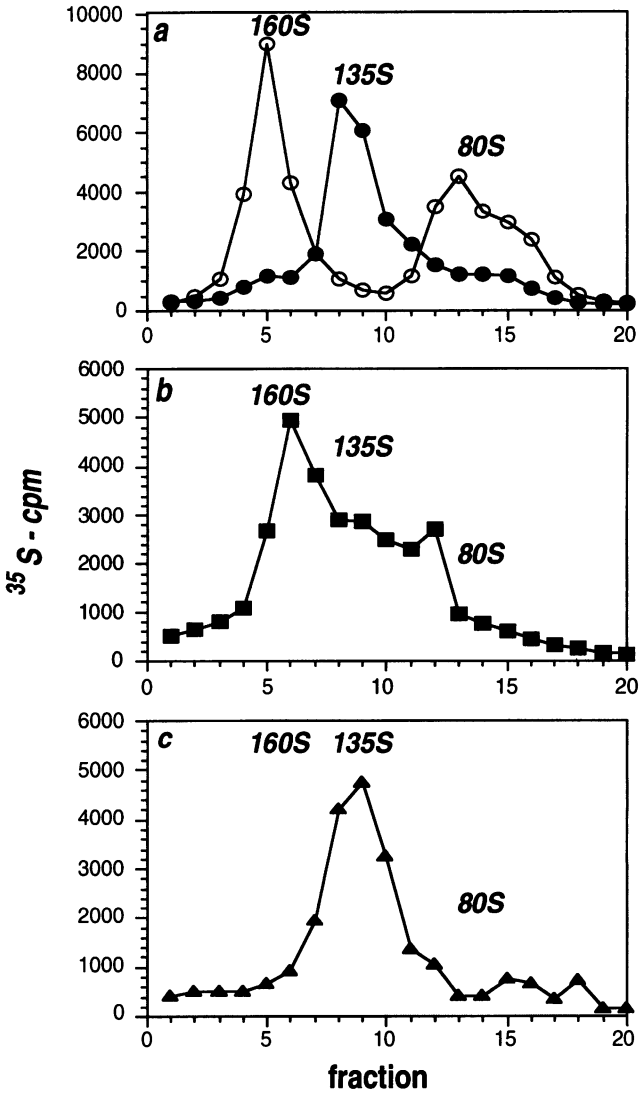

FIG. 2. Poliovirus alteration assays with whole-domain recombinant and control cell lines. Radiolabeled poliovirus strain P1/Mahoney was incubated with the indicated cell lines, and extracts were prepared as described in Materials and Methods. Lysates were overlaid on sucrose gradients; after centrifugation, gradients were fractionated from the bottom. (a) Virus sedimentation markers (open circles) and virus incubated with 20B cells (closed circles); (b) virus incubated with E2 cells; (c) virus incubated with h52neo10 cells. The positions of the 160S, 135S, and 80S peaks are indicated.

the effect of each Pvr mutation on the replication of poliovirus, the time course of virus production at a high MOI was determined (Fig. 4a to d). L cells, the parent line for all of the wild-type and mutant Pvr transformants, are not permissive for poliovirus, while all of the wild-type Pvr transformants give virus yields similar to that for HeLa cells (Fig. 4a). The growth curve in 20B cells, with 102% of the HeLa Pvr surface level, is similar to that in 20B-21 cells, with only 15% of HeLa surface levels (twofold differences in virus yield are not significant in these experiments). These results indicate that poliovirus replication is not affected by a range of wild-type Pvr expression from 15 to 102% of HeLa surface level.

