Parovirus LullI transducing vectors packaged by LullI versus FPV capsid proteins: the VP1 N-terminal region is not a major determinant of human cell permissiveness

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Human cell lines are permissive for LullI, a member of the rodent group of autonomous paroviruses. However, LullI vectors pseudotyped with feline panleukopaenia virus (FPV) capsid proteins can transduce feline cells but not human cells. Feline transferrin receptor (FelTfR) functions as a receptor for FPV. Transfection of Rh18A, a human rhabdomyosarcoma cell line, with FelTfR enabled transduction by vector with FPV capsid. This was not true of other human lines, suggesting restriction at some additional, post-entry, level(s) in human cells other than Rh18A. It seemed a reasonable hypothesis that a second blockage might be in nuclear delivery mediated by the N-terminal region of the minor capsid protein, VP1. We therefore generated virions containing an LullI–luciferase genome, packaged using chimaeric VP1 molecules (N-terminal region of LullI VP1, fused with body of FPV, and vice versa) together with the major capsid protein, VP2, of FPV or LullI. The virions were tested for ability to transduce feline and human cells. Our hypothesis predicted that the N-terminal region of LullI VP1 should allow transduction of human cells expressing FelTfR, while the FPV N-terminal region should not allow transduction of human cells (except for Rh18A). The experimental results did not bear out either of these predictions. Therefore, the VP1 N-terminal region appears not to be a major determinant of permissiveness for LullI, versus FPV, capsid in human cells.

INTRODUCTION

Autonomous paroviruses of the rodent group, including minute virus of mice (MVM), LullI, feline panleukopaenia virus (FPV) and canine parovirus (CPV), contain a single-strand DNA genome of ≅5·1 kb, packaged by two, independently translated capsid proteins (Cotmore & Tattersall, 1987). The major protein, VP2, by itself, is capable of virion formation and DNA packaging. However, the minor protein, VP1, is required for infectivity (Tullis et al., 1993; Maxwell et al., 1995). While VP1 and VP2 share most of their sequence, VP1 has a unique N-terminal region of ≅140 amino acids which includes nuclear localization signals (NLS) (Tullis et al., 1993; Lombardo et al., 2002) and a phospholipase A2 activity (Zadori et al., 2001), believed to be necessary for delivery of an infecting viral genome to the cell nucleus.

We previously derived gene transfer vectors from LullI by substituting foreign DNA for viral coding sequences (Maxwell et al., 1993a, 2002). These recombinant genomes could be packaged by capsid proteins of several of the related rodent paroviruses (Maxwell et al., 1993b; Spitzer et al., 1996). Vectors packaged with the homologous LullI capsid proteins could transduce various human cell lines. However, LullI vectors pseudotyped with FPV capsid proteins were able to transduce feline cells, but not human cells. Display of an integrin binding peptide on the FPV capsid enabled transduction of a human rhabdomyosarcoma cell line (Rh18A) but not of other human cell lines expressing the targeted integrin (Maxwell et al., 2001).

Feline transferrin receptor (FelTfR) functions as a receptor for FPV (Parker et al., 2001). Here, we show that Rh18A cells, but not other human cells, when transfected with an expression plasmid for FelTfR, are rendered permissive for transduction by LullI vectors packaged with unmodified FPV capsid. These observations suggest that transduction by the FPV pseudotyped vector is restricted by receptor binding/cell entry, and also at some subsequent level(s) in human cells other than Rh18A. It seemed a reasonable hypothesis that the second blockage might be in nuclear delivery mediated by the N-terminal region of VP1, and that this region of FPV VP1 might not generally function in human cells, in contrast to the corresponding VP1 region of LullI. To test this hypothesis, we constructed expression plasmids for chimaeric VP1 molecules with the N-terminal region of FPV fused (in the glycine-rich sequence) to the body of LullI and vice versa. These were used, together with expression...
plasmids for VP2 homologous with the respective body regions, to generate virions containing an LuIII–luciferase genome, which were then tested for transduction of feline and human cells. The hypothesis predicts that the LuIII VP1 N-terminal region would be sufficient to enable transduction of human cells in which the entry block had been relieved (by expressing FeTfR), and that the FPV VP1 N-terminal region would allow virions with capsid otherwise composed of LuIII sequence to transduce feline cells, but not human cells except for Rh18A. The experimental results were not in accordance with either prediction. We therefore conclude that the VP1 N-terminal region is not a major determinant of permissiveness for LuIII versus FPV capsid in transduction of human cells.

