# Expression of Mutated Poliovirus Receptors in Human Neuroblastoma Cells Persistently Infected with Poliovirus

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Poliovirus (PV) is able to establish persistent infections in human neuroblastoma IMR-32 cells [Colbère-Garapin *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86, 7590]. During persistent infection, PV mutants are selected that display substitutions of residues in regions of the capsid known to interact with the PV receptor (PVR), a glycoprotein of the immunoglobulin superfamily. The mechanism of persistent infection in IMR-32 cells may therefore involve the selection of mutant PVRs. To test this hypothesis, the sequences of the PVR mRNAs in uninfected IMR-32 cells and in two independent IMR-32 cell cultures persistently infected with the Mahoney strain of PV type 1 (PV1/Mahoney) were determined. The PVR mRNA population of uninfected cells was homogeneous, and no mutation was repeatedly found, whereas that of persistently infected cells displayed missense mutations. Particular mutations were repeatedly detected, and all of them mapped to the N-terminal domain of PVR (domain 1), which interacts directly with PV. These mutations generated several types of PVR variants with the following substitutions: Ala67→Thr alone, Ala67→Thr associated with Gly39→Ser, and Arg104→Gln. Functional analysis of PVR in murine LM cells, stably expressing each of the PVR forms, showed that the PVR forms selected during persistent infection conferred on LM cells partial resistance to PV1/Mahoney-induced lysis. Although adsorption onto PVR seemed to be independent of the PVR form, an analysis of the conformational changes of the capsid during the early steps of the PV cycle provided evidence that the Ser39/Thr67 and Gln104 substitutions almost halved the conversion of 160S infectious particles into 135S A particles associated with the PV–PVR interaction. Altogether, these findings indicate that during persistent infection, specific mutations were selected in the domain 1 of PVR and that these mutations increased the resistance of cells to PV-induced lysis. These results are discussed in view of the position of the mutations on PVR.   © 2000 Academic Press

## INTRODUCTION

An investigation of persistent infections in cell cultures leads to a better understanding of virus–cell interactions and improves our knowledge of the molecular factors involved in the establishment and maintenance of these infections. Many cytolytic RNA viruses are able to infect cultured cells persistently. Studies of the mechanisms of these models of viral infection have shown that there is frequently a coevolution of mutant cells with higher resistance to viral infection and of virus variants with increased virulence. In models of persistent infection with reovirus, murine hepatitis virus (MHV), foot-and-mouth disease virus (FMDV), and murine encephalomyocarditis, coevolution of cell and virus involves steps in the process of viral entry (Baer *et al.,* 1999; Chen and Baric, 1996; Dermody *et al.,* 1993; Martin-Hernandez *et al.,* 1994; Pardoe *et al.,* 1990; Sawicki *et al.,* 1995). We have devel-

oped several models of persistent infections with poliovirus (PV), in particular, in neuroblastoma IMR-32 cells, human cells of neuronal origin, and primary cultures of human fetal brain cells (Colbère-Garapin *et al.,* 1989; Pavio *et al.,* 1996), but the mechanisms by which these persistent infections occur are not yet known.

Studies of persistent infection with PV, a member of the Picornaviridae family, are facilitated by its structure and its multiplication cycle being well characterized (Wimmer *et al.,* 1993). This small virus has a single-stranded RNA genome of positive polarity, enclosed in an icosahedral capsid composed of 60 copies of each of the four viral proteins (VP1–4). The atomic structure of the Mahoney strain of PV type 1 (PV1/Mahoney) has been determined (Filman *et al.,* 1989; Hogle *et al.,* 1985): the major capsid proteins VP1, VP2, and VP3 share a similar structure constituted of an eight-stranded β-barrel core flanked by terminal extensions. The N-terminal extensions, with the small internal protein VP4, create a network on the internal surface of the capsid, and this network probably stabilizes the native virion. Surrounding each of the fivefold axes of symmetry, formed by five copies of the capsid protein VP1, is a deep cleft, called the canyon. The canyon contains the site for PV receptor

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(PVR) binding (Belnap *et al.,* 2000b; Colston and Racaniello, 1994, 1995; He *et al.,* 2000; Xing *et al.,* 2000). On binding to PVR, the PV capsid undergoes a conformational alteration that reduces its sedimentation coefficient from 160S to 135S (Lonberg-Holm *et al.,* 1975). The altered 135S particles, named A particles, have externalized VP4 and the N-terminus of VP1 (Fricks and Hogle, 1990). They are representative of the conformational transitions of the capsid induced by the PVR, although their role in viral uncoating is still debated (Arita *et al.,* 1998; Belnap *et al.,* 2000a; Dove and Racaniello, 1997).

Most reference PV strains, including PV1/Mahoney, are capable of establishing persistent infections in human neuroblastoma IMR-32 cells, whereas the same strains are fully lytic in human epidermoid HEp-2 cells (Colbère-Garapin *et al.,* 1989). This suggests that the cell phenotype plays a crucial role in persistent infection. Furthermore, IMR-32 cells cured of persistent infection are more resistant to PV infection than the original IMR-32 cell line and do not display cytopathic effect (CPE) on secondary infection (our unpublished results), suggesting that during persistent infection, cell variants are selected. Viral variants (PVpi) with novel phenotypic properties are also selected during the persistent infection of neuronal cells (Pelletier *et al.,* 1991). In particular, PVpi exhibit a modified cell tropism. They readily establish persistent infections in the human non-neural HEp-2 cell line, in contrast to the parental reference PV strains (Pelletier *et al.,* 1991). In this model, viral and cellular determinants involved in persistence affect PV–PVR interactions (Calvez *et al.,* 1995a; Duncan *et al.,* 1998; Pelletier *et al.,* 1998).

During persistent infections of either neuroblastoma cells or primary cultures of fetal brain cells with PV1/Mahoney, mutations are selected at two positions, in particular, in capsid proteins between 2 and 6 weeks after infection: one at position 95 of VP1 (Pro→Ser) and the other at position 142 of VP2 (His→Tyr) (Calvez *et al.,* 1995b; Couderc *et al.,* 1994; Pavio *et al.,* 1996). Interestingly, these two residues were also selected in PV1/Mahoney variants adapted to grow on cells expressing mutant forms of the PVR (Colston and Racaniello, 1995). Residue 95 of VP1 is located in the B-C loop, a well-exposed sequence on the virion surface near the fivefold axis, and virions having this mutation convert to the 135S form more readily than PV1/Mahoney *in vitro* (Wien *et al.,* 1997). It was recently suggested that alterations in the B-C loop affect cell entry steps downstream from receptor binding (Belnap *et al.,* 2000b). Residue 142 of VP2 is located on the rim of the canyon in the receptor binding site (Belnap *et al.,* 2000b; He *et al.,* 2000). The His→Tyr substitution at this position has negligible effects on the structure of the virion but may modify the contact point with PVR (Colston and Racaniello, 1995; Wien *et al.,* 1997). The selection of these mutations both in persistently infected neuronal cells and in cells expressing

mutant receptors suggests that interactions between PV and its receptor play an important role in the mechanism of PV persistence in this model.

