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Nomenclature

AAV	Adeno-associated virus
ADV	Aleutian mink disease parvovirus
B19V	Human parvovirus B19
Bp	Base pairs
CPV	Canine parvovirus
DMEM	Dulbecco modified Eagle's medium
FCS	Fetal calf serum
FPV	Feline parvovirus
IF	Immunofluorescence
mAb	Monoclonal antibody
MVM	Minute virus of mice
MVMi	Immunosuppressive strain of MVM
MVMp	Prototype strain of MVM
PCR	Polymerase chain reaction
PPV	Porcine parvovirus
qPCR	Quantitative PCR
SN	Supernatant
ssDNA	Single stranded DNA
SV40	Simian vacuolating virus 40 or Simian virus 40

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Part I

Introduction

1. Introduction

1.1. Tropism

Most parvoviruses, such as MVM, CPV and FPV , show specific host ranges and tissue tropisms. The majority of the parvoviruses are members of those tightly controlled viruses. However, some parvoviruses, as for example many of the AAVs, infect many hosts and a variety of tissues. Understanding controls of these properties for autonomous parvoviruses show great promise for the therapeutic delivery to be controlled or modified in gene therapy applications [1].

To date, two independent strains of the parvovirus MVM have been described to occur in mice. Both strains display disparate *in vitro* tropism and *in vivo* pathogenicity despite differing by only 14 amino acids in their capsid proteins. First, MVMp, the prototype strain, was originally isolated from a contaminated murine adenovirus stock and was shown to replicate efficiently in mouse fibroblasts [2]. Secondly, MVMi, the immunosuppressive strain, was recovered from an infected EL-4 T-cell lymphoma culture [3]. Although MVMi infection may result in pathology of infected mice, it has been shown that the infection more likely interferes with numerous T-cell functions *in vitro*. The infection rather causes problems for the ongoing study the mice are being used for as the immune system will be activated, the activity of T-lymphocytes or B-lymphocytes will be altered and tumor formation may be suppressed [4, 5, 6].

As above-mentioned, it has been shown that the allotropic MVM strains, MVMi and MVMp, differ in their target cell tropism. In order to map the viral sequences responsible for that difference, chimeric viral genomes were constructed *in vitro* from infectious genomic clones of both strains. The differences in the cell tropism between MVMi and MVMp have been mapped to the capsid gene, in particular to the VP2 residues 317 and 321. Both residues are located at the base of the threefold spike of the virion and are involved in controlling the cell tropism of the two distinct MVM strains [7, 8, 9]. Interestingly, residue 321 aligns with residue 323 of CPV, that itself is a critical

determinant for CPV host range tropism. Further residues (VP2 residues 399, 553 and 558) were identified in MVMi to be able to confer fibrotropism to single site-directed mutants. Those residues cluster around the twofold dimple-like depression [10].

In general, tissue tropism determining amino acids were found be located on, or near, the viral surface, mainly by raised structural elements around the threefold axes of symmetry. Differences in tropism and pathogenicity have also been mapped to the capsid proteins of Aleutian mink disease parvovirus (ADV) [11], porcine parvovirus (PPV) [12], CPV [13, 14], and FPV [15] in a capsid region analogous to that observed for MVM. Although the same structural element of viruses is involved in mediating host and tissue tropisms, each appears to be affecting a different mechanism. Host ranges of CPV and FPV are controlled by receptor binding, whereas the cell tropisms of MVM appear to be due to restrictions of interactions with intracellular factors [10, 1].