**METHODS**

**Cell culture and virus production.** Cell lines were grown in RPMI 1640 with 10% NuSerum IV (Collaborative Biomedical Products; Becton Dickinson) at 37°C in an atmosphere of 5% CO₂. Virus production was as described (Maxwell et al., 1993a, 2002), using electroporation to co-transfect the 324K cell line with a plasmid containing an LuIII–luciferase recombinant genome and one or more plasmids encoding capsid proteins and/or nonstructural proteins. Virus stocks, consisting of combined culture medium and freeze–thawed extracts of the electroporated producer cells, were stored at 4°C. In most experiments, virions contained the GLuP38LUC2 LuIII–luciferase genome (Spitzer et al., 1996). In some experiments, virions contained an alternative genome, GLuP4LUC1 (Corsini et al., 1995). For the latter experiments, pLuNS1 was co-transfected into the producer cells as a source of nonstructural protein when necessary (i.e. when the capsid expression plasmids did not also express nonstructural proteins).

**Plasmid construction.** Schematic diagrams of the plasmids are shown in Fig. 1. Recombinant LuIII plasmids containing the luciferase reporter gene were pGLuP38LUC2, in which the luciferase reporter sequence replaced most of the coding sequence for capsid proteins (Spitzer et al., 1996), and pGLuP4LUC1, in which luciferase replaced almost the entire viral sequences encoding nonstructural and capsid proteins (Maxwell et al., 1993a; Corsini et al., 1995).

Construction of helper plasmids is summarized as follows; full details are available by request. Plasmid pF.VP (Spitzer et al., 1996) expresses both VP2 and VP1 of FPV by alternative splicing of a common transcript from the viral P38 promoter, using either of two splice donor sites (D1 and D2, respectively) with a single acceptor (A) (Carlson et al., 1985). Plasmids pF.VP2 and pF.VP1, expressing either VP2 or VP1 alone, have been described (Spitzer et al., 1997). pF.VP2 has a frameshift mutation in the VP1 unique region which prevents VP1 expression; this plasmid expresses VP2 from P38 transcripts with the D1-A splice. pF.VP1, constructed by introducing the alternative D2-A splice at the DNA level, retains codons 1–10 of VP1 prior to the splice, and 11–143 following the splice.

The mRNAs for VP2 and VP1 of LuIII and MVM are generated from P38 transcripts by similar alternative splicing to FPV except for the use of two splice acceptors (A1 and A2). VP2 uses the D1 splice donor and, primarily, the A1 acceptor, while VP1 uses the D2-A2 splice (Clemens et al., 1990). To generate pLu.VP2 and pLu.VP1, we took advantage of previously described MVM constructs containing these splices, derived from cloned cDNA (Tullis et al., 1993). Thus, both of these plasmids were generated from pGLuABE (a plasmid containing a terminally deleted LuIII genome; Hanson & Rhode, 1991) by substituting an MVMp DNA fragment (nt 2071–2650 of wild-type MVMp) including the P38 promoter and either the D1-A1 or D2-A2 splice. As a result of these manipulations, pLu.VP2 expresses authentic LuIII VP2 while pLu.VP1 expresses authentic LuIII VP1 except for two amino acid substitutions from MVM (indicated by asterisks in Fig. 2) in its unique N-terminal region.

