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Early intranuclear replication of African swine fever virus genome modifies the landscape of the host cell nucleus

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ABSTRACT

Although African swine fever virus (ASFV) replicates in viral cytoplasmic factories, the presence of viral DNA within the host cell nucleus has been previously reported to be essential for productive infection. Herein, we described, for the first time, the intranuclear distribution patterns of viral DNA replication events, preceding those that occur in the cytoplasmic compartment. Using BrdU pulse-labelling experiments, newly synthesized ASFV genomes were exclusively detected inside the host cell nucleus at the early phase of infection, both in swine monocyte-derived macrophages (MDMs) and Vero cells. From 8 hpi onwards, BrdU labelling was only observed in ASFV cytoplasmic factories. Our results also show that ASFV specifically activates the Ataxia Telangiectasia Mutated Rad-3 related (ATR) pathway in ASFV-infected swine MDMs from the early phase of infection, most probably because ASFV genome is recognized as foreign DNA. Morphological changes of promyelocytic leukaemia nuclear bodies (PML-NBs), nuclear speckles and Cajal bodies were also found in ASFV-infected swine MDMs, strongly suggesting the viral modulation of cellular antiviral responses and cellular transcription, respectively. As described for other viral infections, the nuclear reorganization that takes place during ASFV infection may also provide an environment that favours its intranuclear replication events.

Altogether, our results contribute for a better understanding of ASFV replication strategies, starting with an essential intranuclear DNA replication phase which induces host nucleus changes towards a successful viral infection.

1. Introduction

African swine fever (ASF) is one of the most endangering notifiable diseases of swine. Endemic in most of African sub-Saharan countries and in the Italian island of Sardinia, it was introduced into Caucasus and then Eastern Europe in 2007, where it remains endemic (reviewed in Costard et al., 2013). Recently, it has been found in EU countries namely Lithuania, Latvia, Ukraine and Estonia (reviewed in Sánchez-Vizcaíno et al., 2015). No treatment or vaccine is available and control of the disease is solely based on rapid and efficient laboratory diagnosis, and on the implementation of strict sanitary measures.

The aetiological agent (African swine fever virus—ASFV) replicates mainly in swine mononuclear phagocyte cells, causing from hyper-acute to chronic and non-apparent forms of disease (reviewed in Blome et al., 2013). ASFV is a complex large DNA virus with a double-stranded genome (170–190 kb) flanked by inverted terminal repeats and closed by hairpin loops (reviewed in Dixon et al., 2012).

Although ASFV replication cycle mainly occurs in viral cytoplasmic factories, several reports have referred the presence of viral genomes' inside the host nucleus and the importance of the nuclear phase for ASFV infection (Brookes et al., 1996; Garcia-Beato et al., 1992; Rojo et al., 1999). Abrogation of viral infections has been previously reported in enucleated Vero cells (Ortin and Viñuela, 1977). Other authors have shown the presence of full-length viral DNA concatemers in nucleoplasmic extracts and small viral genome fragments were pointed as potential nuclear precursors of the larger cytoplasmic sequences.

Although, some early works have identified the presence of ASFV genomes inside the host nucleus (Tabares and Sánchez Botija, 1979; Vega et al., 1994) and the need of this cell compartment for ASFV infection (Ortin and Viñuela, 1977), the intranuclear phase of ASFV remains poorly understood, in particular, the viral DNA replication events. Moreover, recent studies reinforce the importance of the host cell nucleus by showing that ASFV infection can induce

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nuclear lamina disruption (Ballester et al., 2011) and the activation of Ataxia Telangiectasia Mutated Rad3-related (ATR) pathway (Simões et al., 2013) in Vero cells, still no data are available in swine macrophages, the natural target cells of ASFV infection in vivo.

Indeed, studies on virus–host cell interactions at the cellular level have improved knowledge about other viruses subvert both cellular DNA damage response (DDR) and antiviral mechanisms. These cellular surveillance networks can be activated whenever viral genomes are detected inside the host nucleus, in order to limit viral replication and to restrict the use of host factors (reviewed in Lilley and Weitzman, 2010; Weitzman et al., 2010). DDR is composed by a plethora of signal transduction pathways, responsible for regulating cell cycle, DNA replication/repair and for inducing cell apoptosis whenever DNA damage is beyond recovery (reviewed in Jackson and Bartek, 2009; Nikitin and Luftig, 2012). DDR mechanisms can be differentially activated by viruses to favour the fidelity of viral DNA synthesis (Homologous Recombination, HR) or to prompt an error-prone repair which randomly fills the genomic gaps (Non-Homologous End-Joining, NHEJ). While HR mechanisms are mediated both by Ataxia Telangiectasia Mutated (ATM) and Ataxia Telangiectasia Mutated Rad3 related (ATR) signalling cascades, NHEJ machinery is regulated by DNA-dependent protein kinase catalytic subunit (DNA-PKcs) pathway (reviewed in Weitzman et al., 2010). Following cell entry, several viruses can induce phosphorylation of histone variant H2AX at serine 139, named γH2AX (a hallmark of double strand DNA breaks—DSBs), and activate the Replication Protein A 32 kDa subunit (RPA32), that is mainly associated with stalled replication sites. Upon viral DNA recognition, other DDR mediators (e.g. p53) and specific transduction kinase effectors of the Phosphoinositide 3-kinase (PI3K) family (e.g. ATM and ATR), modulate the activity of downstream checkpoint kinase-1 and -2, interfering with the cell cycle and modulating viral replication (reviewed in Nikitin and Luftig, 2012). However, most viruses can also induce mislocalization or even degradation of DDR-related factors to highjack host cell mechanisms and to avoid apoptosis (reviewed in Lilley and Weitzman, 2010).