The PVR, also named CD155, is a member of the immunoglobulin superfamily of proteins (Koike *et al.,* 1990; Mendelsohn *et al.,* 1989). Its cellular function is not yet known. PVR is predicted to contain three extracellular Ig-like domains in the order V-C2-C2, followed by a transmembrane region and a cytoplasmic tail. In HeLa cells, two different bases (G and A) have been found at nucleotide position 199, resulting in an Ala or a Thr residue at amino acid position 67 in the PVR. It was suggested that this difference in PVR mRNA of HeLa cells may be due to an allelic difference (Koike *et al.,* 1990). Four isoforms are produced by alternative splicing, of which only two, PVRα and PVRδ, are functional membrane-bound PVRs (Koike *et al.,* 1990). The N-terminal V-like Ig domain (domain 1) appears to contain the binding site for PV (Aoki *et al.,* 1994; Bernhardt *et al.,* 1994; Koike *et al.,* 1991; Morrison *et al.,* 1994). The three-dimensional structure of PVR bound to PV has been recently determined by cryoelectron microscopy (Belnap *et al.,* 2000a,b; He *et al.,* 2000; Xing *et al.,* 2000). PVR domain 1 is necessary and sufficient for the early steps of the PV cycle. Although the PVR domains 2 and 3 do not directly interact with the PV particle, they seem to be important for efficient PVR function. In addition, cells expressing PVR with mutations in the C″ edge of the domain 1 do not develop CPE during PV infection, suggesting that PV-induced CPE may result from PV–PVR interactions (Morrison *et al.,* 1994). These data suggest that the mechanism of persistent infection in neuroblastoma cells may involve the selection of mutated PVRs.

To investigate this hypothesis, the sequences of PVR mRNAs in two independent neuroblastoma IMR-32 cell cultures persistently infected with PV1/Mahoney were determined. Missense mutations were repeatedly identified in the region encoding domain 1 of the PVR. Their effects on cell resistance to PV-induced lysis and on the kinetics of PV multiplication were then studied in stable murine LM cell lines expressing mutated PVR. PV binding onto these cells and the following conformational transitions of the viral capsid were also investigated. The results are discussed in view of the position of the mutations on the structure of PVR domain 1.

## RESULTS

### Selection of mutations in the mRNA of PVR during persistent infection

To investigate whether missense mutations are selected in the PVR expressed in persistently infected neuroblastoma cells, IMR-32 cells were infected with PV-1/Mahoney, and two independent cultures, called IMR/KK3 and IMR/KK5, were maintained for 2.5 months after infection. Total RNA was extracted from both persistently
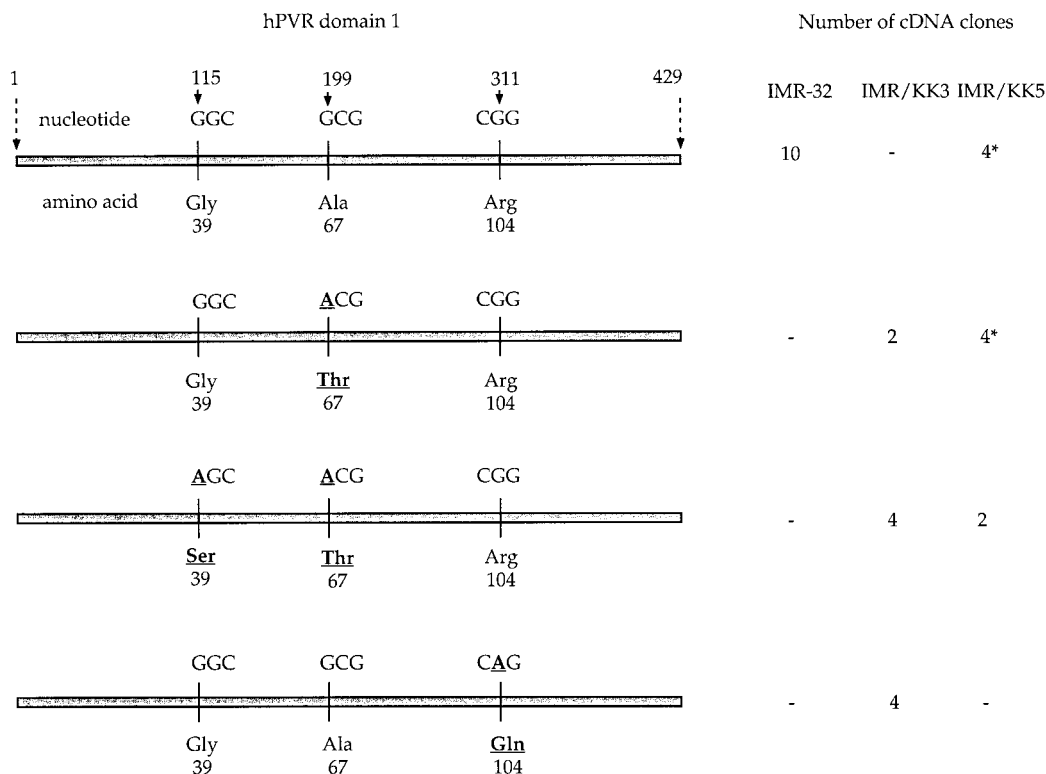
FIG. 1. Localization of missense mutations identified in domain 1 of PVR expressed in IMR-32 cell lines persistently infected with PV. Ten cDNA clones obtained after amplification by RT–PCR of PVR domain 1 mRNA from uninfected IMR-32 cells and from PV persistently infected cell lines, IMR-KK3 and IMR-KK5, were sequenced. The number of clones corresponding to each sequence is indicated. Nucleotide and amino acid substitutions are indicated in bold. ∗, Several substitutions were found in other loci than those indicated; however, each was found only in a single clone.

infected cell cultures and from uninfected IMR-32 cells. The mRNA of the PVRα isoform was amplified in three fragments by RT–PCR, with specific oligonucleotides as described under Materials and Methods. Each fragment was cloned, and the sequence of 10 clones of each was determined.

The PVR sequence expressed in IMR-32 cells at passage 13 was identical to the published sequence (Koike *et al.,* 1990; Mendelsohn *et al.,* 1989) for all of the cDNA clones covering the 5′, central, and 3′ parts of the PVR-encoding region, with the exception of one mutation at nucleotide 173 (T→C) found in only a single clone and resulting in a Val-to-Ala substitution at position 58 of the amino acid sequence (data not shown). All of the 10 clones corresponding to the first domain of PVR exhibited a G at position 199, resulting in an Ala at position 67 of the amino acid sequence (Fig. 1). Two different bases (G and A) have been reported at this nucleotide position in the published sequences (Koike *et al.,* 1990; Mendelsohn *et al.,* 1989), resulting in an Ala and a Thr residue, respectively, at position 67 in the PVR sequence. Therefore uninfected IMR-32 cells expressed only one of these allelic forms. Despite IMR-32 cells being tumor cells (Tumilowicz *et al.,* 1970), only one dominant PVR form was found. To confirm this homogeneity and because we could not clone IMR-32 cells, sequences were deter-

mined at a later passage for IMR-32 cells (passage 55). The same PVR dominant form was found, and the mutation at nucleotide position 173 was not found in this second series of 10 clones.