pLu.CMV.VP2 is an alternative expression plasmid for LuIII VP2 which uses the CMV promoter. It was constructed by substituting only the VP2 coding sequence of LuIII (Fsp−Sspnt 2773–4638) for the β-galactosidase sequence of pCMV-β (Clontech). pLu.CMV.VP2 does not contain any of the VP1 unique N-terminal sequence upstream of codon 137.

pLu.F.VP1 and pF.Lu.VP1 were constructed by first modifying pF.VP1...
and pLuVP1 to generate a novel BamHI site in an homologous Gly-Ser codon pair within the glycine-rich sequence in the VP2 N-proximal region within the VP1 coding sequence. This was done by PCR-based, site-directed mutagenesis of two third-position nucleotides in each case (the desired mutations were confirmed by DNA sequencing). The novel BamHI sites were then used to exchange DNA fragments encoding the VP1 unique regions between the two plasmids.

**Assay for transduction of recipient cells.** Recipient cells were seeded in 12-well plates (5×10⁴ cells per well) 1 day prior to infection with virus samples diluted in medium (0.25 ml total volume per well). After 1.5–2 h at 37°C, with frequent swirling, more medium (0.75 ml) was added and incubation was continued for 2 days before luciferase assay (Promega kit with a TD-20/20 luminometer; Turner Designs).

Transfection with FelTfR (see Figs 3 and 4), 1 day prior to infection, was performed as follows. Cells from subconfluent cultures were suspended with medium at 1–1.5×10⁵ cells ml⁻¹. Samples of cell suspension (100 μl) were mixed with control plasmid or with FelTfR expression plasmid, pBI725 (2 μg in 5 μl water), and electroporated (Maxwell & Maxwell, 1988) at 250 μF with voltage optimized for each cell line. Each electroporated cell sample was diluted with medium (12.5 ml) and distributed equally among wells of a 12-well plate (1 ml per well). Each well thus received 5×10⁴ surviving cells. The plates were incubated at 37°C overnight before infection of the reattached cells as described above. Control experiments with a β-galactosidase reporter plasmid indicated that 30–50% of cells expressed the electroporated gene at this time. Efficient expression of FelTfR was also confirmed directly in 324K and A375 cells, transfected with pBI725, using epifluorescence microscopy after staining the cells with Texas red-conjugated canine transferrin (respectively, 24 and 51% of cells stained).

**RESULTS**

Fig. 2 shows amino acid sequence alignment for the N-terminal regions of the VP1 capsid protein of LuIII and FPV, extending for the first 56 amino acids into the region shared with VP2. While there is high overall sequence conservation in this region (75% identity or similarity), there are also enough amino acid differences to render plausible the hypothesis that the unique region of the two VP1 proteins might differ in interaction with cellular factors in human versus feline cells. The two established VP1 functional motifs, namely the N-terminal NLS, BC1 (Lombardo et al., 2001), and the phospholipase A (sPLA2) domain (Zadori et al., 2001), are highly conserved, but there are considerable differences in other parts of the sequences, particularly within the 40–50 amino acids preceding the start of VP2. Fig. 2 also shows alignment of the C-terminal VP regions (last 87 and 82 amino acids of LuIII and FPV).

To investigate possible functional differences between the two VP1 sequences, we generated expression plasmids for VP1 chimaeras in which the N-terminal regions were exchanged between LuIII and FPV, as diagrammed in Fig. 1.
Fig. 3. Packaging with FPV VP1 and VP2 capsid proteins allows efficient transduction of feline cells, or of human Rh18A cells that express FelTfR, but only inefficient transduction of other human cell lines. Luciferase activity was determined in extracts prepared 2 days after infection of the indicated recipient cells with transducing virus, GLuP38LUC2, packaged using pF.VP as capsid helper. The recipient cells had been seeded with (filled bars) or without (open bars) transfection with FelTfR expression plasmid, pBl725, as indicated, 1 day prior to infection. The luminometer background reading was 0.01–0.03. Note that the ordinate is on a log scale.

