Expression and subcellular targeting of canine parvovirus capsid proteins in baculovirus-transduced NLFK cells

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Abstract A mammalian baculovirus delivery system was developed to study targeting in Norden Laboratories feline kidney (NLFK) cells of the capsid proteins of canine parvovirus (CPV), VP1 and VP2, or corresponding counterparts fused to EGFP. VP1 and VP2, when expressed alone, both had equal nuclear and cytoplasmic distribution. However, assembled form of VP2 had a predominantly cytoplasmic localization. When VP1 and VP2 were simultaneously present in cells, their nuclear localization increased. Thus, confocal immunofluorescence analysis of cells transduced with the different baculovirus constructs or combinations thereof in the absence or presence of infecting CPV revealed that the VP1 protein is a prerequisite for efficient targeting of VP2 to the nucleus. The baculovirus vectors were functional and the genes of interest efficiently introduced to this CPV susceptible mammalian cell line. Thus, we show evidence that the system could be utilized to study targeting of the CPV capsid proteins.

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1. Introduction

Structurally and genetically related to feline panleukopenia virus and mink enteritis virus [1,2], canine parvovirus (CPV) is a type member of autonomous Parvovirus genus [3]. Non-enveloped capsids of CPV are assembled from 60 copies of the viral protein (VP) structural proteins [4]. The virion of CPV has a diameter of approximately 25 nm. It has a $T=1$ icosahedral symmetry. An eight-stranded anti-parallel beta barrel motif is found in the CPV capsid, like in most viral capsid structures [4-6]. The linear single-stranded DNA genome (~5 kb) contains two promoters directing the expression of two non-structural proteins (NS1 and NS2) in the left reading frame, or for the structural proteins (VP1 and VP2) in the right reading frame [1,7]. Full DNA-containing capsids are predominantly composed of a combination of VP2 and VP3 proteins, with a few copies of VP1 in a 9:1 ratio [8]. When compared to VP2, VP1 has additional 143 N-terminal amino acid residues and VP3 is proteolytically cleaved from VP2 by the removal of about 12–15 amino acids from the N-terminus of VP2 [3,9]. Empty capsids form similar amounts as in full capsids in CPV infected cells and have a composition of VP1 and VP2 only in a ratio of 1:10. Previously, it has been shown that VP2 can assemble into capsid-like structures.

The steps of a CPV infection are only partially characterized. The location of host range determinants has been mapped to the surface of the capsid structure [5,10]. It has been reported that CPV and feline parvovirus (FPV) bind to human and feline transferrin receptors, TIRs [11]. CPV enters the cells by a dynamin-dependent, clathrin-mediated endocytic pathway where the capsids finally co-localize with the TIR in perinuclear vesicles [12,13]. One of the last compartments in the CPV entry route has been identified as lysosomes [14]. Once in the cell, capsids penetrate only slowly into the cytoplasm. It has been shown that when anti-capsid antibodies were injected into the cytoplasm even at 6 h after virus inoculation, productive infection was prevented [15]. These results suggest that capsids can remain within the endocytic compartments several hours after uptake. In addition, a potential nuclear localization signal of SV40-type has been identified within the N-terminal part of VP1 [16]. Further, some specific changes in the identified NLS reduced the relative infectivity of the CPV capsids [17]. In the case of minute virus of mice (MVM), both VP1 and VP2 have NL sequences. Two classical NL sequences were mapped near the VP1 specific N-terminus, while a sequence in VP2, rich in basic amino acids, has been shown to be associated with nuclear transport in a conformation-dependent manner [18,19].

Baculoviridae is a family of large enveloped arthropod viruses that contain large circular double-stranded DNA genomes and baculoviruses have a restricted host range limited to few closely related species within a single genus or family [20–23]. A prototype virus of this family is Autographa californica M nucleopolyhedrovirus (AcMNPV) [24] and AcMNPV is commonly used in the baculovirus expression vector system (BEVS).