The comparison of PVR mRNA sequences isolated from persistently infected IMR/KK3 and IMR/KK5 cells with that from IMR-32 cells showed that PVR mRNA from persistently infected cells carried missense mutations in the region encoding domain 1 of PVR (Fig. 1). No mutations were repeatedly found in domains 2 or 3 or in the transmembrane or cytoplasmic regions of the PVR in most of the clones. One missense mutation was found in the transmembrane domain of only one clone isolated from IMR/KK3 cells, and another one was found in the cytoplasmic domain of one clone isolated from IMR/KK5 cells (data not shown).

Among the 10 clones corresponding to the first domain of the PVRs expressed in IMR/KK3, two had the substitution at nucleotide 199 that results in an Ala-to-Thr change at position 67 (Fig. 1), generating the other allelic form found in HeLa cells (Koike *et al.,* 1990; Mendelsohn *et al.,* 1989); four had two substitutions, one at nucleotide 115 causing a Gly-to-Ser change at residue 39 and the other at nucleotide 199 causing the Ala-to-Thr change at position 67; and four had a substitution at nucleotide 311 causing an Arg-to-Gln change at position 104.

The 10 clones derived from IMR/KK5 carried mutations at the same positions as those found in IMR/KK3: four clones had the substitution causing an Ala-to-Thr change at position 67, and two clones had both substitutions causing the Gly-to-Ser change at residue 39 and the Ala-to-Thr change at position 67 (Fig. 1). Several substitutions were found in other loci of PVR domain 1 of IMR/KK5 cells, but each was found in only one clone (data not shown). Because the relevance of mutations detected only once is unclear, we chose to study further only the mutations detected in several clones.

These results indicated that PVR mRNA from uninfected IMR-32 cells was homogeneous and that during persistent infections of IMR-32 cells with PV, particular mutations were expressed in the PVR mRNA. One of them corresponds to an allelic variation. Interestingly, all of these mutations were located in the region encoding domain 1 of the PVR, the domain that interacts with the PV particle.

## Isolation of murine cells transformed by the PVR cDNAs and PVR expression

To examine whether mutated PVRs expressed in persistently infected cells play a role in PV persistence, mutated PVR forms were expressed in murine LM cells that do not have the PVR gene. Mutations corresponding to the PVR forms identified in IMR/KK3 and IMR/KK5 were introduced by site-directed mutagenesis into PVR cDNA. The plasmid pSV2-PVR$\alpha$ (Koike et al., 1990), containing the cDNA encoding PVR$\alpha$ expressed in IMR-32 cells (PVR$_{IMR}$), was used to obtain three different PVRs: PVR-Thr67 with a threonine residue at position 67, PVR-Ser39/Thr67 with a serine residue at position 39 and a threonine residue at position 67, and PVR-Gln104 with a glutamine residue at position 104. All of the PVR cDNAs were used to establish stable transformant LM cell lines expressing the various PVR forms, as described under Materials and Methods. PVR-expressing cells were stained by immunofluorescence with anti-PVR antibodies and sorted by fluorescence activated cell sorting (FACS). Sorting was performed two or three times for each cell culture, until 95–99% of the sample was transformant LM cells expressing PVR. There were some differences in the level of PVR expression on the cell surface between the different cell lines, as previously observed (Morrison et al., 1994). The level of PVR expression on the cell surface of LM/PVR$_{IMR}$ was similar to that on the cell surface of the fully permissive HEp-2 cells, which are commonly used to amplify PV. The level of PVR expression on the cell surface of LM/PVR variants was between one third and two thirds of that on LM/PVR$_{IMR}$. The presence of mutations in the PVR mRNA of these cells was verified. The phenotype of LM/PVR cells, evaluated by immunofluorescence with anti-PVR antibodies and FACS analysis, was stable for 2 months. We thus obtained four
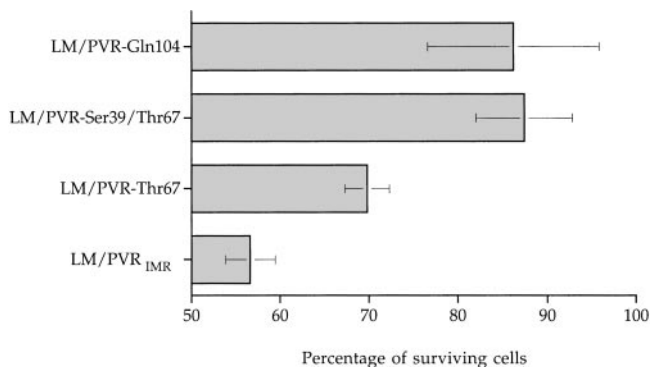


FIG. 2. Percentage of LM/PVR cells surviving after infection with PV. LM/PVR cell lines were infected at an m.o.i. of 10 TCID$_{50}$/cell with PV1/Mahoney, and the percentage of surviving adherent cells was determined after 28 h of incubation. Each value is the average of two independent experiments. The SEM values are indicated.

stable LM cell lines, each expressing a different form of PVR, called LM/PVR$_{IMR}$, LM/PVR-Thr67, LM/PVR-Ser39/Thr67, and LM/PVR-Gln104. Cell clones expressing each of the PVR forms were also isolated.

## Resistance of cells expressing mutant PVRs to PV-induced lysis

To test whether mutated PVRs affect CPE induced by PV, LM cell lines expressing PVR$_{IMR}$ or PVR variants were infected with PV-1/Mahoney. The percentage of surviving cells 28 h after infection was determined. To ensure that each cell was infected independently of the level of PVR expression on the cell surface, cell cultures were infected at a high m.o.i. [10 TCID$_{50}$ (50% tissue culture infectious doses)/cell]. As shown in Fig. 2, a larger percentage of LM/PVR-Ser39/Thr67 and LM/PVR-Gln104 cells (nearly 90%) than LM/PVR$_{IMR}$ cells (56%) survived PV-1/Mahoney infection. LM/PVR-Thr67 had an intermediate phenotype with 70% cell survival. These results were repeatedly obtained and confirmed at an m.o.i. of 0.1 TCID$_{50}$ per cell (data not shown). Similar differences were detectable up to 3 days postinfection (p.i.), after which time the infection led to the selection of a few PVR-negative cells, preventing longer experiments. Comparable results were also obtained with LM/PVR cell clones expressing the same PVR forms (data not shown).