We previously showed that separate expression plasmids for VP1 and VP2 of FPV could be used as helpers to package recombinant LuIII genomes in co-transfected cells (Spitzer et al., 1997). To ensure efficient co-assembly into capsids, the VP1 chimaeras (Fig. 1c) were used together with expression plasmids for VP2 (Fig. 1b), that shared the same entire homologous region of ordered structure in the capsid, downstream of the glycine-rich region (Cotmore & Tattersall, 1987; Tsao et al., 1991). This was achieved by making the junctions of the VP1 N termini in the chimaeras within an homologous Gly-Ser codon pair within the glycine-rich regions, as indicated in Figs 1 and 2. Transducing virions were then generated by a standard co-transfection procedure. The virions contained a recombinant LuIII genome (Fig. 1a) in which a luciferase reporter gene replaced either the major capsid protein coding sequence (GLuP38LUC2) (Spitzer et al., 1996) or the entire viral coding sequences (GLuP4LUC1) (Maxwell et al., 1993a; Corsini et al., 1995).

Fig. 3 shows results obtained when feline or human cell lines were transduced with the vector packaged by capsid proteins consisting entirely of FPV sequence. The human lines were infected, with or without prior transfection with an expression plasmid for FelTfR. We observed comparably high transduced luciferase activity in the CFK feline cells and in the unique human line, Rh18A, expressing FelTfR. There was no activity above the luminometer background in Rh18A, or in other human lines, without FelTfR transfection. The latter transfection did not confer detectable transduction on human HeLa cells, but enabled a low level of transduction of A375 melanoma cells and of the SV40-transformed line, 324K, which varied sporadically (especially in 324K) among replicate infections. These levels of activity were, however, ≥100-fold lower than in Rh18A cells expressing FelTfR (Fig. 3).

Fig. 4 shows transduction results obtained with vector packaged by capsid proteins with the N-terminal region of VP1 derived from LuIII (Lu-F.VP1), but otherwise consisting of FPV sequence (F.VP2). This experiment included control infections with virions generated using a VP2 helper alone (‘No VP1’); these showed no activity above the luminometer background, confirming the previously established requirement for VP1 for transducing activity. Efficient transduction of CFK cells by virions containing Lu-F.VP1 (Fig. 4, leftmost bars) confirmed that this Lu-FPV chimaeric VP1 was fully functional in these feline cells. However, with human cells as recipients, these virions showed essentially the same lack of activity as virions packaged by capsid of entirely FPV origin (Figs 3 and 4). Thus, the Lu-F.VP1 chimaeric molecule did not confer ability to transduce human 324K or A375 cell lines efficiently, with or without expression of FelTfR (Fig. 4).

Results of the converse experiment, with the N-terminal region of VP1 derived from FPV, in a capsid otherwise consisting of LuIII sequence, are shown in Fig. 5. Virions having packaged either of the recombinant genomes, GLuP38LUC2 or GLuP4LUC1, gave similar results (Figs 5a and 5b, respectively). As shown in Fig. 5(a), virions containing GLuP38LUC2, packaged with Lu.VP2 together with F-Lu.VP1, transduced human 324K cells almost as efficiently as the corresponding virions containing Lu.VP1. However, in this experiment we also observed that virions produced in the absence of a VP1 expression plasmid (‘No VP1’ in
Fig. 5a) gave low, but significant transduction (amounting to \(\approx 1\%\) of that seen when either VP1 was included). We attribute this to putative recombination in the producer cells between pGLuP38LUC2 and pLuVP2, generating a minimal amount of a recombinant DNA molecule capable of expressing LuIII VP1 by alternative splicing. This would be possible because GLuP38LUC2 retains unspliced DNA including both splice donors and acceptors, upstream of the luciferase reporter (Fig. 1a). Therefore, to eliminate any possible uncertainty of interpretation due to the presence of this DNA sequence we repeated the experiment in such a way that this sequence was eliminated from the producer system. This was achieved by packaging GLuP4LUC1 (Fig. 1a), instead of GLuP38LUC2, and by using minimal helper plasmids supplying LuIII NS1 and VP2 (pLu.NS1 and pCMV.Lu.VP2 in Fig. 1a and b), together with the same expression plasmids as used previously for LuVP1 or F-LuVP1. With the latter combination, DNA encoding the N-terminal region of LuIII VP1 was absent from the producer system, so that F-LuVP1 was the only possible source of intact VP1, regardless of any recombination events that might occur between the transfected plasmids. As shown in Fig. 5(b), the chimaeric F-Lu VP1 was again clearly functional in enabling efficient transduction of the human cell line, 324K, although its activity was somewhat lower than that of authentic LuIII VP1.