It is also known that viruses can modify the antiviral mechanisms of the host cell by changing the functions and morphology of subnuclear domains. The eukaryotic cell nucleus is highly organized in non-membranous domains, such as promyelocytic leukaemia protein nuclear bodies (PML-NBs), nuclear speckles and Cajal bodies, which can be identified through the immunolabelling of their major protein constituents (Bellini, 2000; Schneider et al., 2008). Whereas, PML-NBs are mainly associated with cell cycle control, apoptosis, immune responses (Everett and Chelbi-Alix, 2007) and are usually disrupted by viruses (Everett, 2006), nuclear speckles and Cajal bodies are functionally related with RNA biogenesis, transcription and splicing events, and are also preferential targets during viral infections, as Adenovirus and Herpesvirus infections (Chang et al., 2011; Salsman et al., 2008).

Herein we report a comparative study on spatial and temporal dynamics of ASFV DNA replication sites during the early phase of infection, using swine MDMs and synchronized Vero cells through the detection of BrdU-labelled DNA molecules. In addition, studies conducted in swine MDMs enable the characterization of viral elicited DDR mechanisms by western blot and immunofluorescence analysis, along with the immunofluorescence characterization of morphological changes occurred in different subnuclear domains during ASFV infection. Although we have recently reported the ATR pathway activation in ASFV-infected Vero cells (Simões et al., 2013), studies on such viral interactions with DDR mechanisms in swine monocyte-derived macrophages (MDMs) have not been published.

2. Material and methods

2.1. Swine monocyte-derived macrophages and Vero cell cultures

Blood samples from healthy crossbred pigs (approximately 6-months old) were collected under aseptic conditions into heparinized flasks during bleeding at the abattoir. Thereafter, blood samples were incubated at 37 °C for 15 min with 0.5% v/v of Dextran T500 solution (Merck Millipore, Darmstadt, Germany) in Hank's balanced saline solution (Gibco, Life Technologies, Karlsruhe, Germany) to allow erythrocyte sedimentation. Collected supernatants were diluted (1:1) in RPMI-1640 medium culture supplemented with 2 mM L-glutamine, non-essential aminoacids, 100 UI/ml Penicillin, 100 μg/ml Streptomycin (all from Gibco), and maintained in culture for 48 h as previously described (Portugal et al., 2009). After incubation non-adherent cells were removed by washing with a pre-warmed phosphate-buffered saline (PBS, Gibco) solution. Adherent monocyte-derived macrophages (MDMs) were harvested after 5 min incubation at 37 °C with 0.05% Trypsin-EDTA (Gibco) and cellular viability determined by trypan blue dye exclusion (>90%). Collected MDMs were seeded at 5.0×10^5 cells/cm2 on 24 or 6-well tissue culture plates (depending on the assays), allowed to adhere for 3 h, washed again to remove non-adherent cells and further maintained with RPMI-1640 supplemented with 20% clarified autologous plasma.

Vero cells (kidney epithelial cells of African green monkey *Cholorocebus aethiops*) were purchased from the European Cell Culture Collection (ECACC, Salisbury UK), and maintained in DMEM (Dulbecco Modified Eagle's minimal essential medium) supplemented with L-Glutamax, 10% foetal calf inactivated serum and non-essential aninoacids (all from Gibco). Cell cultures were grown at 37 °C under a 5% CO_2 humidified atmosphere.

2.2. Viral isolates and infections

The ASFV/L60 isolate was propagated in swine MDMs (Martins et al., 1987), while ASFV-Ba71V isolate was propagated on Vero cells (Carrascosa et al., 2011). Viral titrations were calculated through the observation of cytopathic effect (CPE) at end-point dilutions. For immunoblot analysis, viral infections were carried out at a multiplicity of infection of 2 (MOI of 2), during a 24 h time-course incubation, after 1 h adsorption period. For indirect immunofluorescence studies similar procedure was performed using a MOI of 5. When required, virus inactivation was performed by UV-irradiation for 1 h, using a transiluminator (8 W, Model UVTM25, Mighty Bright, Hoefer Scientific Instruments, San Francisco, USA) as previously described (Gómez del Moral et al., 1999).

2.3. Microscopy analysis

2.3.1. Cell synchronization

Considering that swine MDMs are non-cycling cells, arrested in G_0 and G_1 early stages (Solovei et al., 2004), incorporation of BrdU molecules in these cells will only occur as a result of viral DNA synthesis. In contrast, cultures of continuous Vero cells were synchronized in G_2/M phase by a double-step addition of thymidine (100 μM during 16 h, Sigma–Aldrich, St. Louis, USA), followed by a nocodazole exposure (200 ng/ml during 10 h, Sigma–Aldrich), as previously described (Harper, 2005). This experimental approach was used to inhibit S phase entry thus ensuring that BrdU staining is only due to the viral DNA synthesis and not resulting from cellular DNA replication. Cells obtained by mitotic shake-off were seeded on glass coverslip slides (1.0×10^5 cells/cm^2) and adsorbed for 1 h with ASFV-Ba71V at a MOI of 5.