The growth curves for poliovirus at a high MOI in cell lines

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                               CDR1                    CDR2
        A     A'         B                 C         C'       C"
Mph..DVRVRVLPEVRGRLGGTVELPCHLLPPTTE..RVSQVTWQRL.DGTVVAAFHPSFGVDFP
Pvr..DVVVQAPTQVPGFLGDSVTLPCYLQVPNMEVTHVSQLTWARHGESGSMAVFHQTQG...P
                             ** ******                  * * ***
                              a    b                    c d  g

                                               CDR3
     ___           D           E             F        G
Mph..NSQFSKDRLSFVRARPETNADLRDATLAFRGLRVEDEGNYTCEFATFPNGTRRGVTWLRVI
Pvr..SYSESK.RLEFVAAR..LGAELRNASLRMFGLRVEDEGNYTCLFVTFPQGSRSVDIWLRVL
     ***            ****                       * *   *
      j               i                        e h   f
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FIG. 3. Amino acid alignment of Pvr and Mph. Regions of putative β-strands are indicated above the amino acid sequence by the bars labeled A through G. Gaps in the alignments are indicated by dots between amino acids. The locations of loop structures homologous to Ig CDRs are labeled CDR1, CDR2, and CDR3. The positions of homolog-scanning mutants are indicated by asterisks below the amino acid alignment, and the names of mutants are given in lowercase letters below the asterisk(s).

expressing mutant Pvr are shown in Fig. 4b to e. Poliovirus replication in a, c, e, f, and h cells resembles that in 20B cells (Fig. 4b and d). Early virus yields in the c43-8 cell line are lower than the 20B control, which may be a result of the low surface level of Pvr in c43-8 cells (9% of HeLa level). Another c line, c43-15, expresses 34% of HeLa Pvr level, and its growth curve is normal. The d, g, and i cell lines are resistant to poliovirus infection (Fig. 4c). The f cell lines f23-5 and f61-2 also show slightly reduced early yields of poliovirus. Surface levels of Pvr in these f cells are estimated to be 17 to 19%, but as discussed above, these values must be interpreted with caution because of the possible effects of the f mutation on the epitopes recognized by the antibodies used to detect surface expression. The growth curve of poliovirus at a high MOI in j15-9 cells was different from the others (Fig. 4e). These cells produce 100-fold-lower virus yields at 0 and 2 h than that of the

