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Review

African swine fever virus controls the host transcription and cellular machinery of protein synthesis

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ABSTRACT

Throughout a viral infection, the infected cell reprograms the gene expression pattern in order to establish a satisfactory antiviral response. African swine fever virus (ASFV), like other complex DNA viruses, sets up a number of strategies to evade the host's defense systems, such as apoptosis, inflammation and immune responses. The capability of the virus to persist in its natural hosts and in domestic pigs, which recover from infection with less virulent isolates, suggests that the virus displays effective mechanisms to escape host defense systems. ASFV has been described to regulate the activation of several transcription factors, thus regulating the activation of specific target genes during ASFV infection.

Whereas some reports have concerned about anti-apoptotic ASFV genes and the molecular mechanisms by which ASFV interferes with inducible gene transcription and immune evasion, less is yet known regarding how ASFV regulates the translational machinery in infected cells, although a recent report has shown a mechanism for favored expression of viral genes based on compartmentalization of viral mRNA and ribosomes with cellular translation factors within the virus factory.

The viral mechanisms involved both in the regulation of host genes transcription and in the control of cellular protein synthesis are summarized in this review.

Contents

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1. Regulation of cellular gene transcription by ASFV

1.1. Introduction

Immune response against pathogens depends on the ability of the infectious agent to modulate cytokines and other factors that help raise the inflammatory response and recruit immune cells to the site of infection. Viruses have been shown for a long time to deploy a variety of strategies not only to alter the host metabolism via their signaling proteins but also to hijack cellular signaling pathways and transcription factors to control them to their own advantage.

Gene expression is regulated by the functions of *cis*-DNA elements, enhancers and promoters, which respond to cellular signals. The modulator functions mediated by enhancers and promoters need the combination of DNA sequence units, which bind one or more transcription factors. Structural and functional studies have indicated that transcription factors are complex proteins with different regions to execute specific functions: a DNA-binding domain focusing the protein to a specific DNA, a multimerization domain, and an effector domain to modulate the activation or repression of the transcription (Tjian and Maniatis, 1994). Both the structural complexity of transcription factors, together with the architecture of enhancers and promoters, provide the combinatorial form of gene expression.

The nuclear factor-B (NFkB) is a shared term referring to a class of dimeric transcription factors belonging to the Rel family. In resting cells, NFkB subsists in the cytoplasm as an inactive complex bound to inhibitory proteins of the IkB family (Beg and Baldwin, 1993; Ghosh et al., 1998). IkB proteins undergo phosphorylation in response to a variety of stimuli, followed by ubiquitination and degradation in the proteasome, thus making perceptible the nuclear localization sequence of the transactivating heterodimers and allowing translocation of active NFkB to the nucleus, to bind there to specific regions of the promoters (Brockman et al., 1995; Brown et al., 1995).

Proteins belonging to the nuclear factor of activated T cells (NFAT) are a family of transcription factors that regulate the expression of several inducible genes during the immune response, being expressed in a variety of immune competent cells, including macrophages, as well as in endothelial cells (de la Pompa et al., 1998; Shaw et al., 1995).

The distinguishing characteristic of NFAT is its regulation by Ca^{2+} and the Ca^{2+}/calmodulin dependent serine phosphatase calcineurin. In resting cells, phosphorylated NFAT proteins localize in the cytoplasm and further stimulation they are dephosphorylated by calcineurin, translocated to the nucleus, and turn into transcriptionally active (Timmerman et al., 1996).

Either NFkB or NFAT appear to be attractive targets for common viral pathogens, due to their ability to promote the expression of numerous proteins involved in both innate and adaptative immunity (Li and Verma, 2002). A number of viruses, including hepatitis C virus, immunodeficiency virus, Herpes viruses, and African swine fever virus, have been shown to modulate the activation of NFAT or NFkB (Bergqvist and Rice, 2001; Kinoshita et al., 1998; Miskin et al., 1998; Revilla et al., 1998; Rodriguez et al., 2002; Scott et al., 2001).

