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Differential phosphorylation and n-terminal configuration of capsid subunits in parvovirus assembly and viral trafficking

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ABSTRACT

The T1 parvovirus Minute Virus of Mice (MVM) was used to study the roles that phosphorylation and N-terminal domains (Nt) configuration of capsid subunits may play in icosahedral nuclear viruses assembly. In synchronous MVM infection, capsid subunits newly assembled as two types of cytoplasmic trimeric intermediates (3VP2, and 1VP1:2VP2) harbored a VP1 phosphorylation level fivefold higher than that of VP2, and hidden Nt. Upon nuclear translocation at S phase, VP1-Nt became exposed in the heterotrimer and subsequent subviral assembly intermediates. Empty capsid subunits showed a phosphorylation level restored to VP1:VP2 stoichiometry, and the Nt concealed in their interior. However ssDNA-filled virus maturing at S/G2 lacked VP1 phosphorylation and one major VP2 phosphopeptide, and exposed VP2-Nt. Endosomal VP2-Nt cleavage resulted in VP3 subunits devoid of any phospholabel, implying that incoming viral particles specifically harbor a low phosphorylation status. Phosphorylation provides a mechanistic coupling of parvovirus nuclear assembly to the cell cycle.

1. Introduction

In eukaryotes, the activity of protein kinases contributes to many fundamental processes such as signal transduction, metabolism, and the cell cycle, by catalyzing the transfer of negatively charged phosphoryl moieties predominantly to serine, threonine, and tyrosine amino acid residues (Manning et al., 2002; Ubersax and Ferrell, 2007). Viral proteins may serve as substrates for cellular or virus-encoded protein kinases, their phosphorylation influencing structure and function through conformational changes or direct chemical effects on protein interactions. Understanding the biological effect of phosphate substituents incorporated in virus structural proteins is challenged by their transient nature, technical difficulty to precisely map phosphorylation sites, and the lack of resolution in the 3-D structure of virus particles. Nevertheless phosphorylation of structural proteins of RNA and DNA viruses mediates processes key to their life cycles, such as recognition of cellular factors, assembly of viral components, genome packaging, or viral trafficking (e.g. Sugai et al., 2014; Mondal et al., 2015; Bjorn-Patrick and Roy, 2016; Zhang et al., 2016), and may provide targets for antiviral therapies. Icosahedral DNA animal viruses (such as *Herpesviridae, Adenoviridae, Papillomaviridae, Polyomaviridae, or Parvoviridae*) whose structural components must traffick through the nuclear envelope to assemble and mature, conform a framework of viral systems to explore the diversity of functions played by capsid proteins phosphorylation.

With the aim of constructing a comprehensive virus model on this issue, we chose the *Protoparvovirus* Minute Virus of Mice (MVM), a reference member of the *Parvoviridae* (Cotmore et al., 2014), and an important mouse pathogen (Brownstein et al., 1991; Ramirez et al., 1996; Segovia et al., 1999). Parvoviruses are nonenveloped eukaryotic nuclear viruses containing a 5 kb single-stranded (ss) DNA genome in a 25 nm-diameter icosahedral (T = 1) capsid made from two to three polypeptides. The MVM capsid is composed of about ten subunits of the VP1 (83 kDa) and fifty subunits of the VP2 (64 kDa) proteins (Cotmore and Tattersall, 2014). The 3-D atomic structure of the capsid resolved for many parvoviruses exhibits a common folding of the protein subunits in an eight-stranded antiparallel β-barrel topology, whereas the capsid surface may differ drastically due to prominent loops and