The BEVS has been used to produce a variety of parvovirus-specific proteins. Among these, virus-like particles (VLPs) have been produced for adeno-associated virus [25], Aleutian mink disease parvovirus [26], muscovy duck parvovirus [27], human parvovirus B19, porcine parvovirus [28–30], and canine parvovirus [31–35]. Recently, baculoviral vectors have also been used to efficiently mediate gene transfer and expression in various mammalian cell lines both in vitro [36–43] and in vivo.
In these previous studies, mammalian promoters were used. Improvements in gene transfer and specific targeting to mammalian cells by this system have also been conducted [45, 50–52]. Furthermore, it has been shown that baculoviruses are unable to replicate in mammalian cells and little or no cytotoxicity is detected in the context of baculovirus transduction [36, 53]. An in vitro study for a system involving hepatitis B virus replication [54–56] as well as a system to determine replication competence of virus transcripts [57, 58] have been established using recombinant baculovirus transduction of mammalian cells. Besides cell lines, an in vivo system was used to transfer the hemagglutinin gene of the influenza virus [59]. In addition, it has been shown that recombinant baculovirus can be used in monitoring long-term ex vivo and in vivo expression of transduced viral genes without perturbing the physiological state of the transduced cell [60].

In the present study, we have examined the role of using recombinant baculovirus as tools to study intracellular localization of the CPV VP1 protein with respect to nuclear targeting of its viral structural counterpart VP2. This was carried out by constructing various recombinant baculovirus housing VP1 and VP2 with or without the enhanced green fluorescent protein (EGFP) as a tag. These baculoviral constructs were used to transduce NLFK cells by employing the cytomegalovirus immediate-early (CMV-IE) promoter. Together, we have established a novel and gentle system for introduction of parvoviral proteins and genes into mammalian cells to investigate the nuclear targeting of VP1 and VP2 of CPV.

2. Materials and methods

2.1. Genetic baculovirus constructs

VP1 of CPV was amplified by PCR using p265 (Collin Parrish, Cornell University, Ithaca, NY) as a template with the sense oligonucleotide primer 5'-CAT GGA TTC ATG GCA CCT CCG-3' (BamHI site and start codon underlined) and the antisense oligonucleotide primer 5'-CGG GAA TGC TTA ATA TAA TTT TCT AGG TGC-3' (EcoRI site and stop codon underlined). VP2 cloning with a stop codon followed similar procedures as described previously [31]. For C-terminal fusion of VP1 and VP2 to EGFP, the antisense oligonucleotide primer without a stop codon was 5'-CGG A GGC GAA TGC TTA ATA TAA TTT TCT AGG TG-3' (EcoRI site underlined). PCR products of VP1 and VP2 (with or without a stop codon) were cloned into the corresponding restriction sites of pFastBacI (Gibco-BRL, Grand Island, NY). The resulting plasmids with the stop codons were named pVP1EGFPfastbac, pVP2EGFPfastbac, and pEGFPfastbac.

For C-terminal fusion of VP1 and VP2, the sequence encoding EGFP was amplified using pEgfp-C1 (Clontech, Palo Alto, CA). The oligonucleotide primers for EGFP were 5'-GTC GAA TTC ATG GTG AGC AAG GGC GAG GAG-3' (sense, EcoRI site and translational start codon underlined) and 5'-TCT TCT AGA TTA CTT GGA CTC TCT GTC GCT GCC-3' (antisense, XbaI site and stop codon underlined). PCR products of EGFP were digested with EcoRI and XbaI and cloned into the corresponding restriction sites of pFastBacI (Gibco-BRL). Resulting plasmids without the VP1 or VP2 stop codons were designated pVP1EGFPfastbac, pVP2EGFPfastbac, and pEGFPfastbac.

All plasmid constructions were further digested with appropriate restriction enzymes (italic) and transferred to pFastbacI. Genetic constructs had the polyhedrin promoter (SnaiBI and BamHI) replaced with the cytomegalovirus-immediate early promoter (CMV) (NruI and BamHI). Nucleotides of the fusion protein junction (bold), EcoRI site (underlined), and corresponding amino acids (bold) are outlined.