Additionally, the transcription process is regulated by the transcriptional coactivators CBP/p300, which do not specifically interact with promoter elements of target genes, but they are recruited to promoters by interaction with DNA bound transcription factors. Then, they directly interact with the RNA polymerase II complex. In this regard, it has been demonstrated that CBP/p300 interact with multiple transcription factors, including p53, E2F, CREB, NFAT, NFkB, c-Jun and c-Fos (Bannister et al., 1995; Garcia-Rodriguez and Rao, 1998; Gerritsen et al., 1997). The p300 complex achieves this function through diverse functional domains integrated in its amino-terminal (C/H1 and KIX domains) and carboxyl-terminal regions (C/H2 and C/H3 domains) (see Fig. 5).

1.2. African swine fever virus: a unique viral model of host cell transcription interference

African swine fever virus (ASFV) is a large and complex cytoplasmic DNA virus of icosahedral morphology that has been classified as the sole member of the new *Asfarviridae* family (Dixon et al., 2004; Vinuela, 1985; Yanez et al., 1995). African swine fever (ASF) causes severe economic losses and expansion threats. No specific protection or vaccine against ASF is available so far, regardless of the high hazard that the recent outbreak in the Caucasus in 2007, the subsequent dissemination through Russia and the potential dissemination to neighboring countries represents. ASFV naturally replicates mainly in swine macrophages and monocytes, and, after adaptation, can be growth in a number of established cell lines. It has been recently shown that ASFV uses macropinocytosis to enter cells, both in macrophages and in Vero cells (Sanchez et al., 2012), although other mechanisms involving clathrin has been also reported (Hernaez and Alonso, 2010). The entry of virus into cells has been shown to directly stimulate EGFR, PI3K–Akt, Pak1 and Rac1 activation (Sanchez et al., 2012), which could potentially represent one of the first steps for viral regulation of cellular genes transcription.

Furthermore, it has been analyzed by quantitative PCR and microarrays experiments, the modulation of about 150 cellular mRNA during ASFV infection in pig macrophages. The results showed several differences among the level of expression of relevant cytokines, such as IL-1 and TNF-α, depending on the virulence of ASFV strains used (Zhang et al., 2006).

In connection to this, it has also been reported by quantitative RT-PCR and ELISA tests, differences in the level of several cytokines found in pig macrophages after infection with the attenuated ASFV strain NHV or the virulent strain Lisbon60 (L60). In this study the authors demonstrated that the low-virulent ASFV/NH/P68 induced enhanced expression and production of relevant regulatory cytokines, such as IFN-α, TNF-α and IL-12p40 on porcine macrophages in comparison to the highly virulent ASFV/L60 (Gil et al., 2008).

Moreover, changes in gene expression were observed in Vero cells as a consequence of ASFV infection, when the authors searched for infection-associated proteins to determine target proteins for pathogenesis studies. The alterations in cellular protein profile after ASFV infection was analyzed by two-dimensional electrophoresis and proteomics analysis, allowing the identification of twelve over-expressed cellular proteins. The most significant changes were found in redox-related proteins, nucleoside diphosphate kinases, heat shock proteins, members of the Ran–Gppnhp–Ranbd1 complex and apolipoproteins. These cellular protein modifications were hypothetically engaged by the authors with viral-induced transcriptional modulation mechanisms (Alfonso et al., 2004).

The examination of the complete 170-kbp DNA sequence of ASFV has revealed genes coding for structural proteins, enzymes with functions related to DNA replication, gene transcription and protein modification (Yanez et al., 1995) as well as proteins functionally involved in virus–host interactions (Borca et al., 1998; Miskin et al., 1998; Nogal et al., 2001; Revilla et al., 1998; Rodriguez et al., 2002). Among these genes, A238L ORF contains ankyrin repeats homologous to those described in the IkB family and has been shown to act as a *bona fide* IkB-α viral homolog, because it binds p65–NFkB (Revilla et al., 1998).

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1.3. ASFV gene A238L regulates the activation of transcription factors NFkB and NFAT

One of the mechanisms of viral evasion consists of the ability of the infectious agent to regulate proinflammatory molecules and cytokines that provoke the inflammatory response and the recruitment of immune cells to the site of infection. Work developed by different labs in the past several years has identified NFκB and NFAT as two of the most important factors coordinating such responses (Kopp and Ghosh, 1995). Concerning to this, it has been suggested that differences among the sequences encoding for p65–NFkB found in the wild host (warthogs) and in domestic pigs may influence the virulence of ASFV infection, causing either persistence or acute forms of the disease (Palgrave et al., 2011).

