Virology 432 (2012) 45–56

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Essential role of the unordered VP2 n-terminal domain of the parvovirus MVM capsid in nuclear assembly and endosomal enlargement of the virion fivefold channel for cell entry

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ARTICLE INFO

Article history:
Received 16 March 2012
Returned to author for revisions
24 April 2012
Accepted 24 May 2012
Available online 23 June 2012

Keywords:
Parvovirus
Peptide insertion
Assembly
Cell entry
Capsid cleavage
Virus traffick

ABSTRACT

The unordered N-termini of parvovirus capsid proteins (Nt) are translocated through a channel at the icosahedral five-fold axis to serve for virus traffick. Heterologous peptides were genetically inserted at the Nt of MVM to study their functional tolerance to manipulations. Insertion of a 5T4-single-chain antibody at VP2-Nt (2Nt) yielded chimeric capsid subunits failing to enter the nucleus. The VEGFR2-binding peptide (V1) inserted at both 2Nt and VP1-Nt efficiently assembled in virions, but V1 disrupted VP1 and VP2 entry functions. The VP2 defect correlated with restricted externalization of V1-2Nt out of the coat. The specific infectivity of MVM and wtVP-pseudotyped mosaic MVM-V1 virions, upon heating and/or partial 2Nt cleavage, demonstrated that some 2Nt domains become intracellularly translocated out of the virus shell and cleaved to initiate entry. The V1 insertion defines a VP2-driven endosomal enlargement of the channel as an essential structural rearrangement performed by the MVM virion to infect.

Introduction

Viral capsids are nano-sized macromolecular containers evolved to deliver nucleic acid at specific compartments of the host cells. The traffic of capsids from the surface receptor to the appropriate compartment for disassembly to occur is driven by protein signals, which are exposed to host factors following programmed structural rearrangements in the capsid integrity (Mercer et al., 2010). But natural virus features may be engineered with heterologous peptides inserted at rationally selected capsid protein domains. This strategy is being applied to retarget viruses at specific cell types, use them as carrier of foreign antigens, or to infer capsid functions in the life cycle.

The *Parvoviridae*, eukaryotic nuclear viruses containing a 5 kb single stranded DNA genome in a 25 nm-diameter $T=1$ icosahedral nonenveloped capsid (Tijssen et al., 2011), are major candidates for rational capsid retargeting as their 3-D atomic structure is available from many members (e.g. Agbandje-McKenna et al., 1998; Kontou et al., 2005; Kaufmann et al., 2004; Tsao et al., 1991; Xie et al., 2002) and exhibit a wide host range and complex biology. Common features of the parvoviral capsids are the folding of the protein subunits into an eight-stranded antiparallel β-barrel topology, a β-cylindrical projection encircled by a canyon-like depression that surrounds the fivefold symmetry axes, and a pore at the center of the β-cylinder running between the surface and the interior of the capsid. The capsid surface however may drastically differ among the parvoviruses due to the presence of prominent loops, depressions, or smooth surfaces (Gurda et al., 2010), which confer characteristic functions.

Insertional mutagenesis of the *Parvoviridae* capsid with heterologous peptides yielded varying outcomes. Despite functional constraints and deleterious effects of the heterologous peptides on particles formation and infectivity, the Adeno-Associated Virus (AAV) of the *Dependovirus* could be retargeted (Asokan et al., 2010; Girod et al., 1999; Muller et al., 2003). The identification of permissive sites for large insertions (Warrington et al., 2004) allows obtaining recombinant AAV viruses for multiple ongoing gene therapy applications (reviewed in Vandenberghe et al., 2009). Among the members of the autonomously replicating *Parvovirus* genus, the insertion of the RGD motif on the Feline Parvovirus (FPV) capsid surface severely impaired virus yield, although allowed moderate transduction of certain human tumor