1.2. Structure

1.2.1. Parvoviruses in general

Parvovirus capsids are devoid of a lipid envelope and have an average diameter of 18 to 26 nm. The viral capsid is made up of 60 copies of between two and four structural proteins that overlap each other. For each virus there is one major capsid protein present in the capsid structure. Minor proteins form the same core structure, but differ in the sequence length on their amino termini. The capsid proteins display a T=1 icosahedral symmetry and are variously designated VP1-VP4. Thus, the capsid has a 5-3-2 point group symmetry containing 31 rotational symmetry elements that intersect at the center: six fivefolds, ten threefolds, and fifteen twofolds. Despite the differences in protein forms and the low homology between some of the viruses, several structural elements on the capsid surface are common to most parvoviruses. These include raised cylindrical channels at the fivefold axes surrounded by depressed, canyon-like regions. Further shared surface characteristics are protrusions at the threefold axes, termed as spikes or peaks, and dimple-like depressions at the icosahedral twofold axes. A common feature of parvoviruses is their high resistance to physicochemical treatments. This stability provides an effective protection to the fragile, condensed genome in the extracellular environment ensuring transmission between their hosts. The ssDNA genome consists of approximately 5000 bases, packed as either a positive or, more usually, as a negative

sense strand. At the 5' and 3' ends, the genome harbors palindromic sequences of about 120 to 250 nucleotides, that form secondary hairpin structures which are essential for the initiation of viral genome replication [16, 17, 18, 10, 19, 20, 1].

1.2.2. MVM

Both DNA-containing full and empty particles were crystallized in the monoclinic space group C2. Following data processing and refinement, the resulting electron density map was interpreted with respect to the amino acid sequence of MVMi. The known CPV structure was used as a phasing model since 52 % of the 578 amino acids in VP2 of MVM are identical to CPV. The polypeptide chain of the major structural protein, VP2, could be traced from residue 39 to residue 587 at the C-terminus [21]. The common c-terminal part of the structural proteins has an eight-stranded antiparallel β-barrel topology, frequently found in viral capsid proteins [22]. Large loops between the β-strands of the β-barrel that form the principal surface features, particularly the threefold spikes, and determine host-range tropism were found to be quite dissimilar in MVM and CPV. The first 37 amino acids are not visible in the electron density map. Since the N-VP2 terminal part contains a predominantly poly-glycine conserved sequence, it might be highly flexible. There is density extending along the fivefold channels of the MVMi capsid that was modeled as the glycine-rich N-terminal region [23, 24]. *In vitro*, trypsin digestion of full MVM virions results in a truncated VP3 polypeptide that still contains the glycine-rich sequence. In this way, most VP2 N-termini can be cleaved. These findings suggest that there is a dynamic situation at the fivefold channel. In one model, one in five amino termini are externalized along the fivefold axes and are accessible for cleavage. Newly created, cleaved N-VP3 termini could withdraw into the virion and be replaced at the surface by an uncleaved N-VP2 terminus. [25, 10]. A substantial amount of internal electron density could be related to 10 DNA nucleotides that were previously found in the analysis of the structure of CPV [26, 27]. For MVM, 19 additional DNA nucleotides were identified in a difference electron-density map with respect to the data of empty particles. Thus, 29 ordered, or partially ordered, nucleotides per icosahedral asymmetric unit imply that approximately 34 % of the total genome display icosahedral symmetry. This finding, and the conservation of base-binding sites between MVMi and CPV, identifies a DNA-recognition site on the parvoviral capsid interior [10].

Despite the differences in protein forms and the low homology between some of the viruses, it is now clear that several prominent structural elements on the capsid surface are common to most parvoviruses. These include raised regions at the fivefold axes of symmetry, which in some viruses might form a pore into the capsid, depressed regions (canyons) surrounding the fivefold axes, one or three protrusions at or surrounding the threefold axes of symmetry (threefold spikes or peaks) and depressed regions (dimples) at the twofold axes of symmetry (Figure 1 and Figure 2)

1.2.3.

1.2.4.

1.2.5.

1.2.6.

1.3.

1.3.1.

1.3.2.

2. Methods

2.1. Cell Cultures

A9 ouab^{r}l1 cells, a derivative from the original HGPRT^{-} L-cell line A9 represent a clone resistant to 10^{-3} M ouabain after nitrosoguanidine mutagenesis [28]. NB324K cells are a clone of SV40-transformed human newborn kidney cells [29]. The SV40 large T antigen was detected by immunofluorescent staining with monoclonal antibodies [30]. However, NB324K cells do not produce infectious SV40 spontaneously. Both cell lines, A9 mouse fibroblasts and NB324K cells, were routinely propagated under a minimal number of passages in DMEM supplemented with 5 % of heat inactivated fetal bovine serum at 37 ℃ in 5 % CO_2 atmosphere.