ASFV has developed mechanisms to evade the inflammatory and immunological responses during the infection (Dixon et al., 2004). One of these mechanisms evolves from the function of the viral protein A238L, which contains ankyrin repeats similar to those of cellular IκB. The structural homology between A238L and IkB suggests that this viral product might act inhibiting the NFkB activation, thus modulating the transcriptional activation of genes dependent on NFkB in the infected cells. This hypothesis was first demonstrated by ectopic expression of A238L into Jurkat cells that had been previously stimulated to activate the NFkB pathway. Through the analysis of an NFkB-dependent luciferase reporter gene in cells expressing the viral protein, it was assessed that A238L inhibits the expression of genes under the control of NFkB. No effect of A238L expression was found on an AP-1-dependent reporter gene or using a construct with a mutation in the kB site, thus demonstrating that the inhibition observed was specific for NFkB (Powell et al., 1996; Revilla et al., 1998).

It has been reported that A238L interacts with the p65 subunit of NFkB during the infection, likely explaining the mechanism by which the viral protein inhibits NFkB. This result indicates that A238L is present in a complex together with NFkB (Revilla et al., 1998). In support of this, purified recombinant A238L protein added to nuclear extracts from PMA/ionomycin-stimulated cells inhibited binding of NFkB to target DNA sequences and displaced preformed NFkB complexes from DNA (Revilla et al., 1998). Supershift assays, using antibodies specific for different NFkB family members, demonstrated that formation of p50/p65 heterodimers was inhibited by recombinant A238L, rather than formation of p50/p50 complex (Revilla et al., 1998).

Remarkably, A238L lacks of the residues phosphorylated by IkB kinase, which are needed for the regulation of cellular IκB degradation, suggesting that the viral protein is a natural, constitutive and potent suppressor of NFκB activity. Consistent with this observation, the protein could be neither phosphorylated nor ubiquitinated and therefore resistant to stimulus-induced degradation (Tait et al., 2000).

A second unexpected function for the A238L protein was revealed by using the yeast two-hybrid system to recognize host proteins that join A238L (Miskin et al., 1998). The results showed by this report demonstrated that A238L binds to the catalytic subunit of the serine threonine protein phosphatase calcineurin so inhibiting calcineurin phosphatase activity. Among other functions in the cell, calcineurin modulates the activation of NFAT transcription factor family (Crabtree and Olson, 2002; Lopez-Rodriguez et al., 1999; Rao et al., 1997). The domain necessary for inhibiting calcineurin phosphatase was found at the C-terminus of A238L downstream from the ankyrin repeats, and the PxIxITxC/S motif located in this sequence is essential for binding to calcineurin (Miskin et al., 1998). They have also shown that transfection of a plasmid expressing A238L into cells inhibits expression of an NFAT-dependent luciferase reporter gene (Miskin et al., 1998). It is noteworthy that the critical motif necessary for A238L binding to calcineurin is very similar to that required for NFAT binding to calcineurin (Aramburu et al., 1998) and it is not competed either by cyclosporine A or cyclophilin, signifying that the binding sites are different (Miskin et al., 1998).

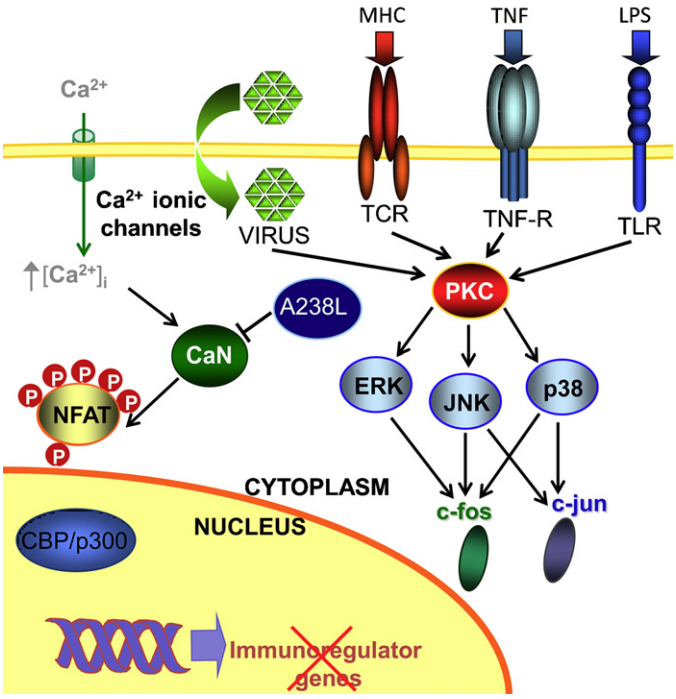

Fig. 1. Inhibition of calcineurin phosphatase activity by A238L viral protein. A238L binds catalytic domain of the serine/threonine phosphatase calcineurin and inhibits its activity impairing NFAT activation and translocation to the nucleus. This inhibition is carried out by the PxIxITxC/S motif located in the carboxyl terminal of A238L (Miskin et al., 1998).