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http://dx.doi.org/10.1016/j.virol.2012.05.025

cells (Maxwell et al., 2001). Heterologous peptides engineered in the most exposed ordered surface loops of virus like particles (VLPs) of the Canine (CPV), Porcine (PPV), and Minute Virus of Mice (MVM) parvoviruses partly or completely impair stability and assembly (Hurtado et al., 1996; Carreira et al., 2004). Nevertheless, the insertion of heterologous peptides at some positions of loop 2 allowed chimeric VLPs of PPV and CPV to be produced in amounts enough to induce specific antibodies against the inserted epitope (Rueda et al., 1999). It should be noted however that VLPs are not adequate particle entities to study virus retargeting, as they assemble at non-physiological high amounts in the cytoplasm of insect cells (Yuan and Parrish, 2001; Riolobos et al., 2010), disregarding the relevance of phosphorylation and signal-mediated nuclear transport of intermediates in the natural parvovirus assembly (Lombardo et al., 2000; Riolobos et al., 2006, 2010).

Unordered capsid domains should better tolerate peptide insertions. The n-termini (Nt) of the capsid proteins (VP) are indeed flexible sequences generally unresolved in the crystal structure of autonomous parvoviruses (Agbandje-McKenna et al., 1998; Kontou et al., 2005; Tsao et al., 1991) and AAV (Xie et al., 2002), with the exception of the n-terminus of the B19 *Erythrovirus* major capsid protein, resolved adjacent to the β-cylinder (Kaufmann et al., 2008). However these unordered Nt sequences play important roles in the virus particle traffic between cellular compartments, as proposed for MVM (Maroto et al., 2004) and subsequently for other parvoviruses (Sonntag et al., 2006; Johnson et al., 2010), which must be taken into account when infectious viruses are manipulated. The Nt of VP1 contains a large unique region (VP1uR) that was shown to be essential for the incoming parvovirus particle to initiate infection (Zadori et al., 2001; Lombardo et al., 2002). Functional domains mapped at VP1uR conferring infectious entry competence to the virus were: (i) a conserved conventional nuclear localization sequence (NLS) that targets the VP1 protein to the nucleus (Lombardo et al., 2002; Sonntag et al., 2006; Vihinen-Ranta et al., 2002); (ii) a phospholipase A2 (PLA2) motif that provides the virus with the means to breach the endosomal membrane (Zadori et al., 2001; Girod et al., 2002; Farr et al., 2005); and (iii) others as yet unclear functions ascribed to clusters of basic amino acids (Lombardo et al., 2002) and to PPXY motives (Cotmore and Tattersall, 2007).

The unordered n-terminal domain of the VP2 major capsid protein (2Nt) is projected outside of the DNA-full parvovirus particle through the fivefold cylinder (Agbandje-McKenna et al., 1998; Kontou et al., 2005; Tsao et al., 1991). These structural data are consistent with the accessibility of 2Nt to proteases and antibodies concomitantly with the maturation of DNA-filled virions (Cotmore and Tattersall, 2007; Cotmore et al., 2010), whereas this domain remains internal in purified empty native capsids (Tattersall et al., 1977), or VLPs (Hernando et al., 2000). Phosphorylation at three 2Nt serine residues by the Raf-1 kinase was required for nuclear translocation of MVM assembly intermediates (Riolobos et al., 2010) and nuclear egress of mature virus in certain cellular hosts (Maroto et al., 2004), but it was not relevant in the initiation of the infection (Maroto et al., 2000). The 2Nt domain may be cleaved from VP2 to form the VP3 protein (Paradiso, 1981) by a chymotrypsin-like protease (Tattersall et al., 1977; Paradiso et al., 1984; Tullis et al., 1992). This cleavage occurs in the pH-dependent entry pathway of the members of the *Parvovirus* genus (Weichert et al., 1998; Ros et al., 2002; Boisvert et al., 2010; Parrish, 2010), soon after virus internalization (Mani et al., 2006). In vitro, VP2/VP3 cleavage triggered a pH dependent structural rearrangement of the MVM virion leading to VP1uR externalization (Farr et al., 2006). However the function of this cleavage in the infection is unclear as it could not be prevented by either mutagenesis of the putative cleavage site (Tullis et al., 1992) or protease inhibitors (Cotmore and Tattersall, 2007), and viral stocks exhibiting variable VP2/VP3 ratios (Tattersall et al., 1976, 1977) or even lacking VP3 subunits (Maroto et al., 2004), were equally infectious.