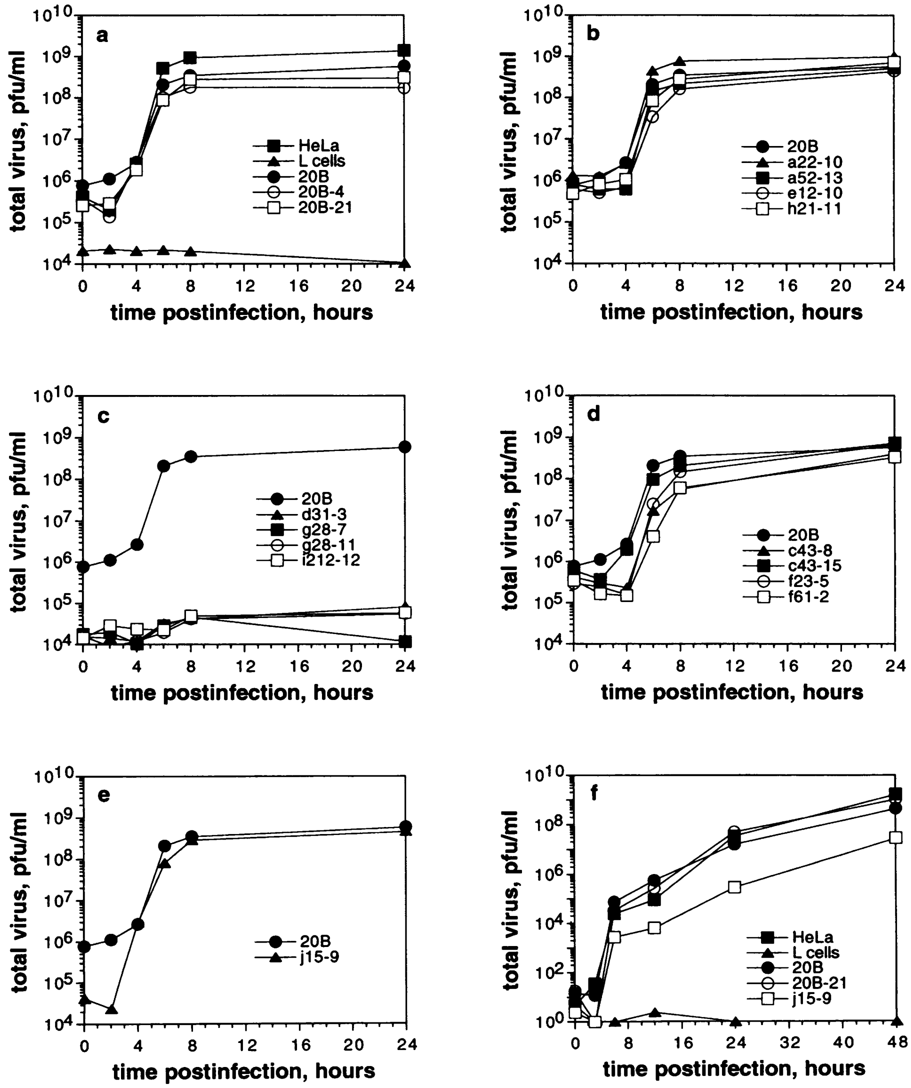

FIG. 4. Poliovirus growth curves on cell lines expressing the Pvr homolog-scanning mutants. Confluent monolayers of the indicated cell lines were infected with P1/Mahoney at a MOI of 20 (a to e) or 0.001 (f), and total virus titer was determined by plaque assay at different times postinfection. All growth curves of infections with similar MOIs were performed in one experiment, but the data are shown in independent graphs for clarity. (a) Growth curves of control cell lines expressing no Pvr (L cells) or wild-type Pvr (HeLa, 20B, 20B-4, and 20B-21 lines); (b) growth curves of 20B control and a, e, and h cell lines; (c) growth curves of 20B control and d, g, and i cell lines; (d) growth curves of 20B control and c and f cell lines; (e) growth curves of 20B control and j cell lines; (f) low-MOI growth curves of HeLa, L-cell, 20B control, and j cell lines.