The consequences of A238L inhibition of calcineurin during ASFV infection can be so far only suggested from knowledge of the effects of cyclosporine A on cell function. A key role for calcineurin is in activating NFAT-dependent gene transcription mainly in T lymphocytes, where the target genes activated by NFAT have been studied and these include several cytokines including IL-2, IL-4, GM-CSF (Rao et al., 1997). Although it has been shown that NFAT is expressed in porcine macrophages (Miskin et al., 1998), the target genes that NFAT is involved in activating in these cells are still unknown. Calcineurin has also been shown to negatively regulate activity of the Elk-1 transcription factor which is phosphorylated by MAP kinase (Tian and Karin, 1999). It has been suggested that A238L could consequently increase Elk1-dependent transcription in ASFV-infected cells. Therefore, many potential benefits for ASFV could result from the inhibition of calcineurin by A238L during the infection (Dixon et al., 2004) (Fig. 1).

1.4. Control of pro-inflammatory and immunomodulatory molecules by A238L

1.4.1. Transcriptional regulation of cyclooxygenase-2 (COX-2)

The inhibitory effect of A238L on NFκB and NFAT transcription factors likely enable the virus to control the expression of immunomodulatory factors dependent on both pathways of signaling in infected macrophages, and thus probably modulating the expression of genes involved in the development of a protective immune response against the virus (Gil et al., 2003; Salguero et al., 2008). Monocytes/macrophages play a key role in the development of the immune response presenting antigens and secreting bioactive molecules. One such secreted product is prostaglandin

E2 (PGE2), a strong lipid mediator of inflammation and modulator of the immune response, whose synthesis is tightly controlled by cyclooxigenase-2 (COX-2) (Janelle et al., 2002). Abundant evidence shows that some viruses regulate COX-2 expression and the production of prostaglandins (PGs) (Fang et al., 2012; Janelle et al., 2002; Murono et al., 2001; Pollara et al., 2012; Steer et al., 2003; Tung et al., 2011).

The COX-2 promoter contains binding sites for the transcription factors NFκB and NFAT/AP-1 (Iñiguez et al., 2000), nuclear factor IL-6/CCAAT-enhancer protein, and cyclic AMP responsive element CRE-binding proteins (Appleby et al., 1994). We have shown that COX-2 transcription is inhibited during ASFV infection, and promoter studies indicate that NFAT sites are involved in this process. We have also found that the viral protein A238L down-regulates COX-2 transcription both during infection in Vero cells and when is ectopically expressed in transfected T cells. Besides, we demonstrated that the inhibition of COX-2 promoter induced by A238L in T cells occurs in a NFκB-independent manner, as the NFκB site is not required for A238L inhibition and p65–NFκB did not recover this inhibition. Results obtained with COX-2 promoter deletion constructs, or with promoter containing distal or proximal NFAT mutated sites, as well as the results of overexpression of NFAT or of a constitutively active version of calcineurin, all together demonstrated that NFAT is the target of A238L-mediated down-regulation of COX-2 promoter (Granja et al., 2004a).

As it has been mentioned above, it is commonly recognized that NFAT activation is controlled at several levels, such as nuclear import and export, DNA binding, and regulation of the transactivating activity (Hogan et al., 2003). Hence, it is well established that nuclear import of NFAT factor requires dephosphorylation by the calcineurin phosphatase. The mechanism by which dephosphorylation mediates NFAT regulation has been clearly established (Beals et al., 1997; Okamura et al., 2000). Removal of five phosphates from a conserved serine-rich sequence located immediately adjacent to the PXIXIT calcineurin-binding motif in NFAT exposes a nuclear localization signal in the regulatory domain and renders an additional phosphoserine residue in the regulatory domain significantly more accessible to calcineurin (Okamura et al., 2000). Thus, the available data demonstrated that dephosphorylation by calcineurin plays a conserved role in activating all four NFAT proteins at multiple levels, including translocation to the nucleus, DNA binding, and transcriptional activity. Inhibition of calcineurin phosphatase activity by A238L both in ASFV-infected macrophages and in Vero cells has been described (Miskin et al., 1998, 2000; Silk et al., 2007). The authors cotransfected IBRS-2 cells with a vector expressing A238L along with an NFAT-dependent reporter gene. They reported a consistent reduction of the NFAT-dependent activity, similar to that described by our lab (Granja et al., 2004a).

