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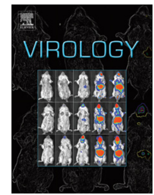

RAB1A promotes *Vaccinia virus* replication by facilitating the production of intracellular enveloped virions

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ABSTRACT

Vaccinia virus (VACV) is a large double-stranded DNA virus with a complex cytoplasmic replication cycle that exploits numerous cellular proteins. This work characterises the role of a proviral cellular protein, the small GTPase RAB1A, in VACV replication. Using siRNA, we identified RAB1A as required for the production of extracellular enveloped virions (EEVs), but not intracellular mature virions (IMVs). Immunofluorescence and electron microscopy further refined the role of RAB1A as facilitating the wrapping of IMVs to become intracellular enveloped virions (IEVs). This is consistent with the known function of RAB1A in maintenance of ER to Golgi transport. VACV can therefore be added to the growing list of viruses which require RAB1A for optimal replication, highlighting this protein as a broadly proviral host factor.

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Introduction

Vaccinia virus (VACV) is the prototypic virus of the Orthopoxvirus genus of *Poxviridae,* a family of large, double stranded DNA viruses which undertake a complex replication cycle entirely within the cytoplasm of an infected cell. Multiple forms of the poxvirus virion are produced during the cycle, and can be differentiated by their cellular location, number of membranes, abundance and function. After entering a cell, via plasma membrane fusion or endocytosis, the VACV virion travels to a perinuclear location to establish a cytoplasmic viral factory (Moss, 2007). These factories produce abundant numbers of intracellular mature virus (IMV), which consists of a core particle surrounded by a single lipid membrane that is embedded with entirely nonglycosylated viral proteins. A small fraction of IMVs (approximately 1% (Payne, 1980)) exit the viral factory and are wrapped by two additional cellular membranes that are embedded with glycosylated viral proteins to form intracellular enveloped virions (IEVs) (Hiller and Weber, 1985). IEVs then travel to the periphery of the cell where their outermost membrane fuses with the plasma membrane, leaving a cell associated virion (CEV) surrounded by the two remaining membranes. CEVs released from the surface are known as extracellular enveloped virions (EEVs). IMVs are robust virions and capable of long-term survival in the environment. In comparison CEVs and EEVs are more labile but crucial for efficient and timely cell to cell spread of VACV in vivo and in vitro (Blasco and Moss, 1992; Smith et al., 2003). Alternative nomenclature refers to IMVs as mature virions, IEVs as wrapped virions, and CEVs and EEVs as extracellular virions (Moss, 2006).

The intricate cell–virus interactions involved in poxvirus morphogenesis are still incompletely understood. High throughput, unbiased, RNA interference screens have been used to identify cellular proteins which are required for poxvirus replication (Beard et al., 2014; Mercer et al., 2012; Sivan et al., 2013; Teferi et al., 2013). Two of these screens identified RAB1A as a strongly proviral host factor (Beard et al., 2014; Sivan et al., 2013). Only a small number of individual cellular proteins were identified in multiple screens, suggesting these particular proteins play a crucial role in the virus life cycle and are therefore worthy of detailed investigation.

RAB1A is a member of the Rab GTPase protein family. This family contains over 60 human Rab proteins which localise to specific intracellular membranes and act as directors and organisers of membrane trafficking including pathways among the ER, golgi, endosomes, lysosomes, phagosomes and autophagosomes (Stenmark, 2009). The most well-known function of RAB1A is to facilitate vesicle trafficking from the endoplasmic reticulum (ER) to the Golgi. This pathway consists of the ER, the ER–Golgi intermediate compartment (ERGIC), and the cis face of the Golgi. Anterograde transport begins at specialised areas of the ER known as ER exit sites (ERES) which produce and release vesicles coated in the membrane coat complex COPII. The small GTPase Sar1 is

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essential for the formation of these COPII vesicles (Donaldson and Jackson, 2011). RAB1A localises predominantly to the ERGIC membrane and recruits the tethering factor p115 to the COPII coated vesicles, facilitating the formation of a fusion complex and thus directing COPII vesicles to the Golgi for delivery of their cargo (Allan et al., 2000). However, in addition to its function in ER to Golgi transport, RAB1A is also involved in early Golgi trafficking (Yamasaki et al., 2009), the motility of early endocytotic vesicles, early endosome to Golgi trafficking (Mukhopadhyay et al., 2011), regulation of the actin cytoskeleton (Kicka et al., 2011), recycling of the integrin protein ITGB1 to the cell surface (Wang et al., 2010) and autophagy (Winslow et al., 2010). RAB1A is therefore a multifunctional protein with roles in varied cellular processes.

Previous work has revealed a role for RAB1A in the life cycles of a number of viruses. RAB1A is required for the trafficking of viral envelope glycoproteins of HIV (Nachmias et al., 2012) and HSV-1 (Zenner et al., 2011), highlighting the protein's role in maintenance of a functional Golgi. In contrast, RAB1A plays a direct role in Hepatitis C virus replication, interacting with the viral protein NS5A and promoting lipid droplet formation (Nevo-Yassaf et al., 2012; Sklan et al., 2007). Examination of known RAB1A functions produces a number of hypotheses for the proviral role of RAB1A in VACV replication cycle. In an analogous manner to HIV and HSV-1, RAB1A may be required to maintain Golgi function which, for poxviruses, is vital for the production of IEVs and therefore CEVs and EEVs (Ulaeto et al., 1995). However, alternative hypotheses exist. RAB1A has been shown to be rapidly recruited to plasma-membrane lipid rafts within 30 min of VACV infection (Schroeder et al., 2012), suggesting a possible role in virus entry. In support of this theory, RAB1A regulates the recycling of ITGB1 to the cell surface (Wang et al., 2010) and this integrin protein has been shown to facilitate VACV entry (Izmailyan et al., 2012). An alternative hypothesis is that RAB1A plays a novel role in VACV replication. This is the case for another cellular protein concerned with vesicle trafficking, golgin-97. Golgin-97 is a tethering factor which facilitates retrograde transport from endosomes back to the trans-Golgi network (Lieu et al., 2007; Lu et al., 2004). Knockdown of golgin-97 in VACV infected cells unexpectedly resulted in disruption of IMV formation and accumulation of immature virions (Alzhanova and Hruby, 2006, 2007). It is unclear why this protein, which facilitates a late stage of the vesicle transport system, should inhibit early stages of VACV morphogenesis.