2.2. Transduction of mammalian cells

Approximately 1×10^6 NLFK cells/ml were grown overnight on coverslips at +37°C. Next day, growth medium was replaced with a mixture of 30 μl DMEM–FCS and 30 μl of virus inoculum (Ac:CMVVP1, Ac:CMVVP2, Ac:CMVVP1EGFP, Ac:CMVVP2EGFP, Ac:CMVVEGFP and Ac:CMVVEGFPVP2 or combinations thereof), containing 1×10^6 plaque-forming units (PFUs) per ml for each coverslip. After incubation (30 min. 37°C), 5 ml DMEM was added to the coverslips. Cells were incubated (+37°C, 48 h), fixed (100% methanol, 6 min, −20°C) and left in PBS at +4°C until immunostaining. Infection of baculovirus-transduced NLFK cells, initially transduced (24 h) with Ac:CMVVP2EGFP, was followed by infection with wild-type CPV [61]. Wild-type CPV-infected cells served as controls. At 48 h post transduction (24 h post-infection), cells were fixed as above.

2.3. Immunostaining of cells

Primary antibodies used for probing proteins were a polyclonal rabbit anti-VP1 antibody, specific for the unique N-terminal sequence of CPV viral proteins (from Collin Parrish), mouse monoclonal anti-capssid antibody [63, 64], and monoclonal mouse anti-non-structural protein 1 (anti-NS1, from Collin Parrish). Secondary antibodies used were alexa-633 (violet) conjugated anti-rabbit antibody and alexa-546 (red) conjugated anti-mouse antibody (Molecular Probes, Eugene, OR). Cells were permeabilized and immunostained and examined with a laser scanning fluorescence microscope (LSM 510, Zeiss) as described [31].

3. Results

Subcellular targeting of the structural proteins of CPV during an authentic infection of NLFK cells is shown in Fig. 2. The proteins were identified using a battery of antibodies specific for VP1 (anti-VP1; [62]), all forms of VP1 and VP2 (anti-VP; from Collin Parrish), or assembled VP2 (anti-capssid; [63, 64]). As can be seen, both VP1 (Fig. 2(a) and (d)) and assembled forms of VP2 (Fig. 2(b), (e) and (h), (k)) were targeted to the nucleus even though they were also present in the cytosol. On the contrary, antibodies recognizing all forms of both proteins detected antigens quite evenly throughout the infected cell (Fig. 2(g) and (j)). The differential interference contrast (DIC, Fig. 2(c) and (i)) and merged (Fig. 2(f) and (l)) images are shown. The roles of VP1 and VP2 in nuclear
targeting were studied by development of a mammalian baculovirus delivery system as described below.

3.1. Individual expression and subcellular targeting of VP1 in baculovirus-transduced NLFK cells

Recombinant baculoviruses housing the individual structural proteins, VP1 and VP2, as well as EGFP and its fusion to VP2 were engineered for mammalian expression (Fig. 1). The subcellular distribution of the individual protein constructs in NLFK cells transduced with the corresponding viruses was studied by confocal imaging (Figs. 3–6) using the battery of CPV specific antibodies presented above as well as by direct viewing of EGFP fluorescence. In addition, the distribution of the fluorescence was quantified using an amount of 150–200 cells in each case. Immunostaining of cells transduced with the recombinant baculovirus, AcCMVVP1, revealed both nuclear and cytoplasmic localization of the VP1 protein (Fig. 3(a) and (d)). The VP1-specific antibody used here did not bind to cells transduced with the VP1-negative control virus, AcCMVEGFP (Fig. 3(g) and (j)). In these cells, EGFP was distributed quite evenly throughout the cell (Fig. 3(h) and (k)). The nuclei were identified by DIC (Fig. 3(c) and (i)) and the merged images of the corresponding images are also outlined (Fig. 3(f) and (l)).