However, the precise mechanism of action of A238L on the process of NFAT translocation was not fully established in those studies. Matsuda et al. (Matsuda et al., 2000) have reported that ectopically expression of A238L induced the cytoplasmic accumulation of GFP–NFAT4 in BHK cells upon stimulation with ionomycin and that the viral protein binds immunophilin in vitro. Yet, no data were available about the subcellular localization of NFAT during ASFV infection. In regard to this, we have reported that both wild type and A238L deletion Ba71V virus induced translocation of NFAT in ASFV-infected Vero cells, clearly indicating that the presence of A238L does not impair NFAT translocation to the nucleus of the infected cell. These apparent differences can be ascribed to the different cellular systems used, mainly to the fact that Matsuda et al. transfected both proteins NFAT and A238L in BHK cells, whereas we have employed both A238L stably expressing T cells and ASFV-infected Vero cells to investigate endogenous NFAT activation, thus adding information about the function of A238L during the infection (Granja et al., 2004a). In addition, a motif similar to

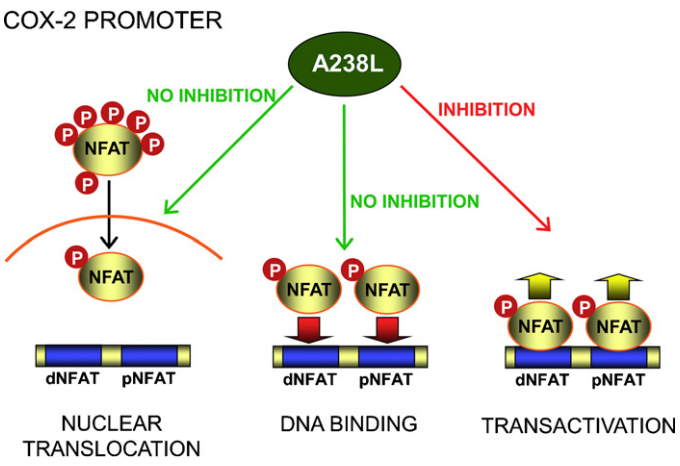

Fig. 2. A238L-mediated inhibition of COX-2 expression induced by NFAT. A238L protein interferes with the transcriptional factor NFAT transactivation, but does not affect either the translocation to the nucleus or binding to specific sequences on COX-2 promoter-DNA binding of NFAT. The word "transactivation" means the events required for the transcription factor recruits the transcriptional machinery necessary for promoter activation and gene expression achievement.

the calcineurin-docking motif of NFAT protein has been found in A238L (Miskin et al., 2000), suggesting that either cellular or viral proteins bind calcineurin at the same site.

Nevertheless, our results showed that modulation of NFAT activity by A238L does not involve either the translocation to the nucleus or DNA binding of this factor to its DNA recognition sequences. Regarding to this, no inhibition of calcineurin by A238L binding peptide was observed (Miskin et al., 2000). Interestingly, this lab further described that the A238L-83 amino acid peptide is able to bind CaN phosphatase (Abrams et al., 2008). It could be also possible that A238L, by binding to calcineurin, might inhibit the activity in some settings but not in others. Moreover, we have shown that A238L robustly inhibits NFAT-mediated transcription by decreasing the activity of its N-terminal transactivation domain both in Jurkat-A238L transfected cells and in ASFV-infected Vero cells (Granja et al., 2004b), providing evidence that A238L efficiently controls COX-2 promoter activity mostly, if not exclusively, through the NFAT response elements. The effects of COX-2/PGE2 inhibition in the pathogenesis of the ASFV infection have not been extensively studied yet, although the deletion of A238L in the non-virulent strain NHV, impairs the protection observed by NHV wt, against the virulent isolate Armenia, an event in which PGE2 could be involved (Y. Revilla et al., unpublished results).