Overall, the results shown in Figs 4 and 5 demonstrate that the N-terminal unique region of LuIII VP1 is not sufficient for transduction of human cells and that this region derived from FPV VP1 is competent in allowing transduction of human cells.

DISCUSSION

There is evidence that determinants of parvovirus species and tissue tropism involve interactions both with cell surface receptors and intracellular factors. Early work with MVMp and MVMi (the fibrotropic and lymphotropic strains of minute virus of mice) demonstrated that both permissive and nonpermissive cells showed similar binding and uptake of these viruses (Spalholz & Tattersall, 1983; Agbandje-McKenna et al., 1998). However, recent studies have clearly shown that CPV evolved from an FPV-related virus during the 1970s and 1980s by acquiring the ability to bind the canine TfR, in contrast with FPV which binds feline, but not canine TfR (Parker et al., 2001; Hueffer et al., 2003a).

Although these results imply that species-specificity of FPV and CPV is primarily determined by their ability to use these cell-surface receptors (Palermo et al., 2003; Govindasamy et al., 2003), the binding and infectivity properties of certain capsid mutants of these viruses (Hueffer et al., 2003b), as well as earlier binding studies (Horiuchi et al., 1992), have suggested possible additional involvement of post-receptor factors in permitting productive infection of canine cells.

Our results indicate that expression of an appropriate cell-surface receptor on human cells, although necessary, is not generally sufficient for transduction by a parvovirus vector with the FPV capsid. We observed that Rh18A cells could be transduced efficiently either via a cell surface integrin (Maxwell et al., 2001), or via the transfected FeTfR (Fig. 3), but this is the only human line we have encountered with these properties. The ability of FPV and CPV to bind or infect human cells has not been examined in detail. Parker et al. (2001) showed that the human transferrin receptor, transfected into hamster or quail cells, conferred binding and uptake of CPV. They also reported that both FPV and CPV could infect human HeLa cells, as indicated by
expression of the nonstructural protein NS1, although the efficiency of expression was not stated. With our vector, packaged by FPV capsid, we observed no transduction of Rh18A cells after transfection with an expression plasmid for human Tfr, as opposed to feline Tfr, nor of HeLa cells (data not shown and Fig. 3). These apparent discrepancies are, at present, unexplained. Regardless, our results imply that, in addition to an appropriate surface receptor, capsid interaction(s) with post-receptor, presumably intracellular, factors may be required for paroviruses to establish productive infection of human cells. At present we do not know whether such interactions may act positively in permitting transduction of Rh18A cells, or negatively in inhibiting transduction of other human cells by vectors packaged with FPV capsid.