We have investigated the suitability of the unordered Nt domains for capsid display of heterologous peptides in an infectious MVM particle. Since our long-term goal is to retarget the genuine oncolytic parvovirus features (reviewed in Rommelaere et al., 2010) to certain tumor cells, two different peptides with affinity for surface receptors of transformed cells were inserted: (i) a single-chain antibody directed against the oncofaetal 5T4 antigen (Shaw et al., 2000), a tumor marker overexpressed in malignant tumors (Fritsche and Mach, 1975); and (ii) the 7-mer ATWLPPR peptide (Binetruy-Tournaire et al., 2000) ligand of the vascular endothelial growth factor receptor type 2 (VEGFR2) implicated in neoangiogenesis (Plate et al., 1992). These peptides were tested for their compatibility with MVM assembly, viral genome encapsidation, exposure on the capsid, and impact on the infectivity of the engineered virions. This study sheds light into the relevance of the configuration of parvovirus assembly intermediates for nuclear transport, and into the orchestrated intracellular capsid structural rearrangement and cleavage that the MVM virion must undergo for cell entry.

Results

Defect of VP2/scAb-5T4 in nuclear translocation and capsid assembly

As a first attempt on MVM retargeting by peptide insertion, the large 286 amino acids long scAb-5T4 polypeptide (Lamikanra et al., 2005) was engineered between residues Met1 and Ser2 of the flexible 2Nt domain in a VP2-only MVMi plasmid (see Materials and methods). This strategy was adopted due to the inability of full-size MVM vectors to encapsidate additional foreign sequences larger than a few hundred nucleotides (Kestler et al., 1999), and to the capacity of VP2 for forming capsids by itself (Tullis et al., 1993), and being trans-complemented by VP1 for nuclear assembly (Lombardo et al., 2002). The recombinant VP2/scAb-5T4 protein was expressed in transfected NB324K cells at the expected size of about 140 kDa (Fig. 1A). To study VP2/scAb-5T4 subcellular distribution and assembly in respect to wtVP subunits, cells were transfected and stained by IF with MVM-specific antibodies (α-VPs, α-VP1, and α-capsid), and with an antibody recognizing the tag of sc5T4-myc. The VP2/scAb-5T4 recombinant protein consistently accumulated as perinuclear cytoplasmic aggregates failing capsid formation (Fig. 1B, upper row), whereas wtVP proteins showed their characteristic efficient nuclear capsid assembly (Fig. 1B, lowest row).

In an attempt to rescue the nuclear uptake of some VP2/scAb-5T4 subunits, we used the capacity of the MVMi capsid proteins to cooperatively interact in the cytoplasm for nuclear import (Lombardo et al., 2000, 2002). Indeed VP1, and more efficiently VP2, wt capsid proteins allowed the co-transport of incompetent VP mutant subunits (Lombardo et al., 2002), in keeping with the VP composition of the two types of trimers that translocated into the nucleus (Riolobos et al., 2006). To this end, VP2/scAb-5T4 was co-expressed with both wtVP proteins, either singly expressed or combined at the VP1:VP2 1:5 ratio found in the physiological assembly intermediates (Riolobos et al., 2006), and their subcellular distribution and capsid assembly phenotype assessed by IF (Fig. 1B, middle three rows). Neither of the wt subunits, whether expressed alone or in combination, was able to significantly facilitate the nuclear import of the VP2/scAb-5T4 protein, as the sc5T4 signal remained cytoplasmic in all cases. On the contrary, the expression of recombinant protein resulted in a significant