3.2. Individual expression and subcellular targeting of VP2 in baculovirus-transduced NLFK cells

Subcellular targeting of the VP2 proteins in NLFK cells transduced with AcCMVVP2 and AcCMVVP2EGFP is portrayed in Fig. 4. Immunostaining of unassembled VP2 or VP2EGFP proteins detected with anti-VP antibodies showed an equal cytosolic and nuclear presence (Fig. 4(a), (e) and (i), (m)). When stained for assembled VP2, a predominantly cytoplasmic location was revealed with little staining observable in the nucleus (Fig. 4(b), (f) and (j), (n)). As compared to the nuclear accumulation distribution of EGFP (Fig. 3(h) and (k)), the fluorescent chimeric version of VP2, VP2EGFP, was found to be in discrete large areas in the cytoplasm when analyzed by direct viewing (Fig. 4(k) and (o)). Similarly, the
localization of assembled VP2 (Fig. 4(b) and (f)) was cytosolic when expressed alone suggesting that the fluorescent fusion partner, EGFP (Fig. 4), did not affect the intracellular localization of VP2. The DIC (Fig. 4(d) and (i)) and merged (Fig. 4(h) and (p)) images are shown.

Together, these results show that the viral vectors were functional and that VP1 displays a pronounced nuclear targeting capacity. However when expressed alone, unassembled VP2 appeared to be distributed quite evenly throughout the cell, whereas it appeared to retain in the cytosol if assembled.

3.3. Co-expression of VP1 and VP2 and consecutive subcellular targeting of VP2

To elucidate the interaction of the VP1 and VP2 proteins, NLFK cells were co-transduced with the recombinant baculoviruses AcCMVVP1 and AcCMVVP2 (Fig. 5). Cells stained for VP1 showed an increase, targeting of the protein to the nucleus (Fig. 5(a) and (d)) as compared to when expressed alone (Fig. 3(a) and (d)). Interestingly, the anti-capsid antibody showed a shift of stained antigens from the cytosol (Fig. 4(b) and (j)) to the nuclear compartment (Fig. 5(b), (e) and (h), (k)). Similarly, an increase in nuclear targeting was seen when cells were probed using antibodies directed against all forms of both structural proteins (Fig. 5(g) and (j)) as compared to VP2 seen in Fig. 4(a) and (e). Again, the nuclei were identified by DIC (Fig. 5(c) and (i)). The merged images are also outlined (Fig. 5(f) and (l)). These results clearly suggest that the transport of the viral proteins into the nucleus is enhanced by the presence of both.

3.4. CPV infection of NLFK cells expressing the VP2EGFP fusion protein

To verify the results described above, we carried out an experiment where NLFK cells transduced with a baculovirus construct harboring the VP2EGFP fusion were further infected with authentic CPV at 24 h post transduction (Fig. 6). Replication of CPV was confirmed using an antibody directed against the non-structural protein, NS1 (Fig. 6(b) and (f)). In the infected cells, where VP1 is produced due to the CPV infection, the fluorescent VP2EGFP fusion protein was clearly observed both in the cytosol and the nucleus, but with a pronounced nuclear targeting (Fig. 6(c), (g) and (k), (o)) when viewing EGFP. In contrast, the VP2EGFP protein was found predominantly in the cytosol in the absence of a CPV infection as shown above (Fig. 4(k) and (o)). Here, the cellular distribution of the CPV structural proteins is further shown using anti-VP (Fig. 6(a) and (e)), anti-VP1 (Fig. 6(i) and (m)), and the anti-capsid antibodies (Fig. 6(j) and (n)). This result suggests that at least VP1 is a prerequisite for targeting of VP2 into the nucleus and that there could be other components of a CPV infection that aid in this.

4. Discussion

Determinants of nuclear targeting of the VP1 and VP2 structural proteins of CPV expressed in NLFK cells were sought by using recombinant baculoviruses as genetic vectors. Here, the structural proteins were expressed under various conditions
aided in the analysis of the cellular distribution of this structural protein without other CPV viral components present. Antigens for VP1 were seen to have both a cytosolic and a nuclear distribution (Fig. 3). As VP1 has shown to possess a potential nuclear localization signal [16], targeting of this protein to nucleus (Figs. 2b and 3a, b; [8,19]) was expected. However, our findings that VP1 did not have a predominant nuclear accumulation are in contrast to other studies where wild-type infectious clones (MVM) were used [19]. The fact that singly expressed VP1 without other viral proteins did not accumulate as efficiently as in wild-type CPV (Fig. 2) suggests that the nuclear localization signal for VP1 is not the only requirement for this protein to have high accumulation in the nucleus. It is significant that in our study VP1 at 48 h did accumulate in the nucleus more efficiently when VP2 was present (see below, Figs. 5 and 6).