The minor capsid protein, VP1, is essential for parovirus infectivity, presumably due to one or more functions of its unique N-terminal region, since the rest of its sequence is shared with the major protein, VP2 (Cotmore & Tattersall, 1987). There is good evidence that this unique region is involved in intracellular trafficking of infecting virions and, specifically, in delivery of the incoming viral genome to the cell nucleus. This function was suggested by the presence of four motifs similar to known NLSs from other proteins (Tullis et al., 1993). These four sequences, rich in basic amino acids, have been designated BC1–BC4 (Lombardo et al., 2002), as indicated in Fig. 2. BC1 and BC2 resemble the NLS of SV40 VP1 while BC2, BC3 and BC4 together conform to bipartite NLS consensus sequences such as found in nucloplasmin (Robbins et al., 1991). Evidence for NLS function is strongest for BC1. Thus, the BC1 sequence, when attached to a heterologous protein (bovine serum albumin), was able to confer nuclear targeting (Vihinen-Ranta et al., 1997). Furthermore, it was shown for CPV that nuclear delivery and infection could be inhibited either by microinjection of antibodies recognizing the VP1 N-terminal region, or by mutagenesis of specific amino acid residues in BC1 (Vihinen-Ranta et al., 2002). More extensive mutagenesis studies with MVM also implicated BC1 in nuclear delivery, as well as elucidating cooperative effects between the VP1 BC sequences and an amphipathic helix sequence near the C terminus of the VP2, termed the nuclear localization motif (NLM) (Lombardo et al., 2000, 2002). These studies suggested that, early during infection, BC1 was most important in nuclear delivery, but with a significant contribution from BC2 (but apparently not BC3 and BC4). Conversely, the NLM appeared to play a major role during virus assembly in effecting nuclear delivery of capsid subunits in a conformation-dependent manner (Lombardo et al., 2000). An additional sequence required for VP1 function encodes an sPLA2 type phospholipase activity that may function in release of incoming virions from endosomes (Zadori et al., 2001). The latter sequence is present between BC1 and BC2, as shown in Fig. 2.

The VP1 N-terminal region presumably functions by interacting with cellular proteins, and these might be species-specific. It was therefore reasonable to hypothesize that such interactions with this region could contribute to species tropism. There are substantial differences in amino acid sequence between LuIII and FPV in the VP1 N-terminal region, as illustrated in Fig. 2. The known functional domains discussed above are well conserved and most amino acid differences lie outside these domains, particularly just preceding the start of VP2. These differences could be important either through novel, direct functional interactions, or by influencing accessibility of the established functional domains. We therefore tested for species-specificity of VP1 function by exchanging the unique N-terminal sequences between VP1 of LuIII and FPV in recombinant virions with capsids otherwise consisting of protein sequence derived entirely from one or the other parental virus. We found that the VP1 N-terminal region of LuIII was not sufficient to enable transduction of human cells by virus with an FPV capsid (even when the cells expressed FeTfr, allowing virus entry). Conversely, the VP1 N-terminal region of FPV was functional in human cells, allowing transduction by virus with capsid otherwise derived from LuIII. These observations effectively refute the hypothesis that this region of VP1 is a major, post-receptor determinant restricting the ability of the FPV capsid, as opposed to the LuIII capsid, to infect human cells. The present results also extend our previous conclusion that the major determinant for CPV transduction of canine cells functions via VP2, rather than VP1 (Spitzer et al., 1997). Thus, for FPV and CPV, the functional determinants of species tropism must presumably be in the sequence shared between VP1 and VP2. As noted above, the NLM near the C terminus of the VP2 of MVM is required for nuclear targeting of capsid components prior to assembly of the capsid in the nucleus. A function of the NLM during initial infection seemed less likely because of the internal location of this sequence within the virion. However, it is possible that the NLM could be exposed after partial disassembly and it might then interact with cellular components in a species-specific manner to direct nuclear targeting. The putative NLM sequences of LuIII and FPV (defined by homology with the MVM motif) differ in 3 of their 11 amino acids. Localization of determinants of species tropism within the NLM or other VP2 sequences of LuIII and FPV should be possible using a targeted, or randomized, sequence exchange approach (Hauck & Xiao, 2003; Bowles et al., 2003) analogous to the one we have applied here to the VP1 N-terminal regions. Elucidating the viral determinants and the mechanisms involved will clearly be relevant in relation to more general understanding of processes that can contribute to emerging viral infections in humans.

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