To detect possible serotype-specific modulation of PV interactions with mutant PVRs, as previously observed (Harber et al., 1995), similar experiments were performed with the PV type 3 strain Leon (PV3/Leon). Again, as in the case of PV1/Mahoney, a larger percentage of LM/PVR-Ser39/Thr67 and LM/PVR-Gln104 cells than LM/PVR$_{IMR}$ cells survived infection (data not shown).

To test whether mutations selected in PV1/Mahoney during persistent infection of IMR-32 cells could compensate for the resistance to cell lysis conferred by mutant PVRs, cell clones expressing the various PVR
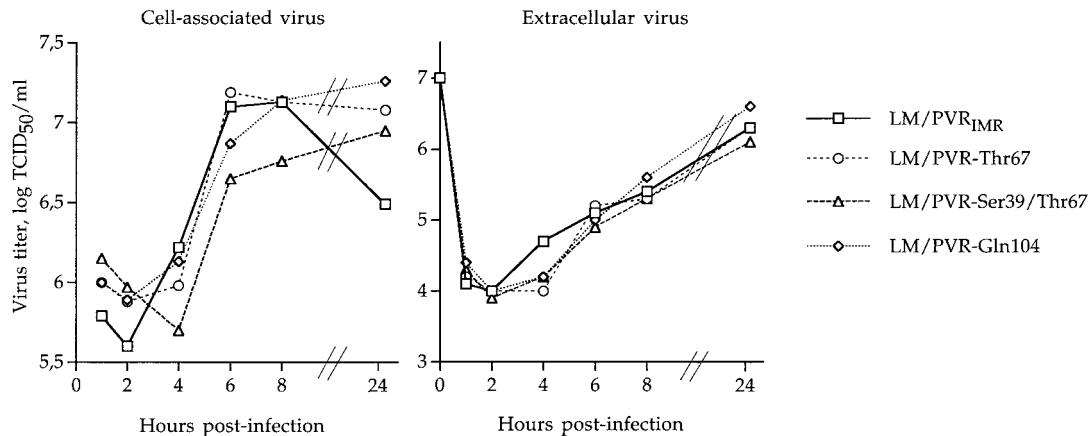
FIG. 3. Single-cycle growth curves of PV1/Mahoney in LM/PVR cell lines. Cells were infected at an m.o.i. of 10 $TCID_{50}$/cell. Cells and supernatants were harvested separately at the indicated times p.i., and then cell-associated and extracellular virus yields were determined by $TCID_{50}$ assay. Each point represents the mean of two separate experiments.

forms were infected with the PV1/Mahoney mutants having either a Pro-to-Ser substitution at position 95 of VP1 (Mah-KKVP1S$_{95}$), or a His-to-Tyr substitution at position 142 of VP2 (Mah-KKVP2Y$_{142}$) (Couderc *et al.,* 1994). The mutant Mah-KKVP1S$_{95}$ was slightly more lytic in LM/PVR$_{IMR}$ cells than the mutant Mah-KKVP2Y$_{142}$ and PV1/Mahoney. With all three viruses, cell clones expressing mutant PVRs were more resistant to lysis than LM/PVR$_{IMR}$ cells (not shown).

Therefore, expression of the mutant PVR forms present in persistently infected neuroblastoma cell cultures conferred on cells a partial resistance to PV-induced lysis.

## Poliovirus multiplication in PVR mutant cell lines

To investigate the effect of PVR mutations on the replication of PV, the time course of virus production was analyzed in LM cell lines expressing each of the PVR forms after infection with PV-1/Mahoney at an m.o.i. of 10 $TCID_{50}$/cell. The amounts of cell-associated and extracellular infectious particles were determined by $TCID_{50}$ assay (Fig. 3). During the eclipse phase, the loss of infectivity appeared maximal 2 h p.i. in LM/PVR$_{IMR}$ and in LM/PVR-Thr67 and LM/PVR-Gln104 cells. However, it was slightly less pronounced in these two cell lines expressing variant PVR. In LM/PVR-Ser39/Thr67 cells, the loss of infectivity seemed to be delayed with a maximum at 4 h p.i. From 6 h p.i., extracellular viral yields were similar in all cell lines. PVR mutations might slightly affect the early steps of infection without affecting viral yields.

The fact that PV1/Mahoney-inoculated cells were truly infected was confirmed by immunofluorescence. Viral antigen, which was not detected at 2 h p.i. in any of the cell lines, was intensely stained 24 h p.i. in 41, 35, 49, and 40% of infected LM/PVR$_{IMR}$, LM/PVR-Thr67, LM/PVR-Ser39/Thr67, and LM/PVR-Gln104 cells, respectively (not shown). This viral antigen therefore corresponded to

newly synthesized viral proteins. Thus cells expressing mutant PVRs were not resistant to infection despite their partial resistance to PV-induced lysis. We then studied PV–PVR interactions further.

## PVR mutations do not affect PV binding to cells

To determine the effects of PVR mutations on the efficiency of virus adsorption, we examined the binding of radiolabeled PV-1/Mahoney to LM/PVR$_{IMR}$ and LM/PVR variant cell lines. Untransfected LM cells were used as a negative control. Because PV undergoes a conformational transition when it interacts with PVR at 37°C (Gomez Yafal *et al.,* 1993), the binding assays were performed at 4°C overnight. LM/PVR$_{IMR}$ cells were incubated with radiolabeled virus at an m.o.i. of 40 $TCID_{50}$/cell. The number of cells was adjusted so as to have identical numbers of PVR molecules available in all assays. Under these conditions, the adsorption of virus onto cells expressing the various PVR forms was similar and reached about 75% of the input virus (Fig. 4). When the adsorption period was shortened (150 min), the adsorption of virus was similar at the surface of LM/PVR$_{IMR}$ and LM/PVR-Gln104 cells (26% of the input virus) and was slightly higher in the case of LM/PVR-Thr67 and LM/PVR-Ser39/Thr67 (30 and 36% of the input virus, respectively). These results indicate that the variant PVR forms had an efficiency of PV binding at least as high as that of PVR$_{IMR}$.