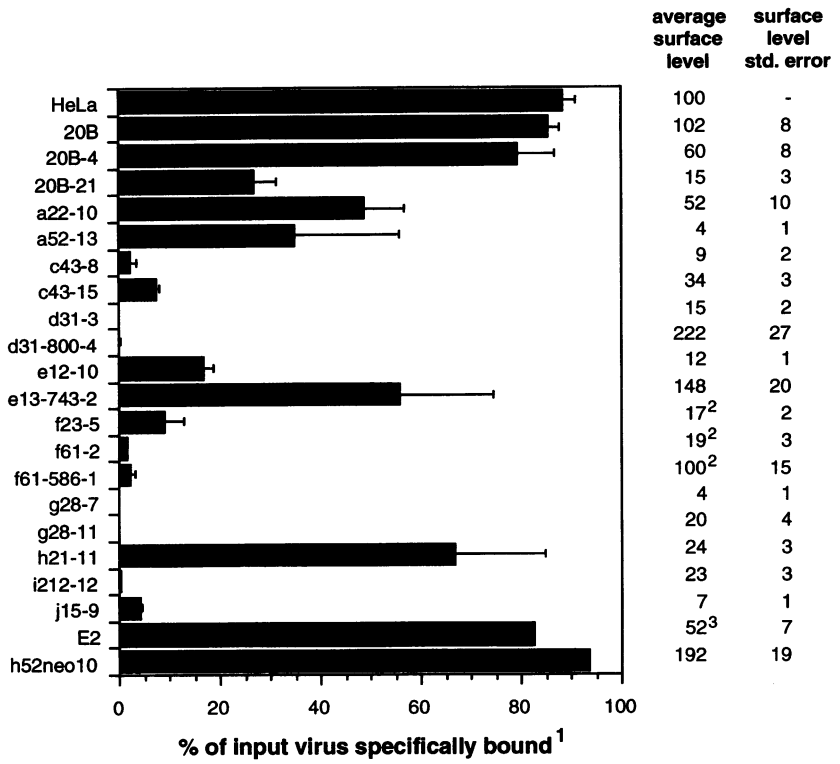

FIG. 5. Radiolabeled poliovirus binding and Pvr surface level expression at high cell concentrations. A total of 1×10^7 cells were mixed with 8×10^{10} particles of radiolabeled P1/Mahoney in a total volume of 0.5 ml and processed as described in Materials and Methods. Specific binding is expressed as a percentage of the total input virus, and the standard error of binding is indicated by the error bars. Surface levels of Pvr and mutant proteins were determined as described in Materials and Methods. The average and standard (std.) error of determinations with 711C and 55D monoclonal antibodies are indicated to the right of the binding graph. Footnote 1, specific binding for HeLa cells was 87 to 95% of the total input counts per minute; nonspecific binding was 1 to 2% of the input counts per minute whether determined by 711C blocking or by virus binding to L cells. Footnote 2, Pvr surface level for f cells is given as determined with monoclonal antibody 55D. Footnote 3, Pvr surface level for E2 cells is given as determined with monoclonal antibody 711C.

20B control, but by 4 h, the virus yield is identical to that of the control. The j15-9 cells also give lower virus yields in low-MOI infection (Fig. 4f): even at 12 and 24 h, virus production is 100-fold lower than in control cells.

To ensure that the Pvr-expressing cell lines did not contain internal blocks to poliovirus replication after uncoating, all of the cell lines were transfected with infectious poliovirus RNA transcripts, and 20 h later, the culture supernatants were sampled, and yields of infectious virus were determined by plaque assay on HeLa cell monolayers. All of the cell lines described in this work produced similar virus yields after transfection with RNA, indicating that there were no internal blocks to poliovirus infection (data not shown).

Poliovirus binding to cells expressing mutant Pvr. To determine the effects of Pvr mutations on virus binding, the binding of radiolabeled P1/Mahoney to wild-type and mutant cell lines was examined. Because poliovirus undergoes a conformational transition when it interacts with Pvr at 37°C, resulting in the release of altered particles into the medium, it is necessary to perform poliovirus binding assays at temperatures below 33°C, which prevents alteration. Binding assays were done at 4°C to minimize effects of endocytosis. At this temperature, virus binding to cells is virtually irreversible, and an estimation of off rates is not possible (10). Our experiments were therefore conducted under steady-state conditions.

It was not possible to determine a dissociation constant (K_d) for each cell line, because the cell concentration needed to obtain 5 to 10% binding of input virus was in excess of 2.5×10^8 cells per ml. Initially, only the a and h mutant-expressing cells bound enough virus at a reasonable cell concentration to allow determination of K_d and direct comparison with the K_d values for control cells (Table 2). The K_ds for mutant Pvr lines a22-10 and h21-11 and for 20B-4 cells were similar.

Because it was not possible to determine the apparent K_ds for all of the remaining cell lines, the ability of these lines to bind type 1 poliovirus was assessed at a single-cell concentration of 2×10^7 cells per ml (Fig. 5). HeLa cells and 20B cells, which express 102% of the HeLa Pvr surface level, bind approximately the same amount of poliovirus, as do 20B-4 cells, which express 60% of HeLa Pvr surface level. 20B-21 cells, which express 15% of HeLa Pvr surface level, bind only 30% as much virus. The a and h mutations do not impair virus binding, since cells expressing the mutant receptors exhibit poliovirus-binding levels that are similar to those of HeLa and 20B cells. The e mutation probably does not impair virus binding, since the e12-10 cell line, which expresses 12% of HeLa cell Pvr, bound nearly as much poliovirus as the 20B-21 cell line did, and the e13-743-2 cell line, with 148% of HeLa cell Pvr level, had virus-binding levels similar to those of the HeLa and 20B controls. Specific poliovirus binding to the d, g, and i cell lines was not detected, despite reasonable levels of Pvr surface expression. These cell lines do not support virus replication (Fig. 4c). The c, f, and j cells appear to have a less severe defect in virus binding. The c43-15 line binds 8% of

