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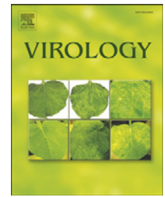

There is an A33-dependent mechanism for the incorporation of B5-GFP into vaccinia virus extracellular enveloped virions

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ABSTRACT

Orthopoxviruses produce two, antigenically distinct, infectious virions, intracellular mature virions and extracellular virions (EV). A33 and B5 are found on EV but not on intracellular mature virions. To investigate the function of A33, a recombinant virus that has A33R deleted and expresses B5R-GFP (vB5R-GFP/ΔA33R) was generated. A comparison of vB5R-GFP/ΔA33R to an analogous virus (vΔA33R) revealed an additional defect in infectious EV production that was not apparent when A33R was present. Characterization of these recombinants revealed that EV produced in the absence of A33 had undetectable levels of B5-GFP. Both recombinants released similar amounts of EV but there were differences in their infectivity. Approximately equal numbers of virions produced by these recombinants were able to bind cells even though EV produced by vB5R-GFP/ΔA33R do not contain B5. These results suggest that in the absence of A33, the cytoplasmic tail of B5 contributes to its incorporation into the envelope of progeny virions.

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Introduction

Vaccinia virus is the best-studied member of the *Orthopoxvirus* genus. It has a double-stranded DNA genome of about 200 kb that is predicted to encode for approximately 200 functional open reading frames (Moss, 2001). Viral replication occurs entirely in the cytoplasm of infected cells in a specialized area known as the viral factory and results in three commonly recognized, morphologically distinct, forms: intracellular mature virions (IMV), intracellular enveloped virions (IEV), and extracellular virions (EV) (Moss, 2006; Smith et al., 2002). IMV is the first infectious progeny virions formed and represents the majority of virions produced by infected cells. IEV are formed by intracellular envelopment of a subset of IMV with an additional double membrane derived from the *trans*-Golgi network (TGN) or early endosomes (Hiller and Weber, 1985; Schmelz et al., 1994; Tooze et al., 1993). IEV are transported to the cell periphery via microtubules and released from the cytoplasm by fusion of their outermost envelope membrane with the plasma membrane (Geada et al., 2001; Hollinshead et al., 2001; Rietdorf et al., 2001; Ward and Moss, 2001a). These released virions have lost one of their IEV membranes but possess one more membrane than IMV and have been called EV. EV can be further classified as being either cell-associated enveloped virions (CEV) or extracellular enveloped virions (EEV) for those virions that have detached from the cell they were produced in. CEV are believed to be important for efficient cell-to-cell spread via actin tails (Reeves et al., 2005; Ward and Moss, 2001a) while EEV mediate greater dissemination of virus (Appleyard et al., 1971; Payne, 1980).

Presently, six proteins encoded by the virus, A33, A34, A36, B5, F12, and F13, have been found to be both exclusive to either IEV or EV and involved in infectious EV production (Duncan and Smith, 1992; Engelstad et al., 1992; Hirt et al., 1986; Isaacs et al., 1992; Roper et al., 1996; van Eijl et al., 2002, 2000). A33 is predicted to play a role in both IEV/EV morphogenesis/egress and subsequent infection. A33R is highly conserved among orthopoxviruses and encodes a 23 kDa type II glycoprotein (Roper et al., 1996). Deletion of A33R results in a reduction in IEV production and an increase in infectious EEV production (Roper et al., 1998). The cytoplasmic tails of A33 and A36 interact and the interaction is required for the incorporation of A36 into IEV and subsequently, actin tail formation (Ward et al., 2003; Wolffe et al., 2001). It has also been reported that A33 interacts with B5 (Perdiguero and Blasco, 2006). The crystal structure of the ectodomain of A33 suggests that it contains an unusual C-type lectin-like domain that may interact with host cells (Su et al., 2010). Here, we have created a recombinant vaccinia virus that has A33R

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deleted and expresses B5-GFP in place of the normal B5. This new virus was more defective than the parental virus vΔA33R. Characterization of these viruses revealed that in the absence of A33, B5-GFP was not found on EV. Quantification of progeny EV produced by these recombinants shows that the absence of A33 only slightly reduces the amount of EV produced in addition to making them less infectious. The removal of B5, in addition to A33, from EV further reduces the infectivity of EV but this reduction is not due to a reduction in the ability to bind cells.

Results

Construction and characterization of a recombinant virus that expresses B5R-GFP and has A33R deleted

A recombinant virus expressing B5R fused to the coding sequence of enhanced GFP in place of normal B5R (vB5R-GFP) has been useful for studying virion egress in living cells (Ward, 2009; Ward and Moss, 2001b). We recently reported an important role for A34 in proper targeting and incorporation of B5 into progeny virions utilizing a recombinant virus that has A34R deleted and expresses B5R-GFP in place of B5R (Earley et al., 2008). In addition to B5 and A34, A33 has been shown to be required for efficient cell-to-cell spread as deletion of A33R results in a reduction in plaque size (Roper et al., 1998). In a similar way, we constructed a recombinant virus that has A33R deleted and expresses B5R-GFP to study IEV/EV morphogenesis in the absence of A33. The new recombinant, vB5R-GFP/ΔA33R, formed plaques that fluoresced green and were noticeably smaller than plaques formed by the parental A33R deletion virus, vΔA33R, (Fig. 1). The two analogous viruses, WR and vB5R-GFP, formed plaques that were similar in size and much larger than those formed by either ΔA33R virus. Plaque size is related to the amount of infectious EV produced and the ability to form actin tails (Blasco and Moss, 1991; Ward and Moss, 2001b). The deletion of A33R abrogates the production of actin tails, therefore, the smaller plaques formed by vB5R-GFP/ΔA33R suggest that the addition of GFP to the cytoplasmic tail of B5 in the absence of A33 is affecting the production of infectious enveloped virions. To test this idea, B5R-GFP was replaced with B5R in vB5R-GFP/ΔA33R to create vB5R/ΔA33R. This replacement restored the plaque size of vB5R/ΔA33R to that of vΔA33R, indicating that the smaller plaque phenotype of vB5R-GFP/ΔA33R is due to the addition of GFP to the cytoplasmic tail of B5.