In conclusion, the data indicate the existence of a new viral mechanism to down-regulate NFAT to modulate the expression of COX-2, which provide a better understanding on the evasion mechanisms used by ASFV (Fig. 2).

1.4.2. Transcriptional regulation of TNF-α promoter by A238L: involvement of CBP/p300

TNF-α is a powerful cytokine secreted by several cell types after viral infection. It has been reported up-regulation of TNF-α gene expression in ASFV-E75-infected macrophages (Gomez del Moral et al., 1999). However, the role that this cytokine could play during ASFV infection or the molecular mechanisms involved in the potential regulation of TNF-α by ASFV remained largely unknown.

It was also demonstrated an inhibition of TNF-α production observed in stimulated macrophages from early times after ASFV infection (Powell et al., 1996). In this work, the authors found involvement of the viral protein A238L on downregulation of proinflammatory cytokine responses in host macrophages after ASFV infection. This hypothesis could be expected since A238L, an early

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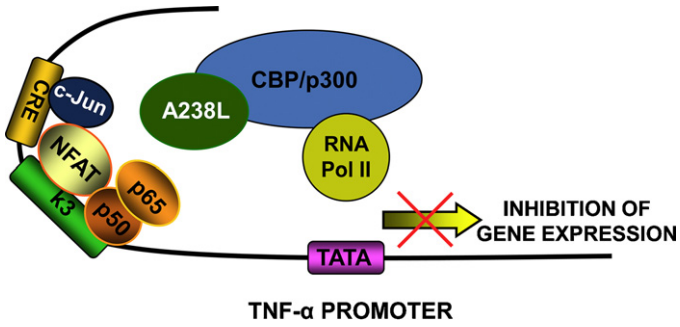

Fig. 3. A238L hampers the recruitment of p300 to the trascription complex. Viral protein A238L interacts with p300 at the nucleus thus impairing its interaction with the transcription factors bound to the specific regions on DNA promoter. The figure represents the transcriptional complex or enhanceosome CRE/κ3 on TNF-α promoter. As a consequence of the blockage induced by the viral protein, the expression of TNF-α is strongly inhibited.

protein synthesized from 6 hpi during the viral cycle, had been shown to inhibit NFκB/NFAT-dependent gene activation. These results were supported by experiments that showed the connection between the expression at mRNA level of cytokines and A238L gene in porcine macrophages infected with ASFV isolates of different virulence (Gil et al., 2003). Furthermore, we identified the cAMP-responsive element (CRE) and κ3 site as responsible of gene regulation during ASFV infection (Granja et al., 2006a). The CRE site has been shown to bind the ATF-2/Jun heterodimer forming a composite element with the κ3 site, which can bind NFκB as well as NFAT factors (Falvo et al., 2000b; Goldfeld and Maniatis, 1989; Leitman et al., 1992; Rhoades et al., 1992). Through these studies, we demonstrated that A238L inhibits TNF-α expression acting at specific DNA binding sites and the transcription factors involved (Granja et al., 2006a).

In order to explain the considerable activity displayed by A238L on different boxes on both COX-2 and TNF-α promoters, we deeply investigated the molecular mechanism subjacent to A238L function. Cellular transcriptional coactivators CBP/p300 are members of a family of transcriptional co-adaptor molecules with distinct functional domains that have been shown to interact with several DNA sequences bound by different transcription factors (Garcia-Rodriguez and Rao, 1998; Gerritsen et al., 1997; Goodman and Smolik, 2000). Furthermore, CBP/p300 has been reported to be bound by several viral proteins such as the adenovirus protein E1A, SV40 large T antigen, and herpes virus E6 and E7 (Arany et al., 1995; Kwok et al., 1996). The consequence of this interaction on the biological effects on p300 functions changes depending on the specific viral proteins and so, both adenovirus E1A and SV40 large T Ag interact with p300 in overlapping locations, large T antigen inhibits, whereas E1A enhances the phosphorylation of p300 (Chakravarti et al., 1999; Goodman and Smolik, 2000). On the other hand, CBP and p300 have been shown to be essential for the optimal transcriptional activity of TNF-α and COX-2 (Deng et al., 2004; Falvo et al., 2000a), two genes controlled by A238L. It is interesting to speculate that a viral gene such as *A238L*, which inhibits the *trans*-activation of NFAT, NFκB, and c-Jun in response to PMA/ion, may have evolved this level of flexibility to accomplish novel patterns of gene regulation to evade the host response. Our results demonstrated that A238L, which localizes in the nucleus of infected cells after PMA/ion stimulation of transfected cells, binds to the CRE/κ3 complex on the TNF-α promoter and displaces the coactivators CBP/p300, thus inhibiting the activation of associated factors such as NFAT, NFκB, and c-Jun (Fig. 3) (Granja et al., 2006a). In support of these data, we found that a deletion mutant of ASFV E70, lacking of A238L gene, increases the synthesis of TNF-α and other cytokines during the in vivo infection (Salguero et al., 2008).