2.1.1. Freezing and thawing of cells

Before use the A9 mouse fibroblasts or NB324K cells were thawed at 37 ℃ and cultured in 5 mL of pre-warmed DMEM supplemented with 5 % FCS. The medium was replaced every 3 to 4 days. In order to freeze the cells for long storage in liquid nitrogen they were passed the day before, to ensure exponential growth. Subsequently, 7.5 % DMSO was added and the cells were frozen slowly at -70 ℃ over night before transfer to liquid nitrogen.

2.2. Virus Stocks

Stocks of MVM without detectable levels of VP3 were propagated on A9 mouse fibroblast monolayers. As soon as the cytopathic effect became evident, the supernatant was collected and pre-cleared from cell debris by low-speed centrifugation. Thereby, intracellular, VP3 rich capsids were discarded. In order to remove low-molecular contaminants, virus containing SN was pelleted through 20 % sucrose cushion in PBS by

ultra-centrifugation. Virus titers were determined by qPCR as DNA-packaged particles per microliter.

2.2.1. Separation of empty and full capsids

Sucrose purified capsids were prepared as previously described in section 2.2, page 6. The virus pellet was resuspended in 10 mL PBS. Caesium chloride was added to a density of 1.38 g/mL adjusted by refractometry (η=1.371) at 4 ℃. The gradient was centrifuged to equilibrium for 24 h at 41000 rpm and 4 ℃ in a Beckmann SW-41 Ti rotor. Gradients were fractionated and tested for intact capsids by dot blot analysis using B7 mAb. CsCl was depleted from the corresponding fractions by size-exclusion chromatography through PD-10 desalting columns and concentrated by ultra-centrifugation when required.

2.3. Freezing bacteria stocks in glycerol

Bacteria were frozen in dry ice. A volume of 700 μL of the bacteria culture that was grown over night in LB-medium was mixed with 300 μL of 50 % glycerol in a cryotube. In order to mix well the glycerol the cryotube was vortexed intensively. Following snap-freeze in dry ice the bacteria were stored at -70 ℃.

2.4. Anion-exchange chromatography

A Mono Q HR 5/5 (Pharmacia) column (5 x 50 mm) was used to analyse viral samples. The Mono Q column was connected to the ÄKTAmicro chromatography system (GE Healthcare) that was operated by the UNICORN control software. The Mono Q column was equilibrated with six column volumes (CV) starting buffer (20 mM Tris-HCl, 1 mM EDTA, pH 7.2). Samples (1 mL) containing at least 10^{10} virus particles in 10 mM Tris-HCl, 1 mM EDTA, pH 8 were applied to the Mono Q column trough a 2 mL loop. After eluting the protein, which did not bind to the column in the starting buffer, a linear salt gradient (0-2 M NaCl) in 20 mM Trsi-HCl, 1 mM EDTA, pH 7.2, was applied. Fractions of 0.185 mL were collected in 96-well plates. Viral genomes in each fraction were quantified by qPCR. All buffers were filtered and degassed before application to the Mono Q column.

2.4.1.

2.4.2.

2.4.3.

2.5.

2.5.1.

2.5.2.

Figure 2.1 shows a photograph of D440N.

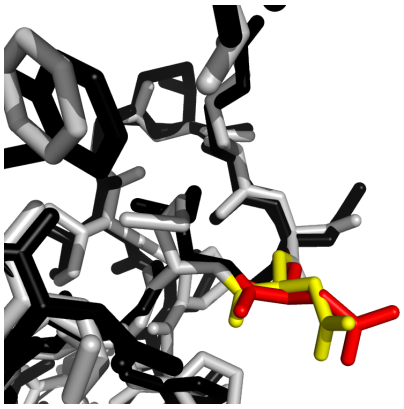

Figure 2.1.: Structure of D440N

Part II

Publication

Part III

Discussion

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