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Vaccinia virus protein A3 is required for the production of normal immature virions and for the encapsidation of the nucleocapsid protein L4

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ABSTRACT

Maturation of the vaccinia virion is an intricate process that results in the organization of the viroplasm contained in immature virions into the lateral bodies, core wall and nucleocapsid observed in the mature particles. It is unclear how this organization takes place and studies with mutants are indispensable in understanding this process. By characterizing an inducible mutant in the A3L gene, we revealed that A3, an inner core wall protein, is important for formation of normal immature viruses and also for the correct localization of L4, a nucleocapsid protein. L4 did not accumulate in the viral factories in the absence of A3 and was not encapsidated in the particles that do not contain A3. These data strengthen our previously suggested hypothesis that A3 and L4 interact and that this interaction is critical for proper formation of the core wall and nucleocapsid.

Introduction

Vaccinia virus, the best studied member of the Poxviridae family, encapsidates its dsDNA genome in a complex and unique structure. The vaccinia virion structure consists of a membrane surrounding a biconcave core with a lateral body in each concavity of the core (Moss, 2013). The core can be divided into two substructures, an external core wall and an internal nucleocapsid. The core wall contains two layers: the outer layer is formed by the proteins A10 (also known as 4a) and A4, while the inner layer is formed by the protein A3 (also known as 4b) (Moussatche and Condit, 2014; Ichihashi et al., 1984; Roos et al., 1996; Pedersen et al., 2000; Wilton et al., 1995). The protein composition of the nucleocapsid is not well defined, however it contains the viral transcription enzymes at a minimum (McFadden et al., 2012; Peters and Mueller, 1963). The L4 protein may serve as a scaffold for the nucleocapsid and the viral DNA is probably part of the nucleocapsid as well (Jesus et al., 2014). The vaccinia virus core is a dynamic structure, representing the environment where early viral transcription occurs upon entry of vaccinia virus in the host cell.

Because vaccinia encodes all the enzymes required for DNA replication and transcription, virus replication occurs essentially independently of the host cell nucleus. All stages of gene expression (early, intermediate and late) and virus assembly occur in the cell cytoplasm (Moss, 2013). The assembly of vaccinia virus particles occurs in areas called viral factories that are cleared of cell organelles (Condit et al., 2006). Briefly, membrane crescents, the first structure visualized in the cytoplasm, grow to form spherical immature virions after association with viroplasm. The immature virions encapsidate one copy of viral DNA and mature to form infectious viruses. The maturation requires cleavage of several viral proteins. During maturation the virions morph from spherical to the classical brick shape and the viroplasm mass is organized into the viral core and the lateral bodies. Even though several players in vaccinia morphogenesis have been characterized, the exact process by which these players orchestrate the arrangement of the viroplasm mass into lateral bodies, core wall and nucleocapsid is still obscure.

The A3 protein, together with A10 and L4, comprises one of the major core structural proteins and is one of the key players required for virus core formation. A3 is expressed during the late phase of gene expression as a 72.5 kDa precursor and is cleaved during virus maturation (Katz and Moss, 1970; Ansarah-Sobrinho and Moss, 2004). Several independent studies have suggested that A3 forms the inner layer of the core wall (Ichihashi et al., 1984; Wilton et al., 1995; Sarov and Joklik, 1972; Pedersen et al., 2000; Moussatche and Condit, 2014). In addition, A3 has the ability to bind DNA (Ichihashi et al., 1984).

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The importance of the A3 protein for the formation of the vaccinia virus core was revealed during the characterization of the thermo sensitive mutant Cts8 (Kato et al., 2004). Analysis of Cts8 showed that, in the presence of a mutated A3, mature viral particles are misshapen and have a defective virus core, which results in a block in viral early transcription. With the intention of extending the understanding of A3 function, we decided to compare the phenotype of Cts8, which encapsidates a mutated form of A3, with a mutant that lacks A3 altogether. For this purpose, we analyzed an inducible mutant in A3L gene. Analyzing both types of mutants is important for obtaining a complete understanding of protein function because in some cases a different phenotype is observed if the protein is absent from infection compared to an infection in which a mutated form of the protein is present (Boyd et al., 2010a, 2010b; Szajner et al., 2003; Mercer and Traktman, 2005).

In this study, we report that in the absence of A3 no infectious viral particles are produced. Under these conditions, cleavage of core proteins during maturation is inhibited, and abnormal immature particles accumulate in the cell cytoplasm. Interestingly, in the absence of A3, accumulation of the L4 nucleocapsid protein in the viral factories is decreased and L4 is not present in the viral particles. This result reinforces the suggestion, raised in our previous studies, that the A3 inner core wall protein interacts with the L4 nucleocapsid protein (Jesus et al., 2014).

Results

Construction and characterization of an inducible recombinant virus in gene A3L

In order to study the function of the vaccinia virus A3 protein, we constructed an inducible mutant in gene A3L using the *E. coli* lac operon system (Zhang and Moss, 1991). The approach chosen is based on the Lac operon inducible system developed by Alexander et al. (1992) and Ward et al. (1995) as modified by Turner and Moyer (1992) and involves the recombination of a PCR fragment into the genome of VACVT7lacOI. The PCR fragment contains sequences homologous to the vaccinia genome flanking a region of the plasmid pVOTE.2, which contains the T7 polymerase promoter under the control of the lac operator and the GPT gene under the control of a vaccinia constitutive promoter as the selective marker. Once recombination occurs, the PCR fragment substitutes the original promoter of A3L with the T7 RNA polymerase promoter and lac operator (Fig. 1A). The parental virus (VACVT7LacOI) contains the lac repressor under the control of a constitutive vaccinia promoter as well as the T7 RNA polymerase gene under the control of the lac operator and a late vaccinia promoter.