B5-GFP is mis-targeted in the absence of A33

The smaller plaque phenotype of vB5R-GFP/ΔA33R compared to vΔA33R suggests that in the absence of A33, the addition of GFP to the cytoplasmic tail of B5 results in a decrease in the amount of infectious EV produced. This could be due to a decrease in the total amount of EV produced, their infectivity or a combination of both. To determine where the defect was occurring, we looked at the localization of B5 in cells infected with our recombinant viruses by immunofluorescence microscopy. Typically, cells infected with vB5R-GFP display three characteristic hallmarks of GFP fluorescence: an accumulation of GFP fluorescence at the site of wrapping in the juxtanuclear region, at the cell vertices, and GFP-labeled virion-sized particles (VSPs) in the cytoplasm (Fig. 2). Cells infected with WR had a similar B5 localization pattern to that seen in cells infected with vB5R-GFP (Fig. 2). In cells infected with either vΔA33R or vB5R/ΔA33R, B5 localized at the site of wrapping and on VSPs (Fig. 2). In contrast, cells infected with vB5R-GFP/ΔA33R displayed a GFP fluorescence pattern different from that seen in cells infected with vΔA33R (Fig. 2). B5-GFP was found throughout the cytoplasm and accumulated in the juxtanuclear region (Fig. 2). In addition, in the absence of A33, there was a distinct reduction in GFP-labeled VSPs in the cytoplasm, suggesting that B5-GFP was not efficiently incorporated into progeny enveloped virions

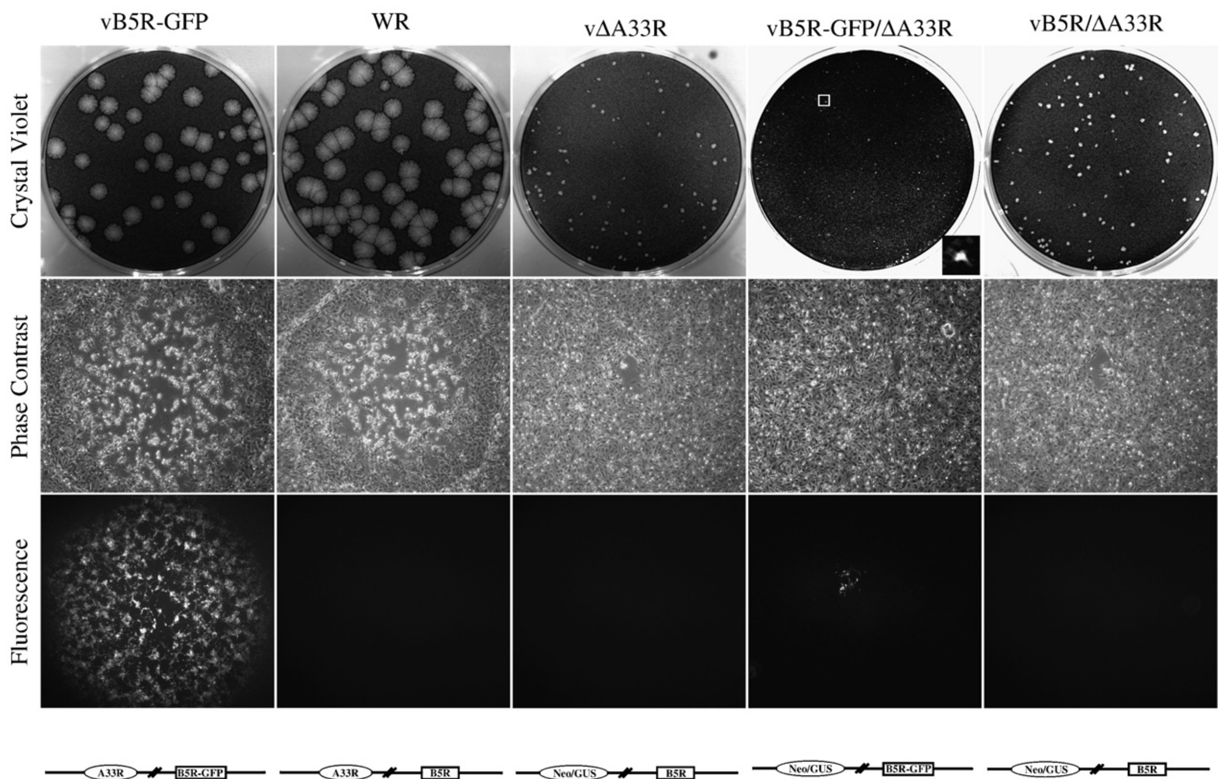

Fig. 1. Plaque phenotypes. Confluent BS-C-1 cell monolayers were infected with the indicated viruses and overlaid with semi-solid media. Two days PI, phase contrast and fluorescence images were captured using a fluorescent microscope. Cell monolayers were stained with crystal violet three days PI and imaged. For vB5R-GFP/ΔA33R, insert shows an enlarged plaque in the boxed area. Schematic representation of the genome of each recombinant virus is shown below the fluorescence images.

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