## PVR mutations affect conformational transitions mediated by PVR

To investigate in more detail the early steps of PV infection in cells expressing mutant PVRs, we studied the conformational transition mediated by the PVR, from 160S native particles to 135S particles (Fricks and Hogle, 1990). Infection was first synchronized for 2.5 h at 4°C by incubating radiolabeled PV-1/Mahoney with LM/PVR
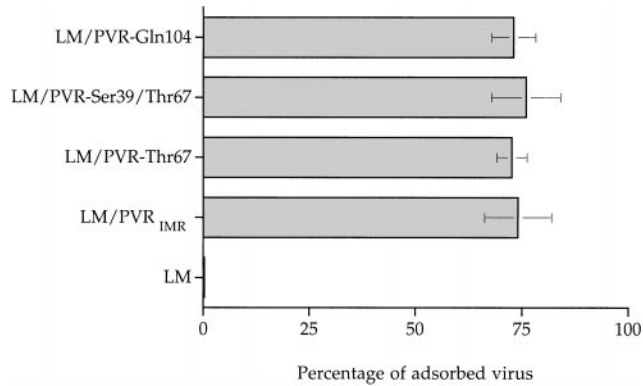
FIG. 4. PV binding onto LM/PVR cell lines. Adsorption was carried out overnight at 4°C with [³⁵S]methionine-radiolabeled PV-1/Mahoney as described under Materials and Methods. PVR-negative LM cells were used as a negative control. Each value is the average of two independent experiments. The SEM values are indicated.

cells. Under these conditions, the binding efficiency of PV1/Mahoney was similar on the four cell lines. Conformational transitions were then induced by incubation at 37°C for 15 min. The distribution patterns of 160S and

135S particles were analyzed by sucrose gradient ultra-centrifugation, and the transitions were evaluated as the ratio between areas under the 135S and 160 peaks (Fig. 5). In LM cells expressing PVR, the 135S/160S ratios for LM/PVR-Gln104 and LM/PVR-Ser39/Thr67 were almost half that for LM/PVR$_{IMR}$. The ratio for LM/PVR-Thr67 was similar to that for LM/PVR$_{IMR}$. Similar differences were obtained when conformational alterations were induced for 40 min (data not shown). This indicates that the major PVR forms isolated from persistently infected IMR/KK3 cells (i.e., PVR-Gln104 and PVR-Ser39/Thr67) were less efficient than PVR$_{IMR}$ in triggering the transitions of PV1/Mahoney.

## DISCUSSION

During persistent PV infection of human cells of neuronal origin, PVpi mutated in regions known to interact with PVR are selected (Borzakian et al., 1993; Couderc et al., 1994; Pavio et al., 1996). This led us to investigate whether the mechanism of PV persistence in these cells involves the selection of mutant PVR. The sequences of the PVR mRNA in two neuroblastoma IMR-32 cell cul-
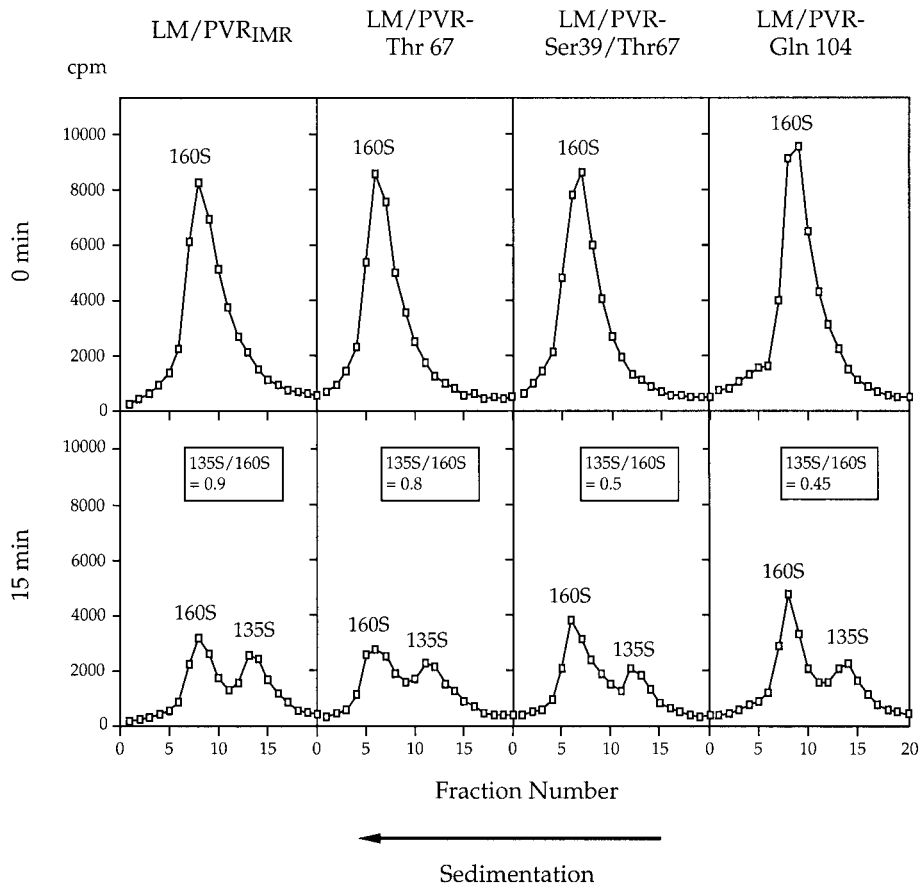


FIG. 5. PV conformational alteration assays in LM/PVR cell lines. Infection of cells with [³⁵S]methionine-radiolabeled PV-1/Mahoney was first synchronized at 4°C, and then infected cells were switched to 37°C for 15 min. The conversion of 160S virions to 135S particles was analyzed after sedimentation through a 15–30% (wt/vol) sucrose gradient. The 135S/160S alteration ratio corresponding to the area under the 135S peak divided by the area under the 160S peak is indicated.

tures (IMR/KK-3 and IMR/KK-5) persistently infected with PV1/Mahoney for 2.5 months were compared with that in uninfected IMR-32 cells. In uninfected cells, at two different passages, all sequences of the clones corresponding to the first domain of PVR cDNA (20 in total), except one with a missense mutation at nucleotide position 173, were identical to the published sequence, with a G at nucleotide position 199 of the mRNA, leading to an Ala at position 67 of the amino acid sequence (Koike *et al.,* 1990; Mendelsohn *et al.,* 1989). The sequences encoding the other domains of the PVR in IMR-32 cells were identical to those of HeLa cells (Koike *et al.,* 1990; Mendelsohn *et al.,* 1989). Thus PVR mRNAs were homogeneous and expressed only one of the two allelic forms found in HeLa cells (Koike *et al.,* 1990). In contrast, the PVR mRNA population of persistently infected IMR-KK3 cultures was heterogeneous: some missense mutations were repeatedly detected, all of which mapped in the first domain of PVR. These mutations lead to (1) the other PVR allelic form with the substitution Ala67→Thr alone and (2) two types of mutant PVRs, one with the substitutions Ala67→Thr associated with Gly39→Ser, and the other with Arg104→Gln. Remarkably, both the substitution Ala67→Thr alone and the double substitution Ala67→Thr and Gly39→Ser were also selected in an independent persistently infected cell culture (IMR-KK5).

The selection of mutations in the domain interacting with PV suggests that the virus is responsible for this selection. There are at least two possible, nonexclusive explanations for the rapid emergence of these mutations: they may already be present in a very small minority of cells in the cell population before infection and would not have been detected in the two series of 10 cDNA clones sequenced at passages 13 and 55, respectively, or they may be induced by the persistent PV infection. The second of these possibilities has previously been suggested in the case of persistent FMDV infections *in vitro* (Martin-Hernandez *et al.,* 1994).