TABLE 3. Poliovirus can exit cells without causing CPE

Cell line	Virus titer[a] (PFU/ml)	CPE[b]
L (Pvr$^-$)	1.05×10^3	–
HeLa	1.50×10^8	+++
20B (wild-type Pvr)	2.97×10^8	+++
Mutants		
a22-10	2.75×10^8	+++
c14-6	4.10×10^7	–
c43-8	6.60×10^7	–
j15-9	2.00×10^8	–
j15-11	1.65×10^8	–

[a] Monolayers of cells expressing wild-type or mutant Pvr molecules were infected with poliovirus P1/Mahoney at an MOI of 10. Samples of supernatant were removed at 48 h postinfection, and contaminating cells were removed by centrifugation. Virus titers were determined by plaque assay on HeLa cells.

[b] All cell lines were observed by light microscopy for CPE. –, no CPE (all cells survived); +++, 100% CPE (all cells destroyed).

input virus despite expressing 34% of the HeLa Pvr surface level. The f23-5 cells bind 9% of input virus and express 17% of the HeLa Pvr surface level, while the f61-586-1 cells bind 2% of input virus and express 100% of HeLa Pvr level. Because the f mutation appears to affect recognition by anti-Pvr monoclonal antibodies, determination of cell surface levels on f cell lines may not be accurate. The j15-9 cell line binds poliovirus poorly, although its surface level is very low, 7% of the Pvr level on HeLa cells. It is therefore difficult to determine conclusively whether this mutation interferes with poliovirus binding.

Mutations in Pvr affect the development of cytopathic effect. During the course of the growth curve experiments and in other, longer-term infection experiments, all cell lines were observed microscopically for the occurrence of CPE. In infections with poliovirus P1/Mahoney at a high MOI, all of the cell lines expressing wild-type Pvr developed CPE by 24 h postinfection, and almost all of the mutant Pvr-expressing lines developed CPE as well. CPE was delayed in lines f23-5 and f61-2, reaching completion only after 48 to 72 h postinfection. CPE did not occur in certain lines, even at 48 h postinfection, despite virus yields in the cell culture supernatant that were in some cases similar to those obtained in HeLa cells (Table 3). These results suggest that poliovirus can be released from cells without apparent CPE.

Pvr model structure. Homology-based model building was used to predict Pvr domain 1 structure (Fig. 6). This provides a convenient three-dimensional framework for interpreting the structural consequences of mutations, which cannot be provided by a sequence alignment alone. Further, sequence and structure alignments of multiple Ig superfamily members better exploit what is known about this well-studied, highly conserved structural motif. For sequences with limited similarity (Pvr and CD4 have 22.3% conserved residues, of which 17.8% are conserved hydrophobic residues), this method of alignment allows greater accuracy than traditional sequence alignments.

The placement of the sheets and loops is the most reliable, with the greatest uncertainty at the two or three residues at the interfaces of these elements. Further, because this model was not the basis for the design of mutagenesis experiments, it provides independent support for the validity of comparing the viral receptors Pvr and CD4. Such a model, however, should not be considered a substitute for a high-resolution structure, and it should be noted that the positions of the main chains of large loops and of all nonconserved side chains are only hypothetical.