Nuclear translocation signals of VP2 of CPV have not been characterized. Previously, it was shown [8] that plasmid-expressed VP2 mutants incapable of assembly into VLPs were targeted to the nucleus as well as the cytosol in A72 cells. This feature of unassembled VP2 to be in the cytosol and nucleus has been seen with VP2 of other paroviruses (MVM; [19], AAV; [25,69]). In our study, VP2 probed with anti-VP was found both in the cytosol and nucleus (Fig. 4), but the antibody detecting assembled VP2 only gave staining in the cytosol. These findings suggest that nuclear transport of VP2 in the assembled form was hampered in the absence of other viral components that would be present in a normal infection. This is in contrast with the results of Yuan and colleagues [8], who showed singly expressed VP2 to accumulate in the nucleus of A72 cells when probed with anti-capsid and anti-VP antibodies at 48 h post-transfection. Use of different cell lines could explain this discrepancy. In the case of minute virus of mice (MVM), it has been suggested that VP2 enters the nucleus as a trimeric complex with VP1 [18,19]. In our study, the assembled VP2 remaining in the cytoplasm is consistent with the inaccessibility of a nuclear transport motif mapped in the internal capsid surface for MVM.

There are a number of possibilities that could contribute to the different intracellular distribution of VP1 and assembled VP2. Rapid accumulation of VP2 in the cytoplasm or binding to intracellular structures could inhibit entry into the nucleus. In support of this possibility, it has been shown that wt CPV has the capacity to bind to lipids [14]. Alternatively, VP2 could lack effective nuclear translocation signals. This is probably not the case, since non-assembled VP2 or VP2 in the presence of other viral genes and viral non-structural proteins has been shown to enter the nucleus [8,70]. We think the most probable explanation to be that interaction of VP2 with VP1 is needed to aid targeting of assembled VP2 into the nucleus. In fact, interaction of structural proteins to aid in nuclear translocation has been previously shown for other viruses [71–73]. The co-transduction experiments (Fig. 5) confirm that there is a cooperation of VP1 aiding VP2 into the nucleus.

To finally confirm the hypothesis that VP1 is necessary for efficient nuclear targeting of VP2, NLFK cells were transduced with a recombinant baculovirus harboring VP2 fused to the N-terminus of EGFP (Fig. 1). The transduced cells were then infected with wild-type CPV and studied by direct viewing of the fluorescent VP2-EGFP fusion protein as well as by immunolabeling of assembled VP2 (Fig. 6). The VP2-EGFP fusion protein was not able to efficiently enter the nucleus (Fig. 3) and thus behaved in a similar way as VP2 alone (Fig. 3). However,
when the transduced cells were infected with CPV, a clear nuclear accumulation of the VP2-EGFP fusion protein was observed (Fig. 6). These results suggest that VP2 needs its structural partner VP1 and/or other components of a wt infection in order to be efficiently targeted to the nucleus.

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References


Fig. 6. Confocal imaging of NLFK cells transduced and infected with AcCMVVP2EGFP and authentic CPV, respectively (see Section 2). Nuclear accumulation of the authentic and recombinant viral proteins was visualized with anti-VP (a), anti-VP1 (i) and anti-capsid (j) antibodies or by direct viewing of the VP2EGFP fusion protein (c and k). DIC (d and i) and merged (h and p) images are shown to display the nucleus and colocalization of the labeled proteins, respectively. Cells were also stained with anti-NS1 (b) against the non-structural protein 1 to confirm CPV infection. Confocal images are 0.7 μm thick midsections of the cell in the same field. Bars 10 μm. The subcellular distributions of viral proteins (e), NS1 (f), the VP2EGFP fusion protein (g and o), VP1 (m), and the capsid-like structures (n) are shown as an average percentage (n > 150) from two independent experiments. Cells were classified into three categories as described in the legend to Fig. 2.