Functional PVR analysis in stable LM cell lines expressing PVR variants showed that cells expressing PVR-Thr67, PVR-Ser39/Thr67, or PVR-Gln104 were more resistant than cells expressing PVR$_{IMR}$ to PV1/Mahoney- and PV3/Leon-induced lysis. This was also observed with PV1/Mahoney mutants having either a Pro-to-Ser substitution at position 95 of VP1 or a His-to-Tyr substitution at position 142 of VP2 (not shown). Although the role of intracellular factors specific to neuroblastoma cells cannot be excluded, these results seem to indicate that PVR mutations selected in persistently infected neuroblastoma cells contribute to the resistance of these cells to PV-induced lysis. It is therefore probable that these mutations were selected because they conferred this resistance. PV entry into the cell via mutant receptors may reduce or delay the initiation of CPE. These results are in accordance with a previous study suggest-

ing that PV-induced CPE may result from PV–PVR interactions (Morrison *et al.,* 1994). Because mutations in the PV capsid are selected during the first weeks of the persistent infection in IMR-32 cells (Calvez *et al.,* 1995b), and two of them confer adaptation to mutant receptors (Colston and Racaniello, 1995), it is likely that PVR mutations were selected early during infection. Thus PVR mutations are probably not the most important factor at the time of infection, when IMR-32 cells expressed PVR$_{IMR}$ conferring a high susceptibility to PV, but may contribute to the mechanism of persistent infection in the weeks after infection.

The partial resistance of LM cells expressing PVR variants was not associated with a decrease in PV yields, indicating that PV growth and liberation were not affected by the mutant PVRs, and that these processes could occur despite delayed cell lysis. There have been similar observations in the HEp-2 cell model of persistent infection (Calvez *et al.,* 1995a) and in murine transformant cells expressing PVR molecules mutated on the C″ edge (Morrison *et al.,* 1994). In an attempt to understand how virus–receptor interactions modulate PV-induced lysis, we studied the early steps of the viral cycle involving PVR, in the various LM/PVR cell lines. Binding experiments suggest that PV adsorption onto mutant PVRs is not affected. The analysis of postbinding conformational changes mediated by mutated PVRs showed that the Ser39/Thr67 and Gln104 substitutions almost halved the efficiency of the 160S-to-135S alteration. Although the role of 135S A particles is still unclear, their formation is often associated with virus uncoating (Belnap *et al.,* 2000a; Curry *et al.,* 1996).

The selection of PVR mutants conferring a less efficient PV particle alteration is in accordance with the selection of PVpi with an enhanced particle alteration (Pelletier *et al.,* 1998) in such a way that they may compensate mutant PVR defects. The residue Thr67 alone, corresponding to one of the allelic forms in HeLa cells (Koike *et al.,* 1990), did not seem to affect the formation of 135S A particles. This residue also had a less significant effect on cell resistance to lysis. Possibly, the transition assays may not be sensitive enough to reveal very small differences in the efficiency of conformational modifications, such as that which may exist between PVR$_{IMR}$ and PVR-Thr67.

The sites of the substitutions in the structure of PVR domain 1 (Morrison *et al.,* 1994) may identify residues involved in PV–PVR interactions (Fig. 6). Residue Ser39 is located in β-strand A′, which has not been identified as a region of direct contact with PV (Aoki *et al.,* 1994; Belnap *et al.,* 2000b; Bernhardt *et al.,* 1994; He *et al.,* 2000; Morrison *et al.,* 1994; Xing *et al.,* 2000). Residue Thr67, in β-strand C, is adjacent to amino acids 68–72, which were found to make contact with the virus (He *et al.,* 2000). Residue Gln104 is located in the D-E loop, which could interact with the virus (Aoki *et al.,* 1994;
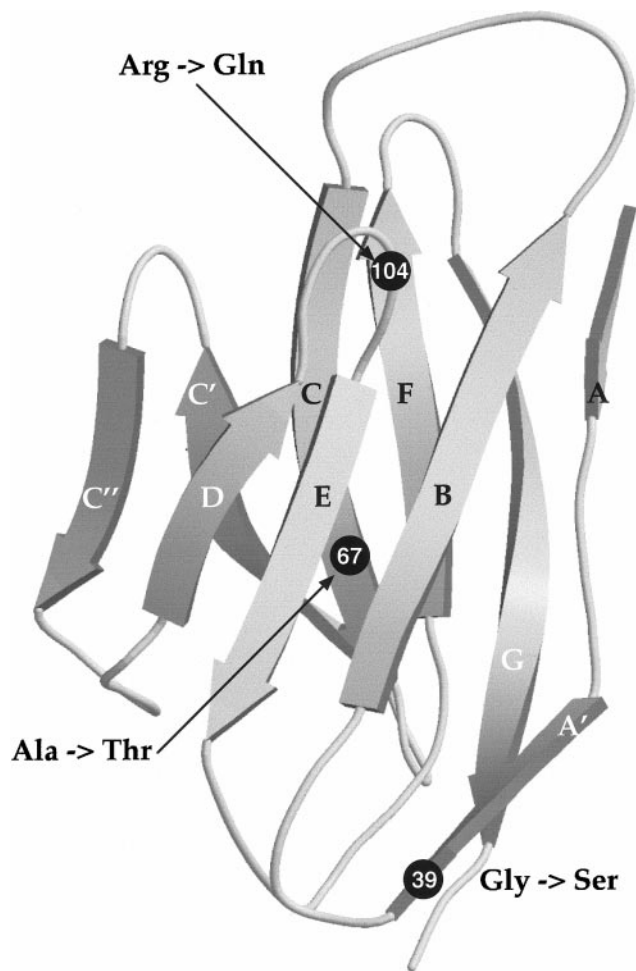
FIG. 6. Structural model of PVR domain 1 showing amino acid substitutions selected in PV persistently infected IMR-32 cell lines. The structure of domain 1 (residues Val29 to Leu142) was adapted from He *et al.* (2000) (PDB ID: 1DGI) by adding the backbone and side chain atoms with programs Midas-Plus (Ferrin *et al.,* 1988) and Sybyl (Tripos, Inc.). The ribbon diagram was calculated with Molscript (Kraulis, 1991) and rendered with Raster3D (Merritt and Murphy, 1994). The β-strands are labeled with uppercase letters, and the substituted amino acids are represented as black, numbered spheres.

Belnap *et al.,* 2000b; Bernhardt *et al.,* 1994; He *et al.,* 2000; Morrison *et al.,* 1994). Furthermore, Asn105, adjacent to Gln104, carries a carbohydrate chain that presents steric hindrance for PV attachment (Bernhardt *et al.,* 1994). Despite their proximity to regions in contact with the viral surface, neither residue Thr67 nor residue Gln104 was found to affect PV binding. Our results, however, identified three residues: the pair Ser39/Thr67 and Gln104, involved in the conformational changes leading to 135S A particles.