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depressions which confer characteristic functions (Tsao et al., 1991; Agbandje-McKenna et al., 1998; Xie et al., 2002; Kaufmann et al., 2004; Kontou et al., 2005; Gurda et al., 2010). The N-terminal (Nt) sequences of the parvovirus capsid proteins (VPs) are flexible sequences with generally unresolved structure in the crystals, with the exception of Nt of the human B19 major capsid protein (Kaufmann et al., 2008). However these unordered Nt can be alternatively exposed on the surface of the capsid in controlled processes to serve as trafficking signals at different stages of the viral life cycle (Maroto et al., 2004; Valle et al., 2006). The unique VP1 Nt sequence (1Nt) contains diverse protein motifs required by the incoming virus to initiate infection, such as phospholipase A2 (PLA_2) activity (Zadori et al., 2001; Farr et al., 2005), nuclear localization sequences (NLS) (Vihinen-Ranta et al., 2002; Lombardo et al., 2002; Sonntag et al., 2006; Johnson et al., 2010; Boisvert et al., 2014), and other functionally uncharacterized domains (Tullis et al., 1993; Lombardo et al., 2002; Popa-Wagner et al., 2012; Porwal et al., 2013). The VP2 Nt sequence (2Nt) localized in the interior of empty capsids can be externalized by heat in vitro (Hernando et al., 2000; Carreira et al., 2004; Riolobos et al., 2010), but in DNA-filled virions some 2Nt are projected outside the capsid (Cotmore and Tattersall, 2007; Plevka et al., 2011; Subramaniana et al., 2017) presumably through the fivefold cylinder (Tsao et al., 1991; Agbandje-McKenna et al., 1998). The 2Nt serves as a signal for active nuclear export of mature MVM prior to cell destruction, an activity that may be crucial for successful viral dissemination in tissues (Maroto et al., 2004). Additionally, although devoid of recognized import signals, some intracellular exposure-competent 2Nt are required for the incoming virus to initiate infection (Sánchez-Martínez et al., 2012; Castellanos et al., 2013). 2Nt externalization presumably enlarges the capsid pore in the endosome (Sánchez-Martínez et al., 2012), facilitating its cleavage-off VP2 subunits observed in the pH-dependent entry pathway of many parvoviruses (Tullis et al., 1992; Mani et al., 2006; Boisvert et al., 2010; Parrish, 2010), a dynamic process leading to 1Nt externalization (Cotmore et al., 1999; Farr et al., 2006; Cotmore and Tatersall, 2014; reviewed in Ros et al., 2017).

In productively infected cells, parvovirus capsid assembly takes place in the nucleus (Hoque et al., 1999; Lombardo et al., 2000), involving orchestrated interactions among capsid subunits. In MVM, assembly begins with the formation of two trimeric intermediates in the cytoplasm at stoichiometric amounts, a homotrimer (3VP2) and a heterotrimer (1VP1/2VP2) (Riolobos et al., 2006). Isolated trimers were nuclear transport competent in permeabilized cells without any other viral component (Riolobos et al., 2010; Gil-Ranedo et al., 2015). Trimers are translocated into the nucleus driven by a structured protein motif (NLM) displaced on the inner capsid surface as an amphipathic beta-strand (Lombardo et al., 2000). Similar non-conventional nuclear transport sequences localized in partially overlapping or contiguous homologous amino acid sequence of the NLM (Valle et al., 2006), were required for nuclear capsid assembly of the human B19 (Pillet et al., 2003), and porcine PPV (Boisvert et al., 2014) parvoviruses. These structured transport motifs may constitute a quality control mechanism for the assembly pathway, precluding the nuclear import and assembly of misfolded subunits or trimers with unbalanced VP1 content (Lombardo et al., 2000). Within the nucleus, the MVM trimers interact through a few residues via hydrophobic and hydrogen bonds (Reguera et al., 2004), and must undergo conformational rearrangements to their final configuration in the capsid (Riolobos et al., 2006, 2010). In AAV2, an assembly-activating protein (AAP) encoded by the viral cap gene was essential for capsid assembly (Sonntag et al., 2010) presumably adapting the conformation of the VP subunits (Naumer et al., 2012).