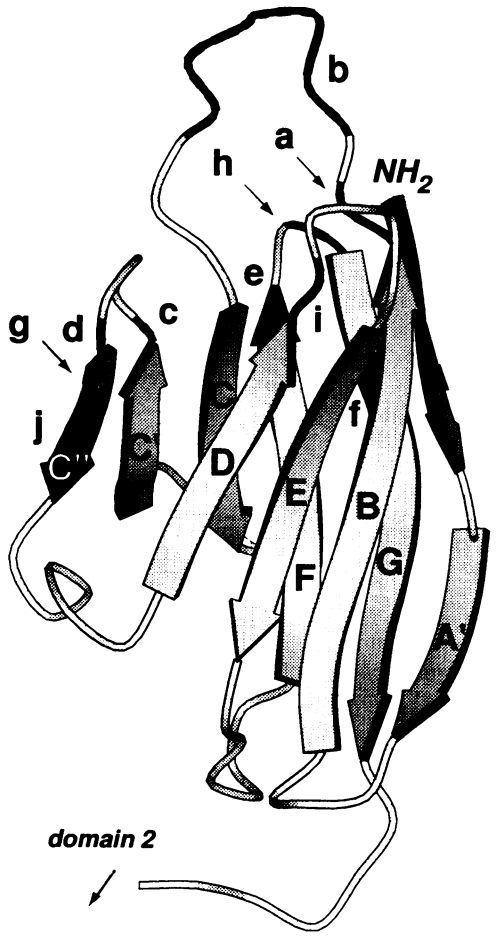

FIG. 6. Structural model of domain 1 of Pvr. The structure of domain 1 was predicted as described in Materials and Methods. The ribbon diagram was constructed with Molscript, and the labels were added with Adobe Illustrator. The predicted structural locations of the mutations are shown in black and labeled with lowercase letters. The β-strands are labeled in capital letters.

DISCUSSION

Results presented here and in our previous work demonstrate that replacement of domain 1 of Mph with domain 1 of Pvr is sufficient to confer poliovirus receptor activity on the Mph protein (36). However, E2 cells, which express this chimeric protein, do not produce poliovirus with the same efficiency as 20B cells, which express Pvr. Poliovirus growth appears normal in cells expressing a chimeric receptor in which domains 1 and 2 of Mph are replaced with the domains from Pvr, suggesting that both Pvr domains 1 and 2 are necessary for full poliovirus receptor activity. The importance of the second Ig-like domain during infection has also been demonstrated for other virus receptors of the Ig family, such as CD4 (9) and ICAM-1 (52, 53).

What role might Pvr domain 2 play in poliovirus infection? Saturation binding studies reveal that the apparent K_d of poliovirus for E2 cells, which express Mph with domain 1 from Pvr, is not different from that of Pvr. However, because the

determinations of K_d were performed at 4°C, a temperature at which binding is virtually irreversible, a true equilibrium has not been achieved, and therefore K_d is a measure only of the association rate under steady-state conditions (33). The dissociation rates of poliovirus in E2 cells and HeLa cells may be different, which may account for the replication defect of poliovirus in E2 cells. Alternatively, Pvr domain 2 may regulate the conformational transitions of the virion that occur when poliovirus interacts with Pvr (14, 20). The finding that E2 cells are defective in virus alteration while h52neo10 cells are not supports this hypothesis. Involvement of virus receptors in events after binding has been demonstrated for CD4, the receptor for HIV-1. A monoclonal antibody directed against CD4 domain 2 is capable of blocking HIV infection at a step after initial gp120-CD4 binding (6, 34).

The close sequence homology of Mph and Pvr enabled the identification of some Pvr residues important for virus interaction by substitution of Mph sequences into Pvr. Mutations a, e, and h had no effect on virus binding and minimal effect on the ability of cells expressing the mutant receptors to support poliovirus replication. Mutations d, g, and i abolished both virus binding and virus replication. The c mutation reduced but did not eliminate binding, but this reduction did not alter replication in c cells. The effect of the f mutation on virus binding was difficult to interpret, but this mutation clearly had little effect on virus replication in f cells. The j mutation reduced virus binding and virus replication.