Our results indicate that specific mutations are selected in the first domain of the PVR during the persistent infection of human neuroblastoma cells. These mutations conferred on cells an increased resistance to cell lysis, which may give them a selective advantage during long-term infections. This resistance correlated with

poorly efficient transition processes. Neuroblastoma cells probably coevolve with the virus during persistent infection. Such coevolution has been observed in persistent infections with several other RNA viruses (Chen and Baric, 1996; Dermody *et al.,* 1993; Martin-Hernandez *et al.,* 1994), but it seems to occur later in this PV model than in that of FMDV (Martin-Hernandez *et al.,* 1994), in which it occurs in the first hours of infection. The emergence of mutant PVRs in persistently infected cells further reveals the diversity of the mechanisms underlying PV persistence in different cell lines: down-regulation of PVR expression, poorly efficient host-cell shutoff, and viral determinants of the capsid (Borzakian *et al.,* 1992; Carp, 1981; Duncan *et al.,* 1998; Kaplan and Racaniello, 1991; Lloyd and Bovee, 1993). The neuroblastoma cell model may be particularly valuable for investigating the way in which PV induces CPE and cell lysis. The cascade of events after the interaction of PV with its receptor and their involvement in these phenomena remain to be investigated.

## MATERIALS AND METHODS

### Cells and virus

Human neuroblastoma IMR-32 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Murine thymidine kinase-negative LM cells, which do not have the PVR gene, its derivatives, and HEp-2 cells were grown in DMEM containing 10% newborn calf serum. The neurovirulent PV type 1 strain Mahoney (PV1/Mahoney), Mahoney mutants Mah-KKVP1S$_{95}$ and Mah-KKVP2Y$_{142}$ (Couderc *et al.,* 1994), and the PV type 3 strain Leon (PV3/Leon) were amplified on HEp-2 cells, and viral titers were determined on these cells by measuring TCID$_{50}$. Two independent persistently infected cultures, IMR/KK-3 and IMR/KK-5, were established after infection of IMR-32 cells with PV1/Mahoney at an m.o.i. of 10 TCID$_{50}$/cell at 37°C. Cells were grown in DMEM with 2% FCS for the first week p.i. and thereafter in DMEM with 10% FCS. The culture medium was changed weekly until the culture reached confluence. Cells were then trypsinized and divided once per week.

### RNA isolation, RT–PCR, and cDNA cloning and sequencing

Total RNA was isolated from uninfected IMR-32 cells and from IMR/KK-3 and IMR/KK-5 cells at 2.5 months after PV1/Mahoney infection (four to six passages), using the RNA PLUS kit (Bioprobe, Montreuil-sous-Bois, France). RNA was precipitated twice with isopropanol and stored at −70°C. PVRα mRNAs were reverse transcribed as three fragments using specific primers annealing at nucleotide (nt) positions 696–723 (fragment 1), 1157–1175 (fragment 2), and 1296–1311 (fragment 3). Re-

verse transcriptions were performed as follows: total RNA (5 $\mu$g) and 15 pmol of primers were heated to 80°C, allowed to cool to 40°C within 30 min, and incubated at 40°C for 45 min. Reaction mixture and Superscript reverse transcriptase (Gibco Life Technologies, Eragny, France) were then added according to the manufacturer's instructions, and the mixtures were further incubated for 60 min at 40°C. cDNA preparations were denatured with 0.2 M NaOH for 10 min at 40°C; neutralized with 0.5 M Tris–HCl, pH 7.5; precipitated overnight with ethanol; and resuspended in 30 $\mu$l of water. Each cDNA fragment was then amplified with a pair of phosphorylated internal primers corresponding to nt $-5$–11 and 405–424 for fragment 1, nt 365–385 and 1100–1123 for fragment 2, and nt 1022–1045 and 1257–1277 for fragment 3. PCRs were carried out with 5 $\mu$l of the cDNAs, 45 pmol of each primer, and AmpliTaq Gold DNA polymerase (Perkin–Elmer Cetus, Norwalk, CT) according to the manufacturer's instructions in a Perkin–Elmer Cetus thermocycler as follows: 10 min at 94°C, 35 cycles of 20 s at 94°C, 20 s at 55°C, and 1 min 30 s at 72°C and then 8 min at 72°C. PCR products were purified by agarose gel electrophoresis and ligated to pUC 19 (Biolabs) linearized with *Pvu*II. For each PCR product, 10 clones were amplified, and the inserts, were sequenced using BigDye Terminator Cycle Sequencing kit (Perkin–Elmer Cetus) according to the manufacturer's instructions. Sequences were analyzed with the ABI PRISM 377 DNA Sequencer (Perkin–Elmer Cetus).

## Site-directed mutagenesis

Substitutions in the PVR gene were introduced in the expression vector pSV2PVR$\alpha$ (Koike *et al.,* 1990), containing the same cDNA sequence of human PVR$\alpha$ as that expressed in IMR-32 cells (kindly provided by A. Nomoto and S. Koike, Japan), with the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) and specific primers. Each substitution was introduced with a pair of complementary oligonucleotides: 5′-CGCCCAAGAAGC$\underline{T}$GGGCACCTGGGTGG-3′ and 5′-CCACCCAGGTGCCC$\underline{A}$GCTTCTTGGGCG-3′ for the A-to-G substitution at nt 115, 5′-CACCATGCCGCG$\underline{C}$-CCAAGTCAGCTG-3′ and 5′-CAGCTGACTTGG$\underline{G}$CGCG-GCATGGTG-3′ for the C-to-T substitution at nt 199, and 5′-CAGCGAGGCATT$\underline{C}$TGCAGCTCCGCG-3′ and 5′-CG-CGGAGCTGCA$\underline{G}$AATGCCTCGCTG-3′ for the G-to-A substitution at nt 311. Automated sequencing as described here was used to verify correct mutagenesis.

## Cell transfection

One-day-old subconfluent cultures of LM cells in 25-cm$^2$ T-flasks were transfected with 1.5 $\mu$g of each of the plasmids carrying the human PVR cDNA and 0.5 $\mu$g of the plasmid pAG60 (Colbère-Garapin *et al.,* 1981) carrying a selective marker conferring resistance to the anti-

biotic G418 (geneticin; GIBCO BRL, Gaithersburg, MD) using LipofectAMINE Plus reagent (GIBCO BRL) as described by the manufacturer. Twenty-four hours after transfection, a selective medium containing a high dose of G418 (690 $\mu$g/ml geneticin) was added to the cells to select for high expression of the transgenes. At 1 month post-transfection, the geneticin concentration was reduced to 370 $\mu$g/ml. G418-resistant colonies were either pooled by trypsinization and PVR-positive cells were selected by FACS or isolated and grown as cell clones. PVR-positive clones were identified by FACS analysis. The presence of mutations in the PVR mRNA of the pools of cell colonies was verified by automated sequencing after RT–PCR amplification as described earlier.