The steps of the parvovirus life cycle (gene expression, nuclear translocation of proteins, capsid assembly, genome replication and encapsidation) are tightly coupled to the host cell cycle progression (Gil-Ranedo et al., 2015). Importantly, parvovirus MVM gene expression in synchronous infection occurred at G1/S, implying that transcription does not require a previous viral DNA amplification, which indeed occurred later at S/G2 phase (Gil-Ranedo et al., 2015). Capsid formation is particularly sensitive to cell cycle regulation, which is exerted at the level of non-conventional nuclear transport route(s) accessed by the assembly intermediates (Gil-Ranedo et al., 2015). The coupling of parvovirus assembly to the cell cycle may largely rely on control of capsid subunits phosphorylation. In MVM infection, VP1 and VP2 structural subunits assembled into empty capsids were post-translationally modified through a complex and VP-specific pattern of phosphoserine and phosphothreonine residues (Maroto et al., 2000). The 3-D structure of virus-like particles (VLPs) lacking phosphorylation (Hernando et al., 2000; Riolobos et al., 2010) that assembled in the cytoplasm (Riolobos et al., 2010; Yuan and Parrish, 2001) indicated that, at least for the VP2-only capsid, subunit phosphorylation is not important for icosahedral T = 1 ordering. However, nuclear transport of the VP2 homotrimer requires cytoplasmic phosphorylation by the Raf-1 kinase (Riolobos et al., 2010), although this phosphorylation was not sufficient to explain its cell cycle-regulated transport (Gil-Ranedo et al., 2015). Raf-1 phosphorylation targets serine residues of 2Nt (Maroto et al., 2000 and 2004), but localization of the many other phosphorylation sites in the VP1 and VP2 capsid subunits and their functions in the steps of the viral life cycle are unknown.

In this report, we have further investigated the phosphorylation and Nt configuration of the MVM structural proteins found in assembly intermediates and viral particles. Our focus was mainly on the less-studied VP1 subunits and their involvement in cell cycle-dependent VPs nuclear transport, capsid assembly, and MVM genome packaging. We show that VP1 subunits are hyperphosphorylated in cytoplasmic assembly intermediates, but are subjected to an orchestrated dephosphorylation programme during assembly that correlates with changes in Nt configuration. Furthermore, empty and DNA-filled virus particles drastically differed in the phosphorylation status of their VP1 and VP2 protein subunits. These data are integrated into an assembly and viral trafficking unified model of potential general interest to understand the life cycle of icosahedral nuclear viruses.

2. Materials and methods

2.1. Virus and cell culture

The prototype (p) strain of the *Protoparvovirus* Minute Virus of Mice (MVMp; Crawford, 1966) was used in this study and referred as MVM. The NB324K simian virus 40-transformed human newborn kidney cell line highly susceptible to the MVM strains (Gardiner and Tattersall, 1988), and a constitutively VPs-expressing stably transfected clone (Gil-Ranedo et al., 2015), were maintained under minimal number of passages in Dulbecco Modified Eagle Medium (DMEM) supplemented with 5% heat-inactivated foetal calf serum (FCS; Gibco BRL). Wherever indicated in the text, infected or transfected cells were synchronized at G1/S with the DNA polymerase-α antagonist aphidicolin, or at G1 upon transfection by density arrest, as described (Gil-Ranedo et al., 2015). Viral stocks used for infections were prepared from large-scale transfection using the pMM984 infectious plasmid (Tattersall and Bratton, 1983), and purified devoid of empty capsids as described (Sánchez-Martínez et al., 2012). Infectious virus titers and reliable plaque sizes were obtained by optimizing previously described plaque assay methods (Tattersall and Bratton, 1983; Rubio et al., 2005) as follows. NB324K cells were dispersed by carefully flushing three times through a 22G needle, and seeded at a density of 2×10^5 cells/P60mm dish. Virus dilutions and cell inoculation (0.25 ml of virus sample per P60mm dish) were performed the next day in complete PBS supplemented with 0.1% FCS. After virus adsorption (1 h at 37 °C under constant shaking) cells were overlaid with 7 ml/P60mm dish of a freshly prepared media composed of DMEM, 10% FCS, non-essential amino acids, and 0.7% low melting agarose (SeaPlaque™ Lonza, prepared in ddH_2O). Plaques were developed six days afterwards by fixing with 10% formaldehyde, and staining with 0.1% crystal violet prepared in 4% formaldehyde.