Initially, we compared viral plaques formed by vA3i in the absence and presence of IPTG to the viral plaques formed during a wild type infection in a plaque assay. Monolayers were infected with serial dilutions of the viruses, incubated at 37 °C in the absence or presence of IPTG for 7 days and stained with crystal violet. Fig. 1B shows that vA3i plaques formed in the presence of inducer are somewhat smaller than wild type plaques. In the absence of inducer, no viral plaques were visualized.

To analyze virus growth during one replication cycle, cells were infected with WR or vA3i at an MOI of 10, incubated at 37 °C in the presence or absence of IPTG and harvested after varying times of infection (Fig. 1C). In the presence of IPTG vA3i grows slower than the wild type virus, consistent with the smaller plaque sizes observed in this condition; however, the mutant reaches wild type titer levels after 48 h of infection. By contrast vA3i does not grow in the absence of IPTG. The data from Fig. 1 confirm that vA3i is dependent on IPTG to produce infectious particles.

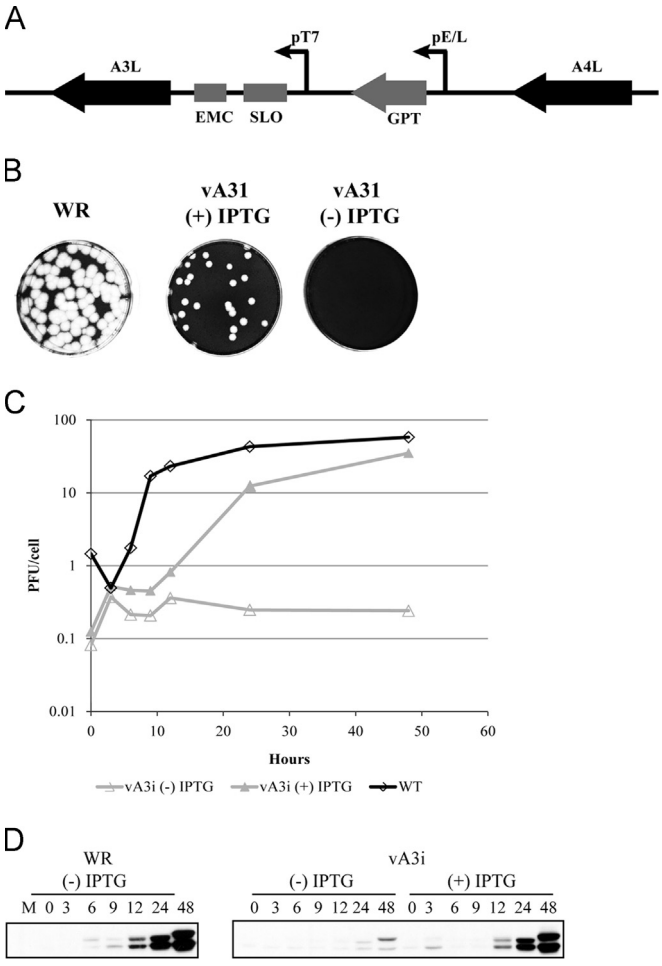

Fig. 1. Initial characterization of vA3i. (A) Illustration of the final genome structure in the region between A3L and A4L genes in vA3i. The viral A3L and A4L genes are represented by black arrows. pT7 and pE/L represent the bacteriophage T7 and synthetic vaccinia virus early/late promoters, respectively. GPT=guanine phosphoribosyl transferase. EMC=the encephalomyocarditis virus independent ribosome entry site. SLO=modified (stem-loop) lac operator. (B) Viral plaques phenotype. Cells were infected with WR or vA3i, incubated at 37 °C in the presence or absence of IPTG and stained with crystal violet after 7 days. (C) One-step growth curve. Cells were infected with WR or vA3i at an MOI of 10 and incubated at 37 °C in the presence or absence of IPTG. After varying times of infection, cells were harvested and the virus titer was determined by plaque assay in the presence of IPTG. (D) Accumulation of A3 during infection. Cells were infected at an MOI of 10 with WR or vA3i and incubated in the presence or absence of IPTG at 37 °C. At varying times post-infection, cells were harvested and samples were analyzed by SDS-PAGE and Western blot with antibodies against A3. The numbers above each lane indicate hours post-infection.

Accumulation of A3 during infection

In order to determine if the expression of A3L was repressed in the absence of IPTG, we infected cells with WR or vA3i at an MOI of 10 and incubated at 37 °C in the presence or absence of IPTG. At varying times post-infection, cells were harvested and the samples analyzed by Western blot. Because A3 is processed during virus maturation, two bands are observed in the Western blot, corresponding to the uncleaved and cleaved A3. In the presence of IPTG, A3 accumulates slower than in the wild type infection, with A3 first appearing after 12 h versus after 6 h in infections with WR (Fig. 1D). This slower accumulation of A3 is consistent with the slower rate of virus growth observed in the one-step growth experiment. In addition, there is a slight accumulation of A3 even when IPTG was not added to the cells. This small amount of A3 in the absence of IPTG is, however, not enough to permit virus growth (Fig. 1C). Finally, the small amount of A3 present at 48 h