## Immunofluorescence staining and FACS analysis

LM cell PVR transformants were trypsinized and immunofluorescence staining was performed to determine the level of PVR expression on the cell surface. All staining and washing steps were performed at 4°C in staining buffer (SB) containing PBS with 1% filtered FCS and 0.1% sodium azide. Cells ($10^6$ for fluorescence analysis and $2 \times 10^7$ for cell sorting) were incubated for 30 min on ice with 50 $\mu$l (for fluorescence analysis) or 500 $\mu$l (for cell sorting) of anti-PVR monoclonal antibody (mAb) 404-19 (Lopez *et al.,* 1999) (kindly provided by M. Lopez, France) or 280 (Minor *et al.,* 1984) (kindly provided by P. Minor, United Kingdom). A mouse IgG$_1$ $\kappa$ antibody (Sigma Chemical, Poole, Dorset, UK) was used as a negative control. Cells were washed once with 5 ml of SB and incubated with a sheep anti-mouse IgG conjugated with fluorescein isothiocyanate (Sanofi Diagnostic Pasteur, Montpellier, France) for 30 min on ice. Cells were washed once again as described above and resuspended in 500 $\mu$l (for fluorescence analysis) and 6 ml (for cell sorting) of SB. Stained cells were then subjected to FACS analysis with a Becton Dickinson (Le Pont de Claix, France) FACScalibur machine, and transformant LM cells were sorted to obtain highly enriched PVR-positive cultures. This procedure was repeated two or three times until the percentage of PVR-positive cells was 95–99%. Relative densities of PVR on the cell surface were evaluated by comparing the mean fluorescence per cell for the various transformant LM cells stained with the same antibody in the same assay. The level of PVR expression on the cell surface of LM/PVR mutants was 1.5–3 times lower than that on LM cells expressing the PVR form of IMR-32 cells (LM/PVR$_{IMR}$). These differences were similar with both anti-PVR antibodies used. We took these differences into account for experiments as indicated here.

We tested for the viral antigen in the various transformant LM cells infected with PV1/Mahoney at an m.o.i. of 10 TCID$_{50}$/cell. The cells were grown on coverslips, infected, and fixed in 3% paraformaldehyde 2 or 24 h after

infection. The C3 mAb (Blondel *et al.,* 1983), or a mouse IgG$_1$ $\kappa$ antibody (Sigma), was used for indirect immuno-fluorescence, with a sheep anti-mouse IgG conjugated with fluorescein isothiocyanate (Sanofi Diagnostic Pasteur), as described previously (Borzakian *et al.,* 1992). Uninfected cells were used as negative controls.

### Resistance of cells to virus-induced lysis

LM-cell PVR-transformants ($10^6$) were seeded onto wells of 24-well plates and infected 18 h later. At the time of infection, cells were counted, and for each cell type, the m.o.i. was adjusted to 10 TCID$_{50}$/cell. After 30 min of adsorption at 37°C, cells were washed twice, and 1 ml of DMEM containing 2% FCS was added to each well. After 28 or 48 h of infection, the number of adherent cells in infected wells was determined as a percentage of that in uninfected wells using a Coulter Counter (Beckman-Coulter). As previously found (Calvez *et al.,* 1995a), and verified here by trypan blue exclusion, adherent cells in both infected and uninfected wells were alive. The percentage values obtained therefore represent the percentages of cells surviving infection.

### Virus growth cycles

Duplicate 24-well plates containing cell monolayers were infected at an m.o.i. of 10 TCID$_{50}$/cell as described earlier and incubated at 37°C for 1, 2, 4, 6, 8, or 24 h. At each time, extracellular virus was harvested, and the corresponding cell-associated virus was recovered by freezing and thawing the cells. The infectivity of clarified virus suspension was determined by TCID$_{50}$ assay.

### Preparation of radiolabeled virus

Virus was labeled with [$^{35}$S]methionine (1400 Ci/mmol; ICN, Orsay, France) and purified on cesium chloride density gradient by isopycnic centrifugation as previously described (Blondel *et al.,* 1983). The yield of mature virions was measured optically, assuming $9.4 \times 10^{12}$ particles/unit of optical density at 260 nm (Rueckert, 1976). The specific activity of radiolabeled virus was $8.25 \times 10^{-7}$ cpm/particle, and the particle/TCID$_{50}$ ratio was 300.

### PV binding assays

Cells in suspension were incubated with [$^{35}$S]methionine-radiolabeled virus in a total volume of 150 $\mu$l of ice-cold DMEM–2% FCS on a side-to-side rocking platform at 4°C overnight. For LM/PVR$_{IMR}$ cells that express the highest level of PVR, radiolabeled virus was added to $1.5 \times 10^6$ cells (m.o.i. = 40 TCID$_{50}$/cell). For the LM/PVR mutants expressing a lower level of PVR, the same amount of radiolabeled virus was added, but the number of cells was adjusted so as to have the same ratio of

virus to PVR molecules in each assay, according to the mean fluorescence values obtained by FACS with anti-PVR mAb 404-19 or 280 before each binding experiment. After the adsorption period, unattached virus was removed by two washes with ice-cold DMEM–2% FCS. Cell-associated radioactivity was then determined by scintillation counting and is expressed as a percentage of the radioactivity present in the inoculum.

### PV conformational alteration assays

Cells were trypsinized, washed with ice-cold DMEM–2% FCS, and then incubated with [$^{35}$S]methionine-radiolabeled virus in a total volume of 2 ml of ice-cold DMEM–2% FCS on a side-to-side rocking platform at 4°C for 2.5 h to synchronize the infection. For LM/PVR$_{IMR}$ cells that express the highest level of PVR, radiolabeled virus was added to $10^7$ cells (m.o.i. = 100 TCID$_{50}$/cell) and as for the binding assay, the number of cells was adjusted for LM/PVR mutants so as to have the same ratio of virus to PVR molecules in each assay. After the adsorption period, unattached virus was removed by two washes with DMEM–2% FCS. Alteration was initiated by adding 5 ml of DMEM–2% FCS preheated to 37°C, and cells were agitated for 15 or 40 min at 37°C. Cells were then washed twice with ice-cold DMEM–2% FCS and once with ice-cold PBS. Cells were pelleted, resuspended in 450 $\mu$l of 140 mM NaCl, 50 mM Tris, pH 8.0, and lysed by the addition of 50 $\mu$l of 140 mM NaCl, 50 mM Tris, pH 8.0, 10% Nonidet P-40, and 1% SDS, as described previously (Calvez *et al.,* 1995a). After incubation for 20 min on ice, the cell lysates were clarified by centrifugation for 5 min in an Eppendorf centrifuge and layered onto 15–30% (wt/vol) sucrose gradients prepared in PBS, which were then centrifuged in a Kontron TST41.14 rotor for 2 h at 40,000 rpm at 4°C. Gradients were fractionated, and the radioactivity per fraction was counted.

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