To determine the approximate location of these mutations in Pvr domain 1, a structure model was constructed on the basis of homologies with known structures of the Ig superfamily (Fig. 6). Mutations d and g, which abolish virus binding, are located on the C″ strand, on one side of domain 1. Mutation c, which had a less severe effect on binding, is located in the C′-C″ strand. Mutation j, located on the C″-D loop, reduced virus binding and replication. Mutations a, e, and h, which did not alter binding or replication, are located on the other side of domain 1. These results suggest that at least the C″ ridge is an important component of the binding site for poliovirus. The binding site of HIV-1 on CD4 has been localized to the C′-C″-D strands and the loops between them, which contain most of the amino acid residues necessary for HIV gp120 binding (49). Amino acid 43 of CD4 is particularly crucial for HIV-1 binding, and certain amino acid substitutions at this position abolish interaction with gp120 (3, 33). A single amino acid change at the homologous residue in Pvr, Q-55→F, also abolishes poliovirus binding. The bulkier, hydrophobic side chain of Phe may interfere with contact between poliovirus and Pvr. The homologous residue in CD4 is believed to play a major role in interaction with gp120. Poliovirus and HIV therefore appear to bind spatially similar faces of their Ig V-like receptors. Whether other regions of Pvr are involved in poliovirus binding cannot be determined from this work, because homolog-scanning mutagenesis does not evaluate the contribution of conserved residues. Additional mutagenesis will be required to identify other regions of Pvr involved in virus binding and entry. Ultimately, high-resolution structure of the virus-receptor complex will be required for a thorough understanding of the phenotypes of Pvr mutants.

The j cell line exhibits a novel profile during the eclipse period of high-MOI infection, producing reduced virus titers at 0 and 2 h postinfection and wild-type levels by 4 h. Results of radiolabeled virus binding indicate that the j mutation decreases virus binding, providing one possible explanation for the early alteration in its growth profile. However, it is not clear how this initial defect is overcome in high-MOI infections. One possible explanation is that only a small number of virions need to enter the cell to result in normal virus yields by 4 h. The fact that the eclipse period becomes protracted in infections with very low MOIs argues against this explanation. Another possibility is that the j mutant allows for accelerated conformational transitions of the poliovirus virion during the process of cell entry, a phenotype which would be consistent with the low virus titer at the 0 h time point. This hypothesis is currently being tested by assessing the kinetics of poliovirus alteration induced by j cells.

The finding that cells expressing Pvr containing the c or j mutations are apparently not lysed by poliovirus infection has two possible explanations. First, not all the c or j cells may be infected, even at high MOI, which would lead to an apparent absence of cell killing. This explanation seems unlikely, given the high yields of poliovirus in some mutant cell lines, but the results of infectious centers assays should address this possibility. A more interesting alternative is that poliovirus-receptor interactions control the course of cell killing and the c and j mutations in some way disrupt this activity. Binding of poliovirus to Pvr might initiate a signaling cascade that ultimately leads to cell death. In a similar manner, separate cross-linking of CD4 and the T-cell receptor at the cell surface leads to cell death by apoptosis (37). Alternatively, interaction of poliovirus with receptor molecules within the cell might provide signals that induce cell death. The CD4-gp120 system provides a precedent for such an interaction. HIV gp120 syncytium induction can be prevented in vitro by intracellular expression of CD4 molecules that have been modified to contain a signal for retention in the endoplasmic reticulum (4, 5). The retained CD4 molecules "trap" newly synthesized gp120 molecules and rescue the cell from syncytium formation. Perhaps the c and j Pvr mutants within the cell are responsible for abrogation of cytopathic effect.

It has generally been believed that cell lysis is an event triggered late in poliovirus infection to aid in release and spread of progeny virions (42). However, several otherwise lytic viruses have been shown to be released from cells in the absence of cell killing. Infection of human K562 cells by poliovirus does not result in CPE, although virus is released from infected cells (28). Simian virus 40 is released from several cell types long before cell lysis occurs (8), and minute virus of mice can be released from cells without cell lysis (46). Precisely why these viruses do not damage their hosts, and how they escape infected cells, remains to be elucidated.

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