Given the multiple plausible hypotheses for the role of RAB1A in VACV replication, we investigated the stage of the viral life cycle for which RAB1A is required.

Results

RAB1A is a proviral cellular factor in VACV replication

RAB1A was identified as a strongly proviral cellular protein in two independent, high-throughput siRNA screens of VACV replication (Beard et al., 2014; Sivan et al., 2013). To investigate this result further HeLa cells were mock transfected or transfected either with a non-targeting negative control siRNA (RSCF) or siRNA targeting RAB1A. A siRNA SMARTpool containing four different siRNAs all targeting RAB1A was used to enhance the magnitude and specificity of protein knockdown. After 48 h of transfection cellular proteins were collected and the level of RAB1A protein in the cell lysates was compared using western blotting. The level of RAB1A was substantially reduced in cells treated with siRNA targeting RAB1A (Fig. 1A), confirming the efficacy of the siRNA knock down. The impact of RAB1A on VACV replication and spread was then examined using VACV-A5L-EGFP, a VACV strain which has the A5 viral capsid protein tagged with EGFP, thereby enabling virus growth to be estimated by measuring fluorescence levels (Beard et al., 2014; Carter et al., 2003). HeLa cells were mock transfected or transfected with non-targeting RSCF siRNA (negative control), siRNA targeting PRK-AB1 (positive control) which is required for efficient VACV replication (Moser et al., 2010), or the RAB1A siRNA SMARTpool. In addition, the SMARTpool was deconvoluted to the four constituent siRNAs and each tested individually. After 48 h the cells were infected with VACV-A5L-EGFP at a low MOI of 0.1 and fluorescence measured after a further 48 h, allowing multiple rounds of virus replication to have occurred (Fig. 1B). Cells were also examined using light microscopy for evidence of any cytotoxic effect of the siRNAs with none detected (data not shown). Fluorescence levels were comparable in the negative control samples (mock and RSCF transfected), but significantly reduced in the wells treated with positive control siRNA (PRK-AB1), the RAB1A SMARTpool and three of the four deconvoluted RAB1A siRNAs.

To assess the effect of RAB1A depletion on viral growth kinetics a multistep growth curve was carried out using both fluorescence levels and viral plaque titration to measure virus replication. Cells were mock transfected or transfected with siRNA targeting the herpesvirus protein VP16 (negative control) or the RAB1A siRNA SMARTpool. After 48 h cells were infected with VACV-A5L-EGFP at an MOI of 0.1 followed by measurement of fluorescence and viral titres at 12 h intervals. EGFP expression was comparable in the negative control samples (mock and VP16) but significantly lower at 36 and 48 hpi in cells with reduced levels of RAB1A (Fig. 1C). Virus titration (Fig. 1D) revealed a statistically significant reduction in the amount of infectious virus at 36 and 48 hpi in cells lacking RAB1A compared with control cells. Overall, these results substantiate the previously reported evidence from the high throughput siRNA screens that RAB1A is required for efficient multicycle growth of VACV (Beard et al., 2014; Sivan et al., 2013).

RAB1A is required for optimal production of EEVs but not IMVs

In order to determine the stage of the VACV life cycle for which RAB1A is required we carried out a onestep growth curve measuring fluorescence levels and viral titres at regular intervals. HeLa cells were mock transfected or transfected with siRNA targeting either VP16 or RAB1A, and after 48 h infected with VACV-A5L-EGFP (MOI = 5). In contrast to the multistep growth curve, EGFP levels in RAB1A, VP16 and mock transfected cells were comparable at all time points in the onestep growth curve (Fig. 2A). EGFP expression by the VACV-A5L-EGFP virus is under the control of the A5 promoter which is expressed both early and late in the viral transcriptional cascade (Yang et al., 2011). Therefore this suggests that all stages of viral replication up to and including late gene expression are unaffected by loss of RAB1A. Titration of the cell associated fraction (which consists almost entirely of IMVs) revealed comparable virus titres in control and RAB1A siRNA transfected cells at all time points, in agreement with the uniform fluorescent results (Fig. 2B).

To examine specifically the impact of RAB1A on later stages of VACV replication we titrated the amount of virus present in the supernatant of cells with normal or reduced amount of RAB1A. Neutralising antibody to IMVs was added to the supernatant fraction prior to titration to ensure that only EEVs that had been released from the cell were titrated. We carried out a onestep growth curve four times (four biological replicates) and at 24 hpi identified an average 0.5 $\log_{10}$ reduction in the amount of virus in the supernatant fraction from cells in which RAB1A was knocked down in comparison to control cells ($P < 0.05$) (Fig. 2C), indicating that fewer EEVs are produced from cells lacking normal levels of RAB1A.

The number of CEVs and EEVs produced by an infected cell influences the size and morphology of the viral plaque, and the

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