1.4.3. Downregulation of iNOS promoter by A238L: a viral mechanism that concurrently blocks CBP/p300 and NFκB

Further to the results described above, we investigate other components of the inflammatory cascade that could be involved in ASFV pathogenesis, possibly controlled by A238L and involving p300. The expression of iNOS in macrophages is induced by lipopolysaccharides (LPS) and inflammatory cytokines, such as interferons (IFNs), and is modulated by a number of transcription factors, including NFκB. By using a recombinant ASFV lacking of the A238L gene, we found that A238L strongly down regulates iNOS promoter activation, as well as the levels of iNOS transcripts (Granja et al., 2006b). In this work we showed that A238L down-regulates iNOS transcription by interfering NFκB-mediated activation of the promoter. We also found that overexpression of the p65 subunit of NFκB neutralized the inhibitory effect of the viral protein. The complex regulation of iNOS gene transcription includes the interaction of these transcription factors with the coactivators CBP/p300. This idea was reinforced since we demonstrated that A238L-mediated inhibition of iNOS promoter activity and iNOS level were reverted by overexpression of p300. Both p300 and CBP contain a histone acetyltransferase (HAT) enzymatic activity that regulates gene expression through acetylation of the N-terminal tails of histones. In addition to modifying histones, p300/CBP directly acetylates several transcription factors, including p65 and p50 (Bannister and Miska, 2000; Berger, 1999). It is remarkable that, in our hands, the p300 HAT deletion mutant construct was unable to restore the iNOS protein level inhibited by A238L, suggesting that iNOS inhibition by A238L might be related to the acetylase activity of p300. Our results also indicated that p300 and p65 were displaced from the iNOS enhanceosome in cells expressing the A238L protein. In agreement with this, increased doses of p65 as well as of p300, not only induced iNOS promoter transcription but, more importantly, reverted the inhibition of iNOS promoter induced by A238L, supporting the involvement of these proteins in the control of iNOS by the viral protein. It is known that p65 subunit of NFκB interacts with p300 to recruit this coactivator to the transcriptional activation complex on iNOS promoter. As described in our work, the presence of A238L impaired this interaction, suggesting that the viral protein might suppress the transcriptional activation of the iNOS/p65 signal transduction pathway by competing with p65 for binding to p300.

Taken together, the data presented by Granja et al. (2006a,b) established a new viral mechanism of p300 transcription coactivator activity downregulation to modulate iNOS activation. It is important to note that the sustained high output of nitric oxide accounts for its antimicrobial effects on a variety of pathogens, including viruses (Karupiah et al., 1993). Thus, the regulation of the iNOS promoter activity by A238L might be an important checkpoint in the virus cycle, which could affect the virulence of the virus. In contrast, it has been reported that deletion of the ASFV A238L gene from the highly virulent Malawi Lil-20/1 strain does not affect the virulence phenotype in domestic pigs (Neilan et al., 1997). Taken into account the results obtained in vivo, it can be speculated that immunomodulation by A238L could play a significant role in subacute and chronic infections both in wild and in domestic pigs, rather than in acute ASF, which cause a fulminating death of the animal after 5 days of infection.

1.4.4. Molecular mechanism of inhibition of CBP/p300 transcriptional pathway by A238L: role of p300–Ser384 as a new regulator of the transactivation controlled by ASFV

Many signal-activated pathways assemble in the transcriptional coactivator proteins CBP and p300, which join together these signals to coordinate and promote the expression of specific sets of genes in response to diverse physiological stimuli (Goodman and Smolik, 2000; Vo and Goodman, 2001).

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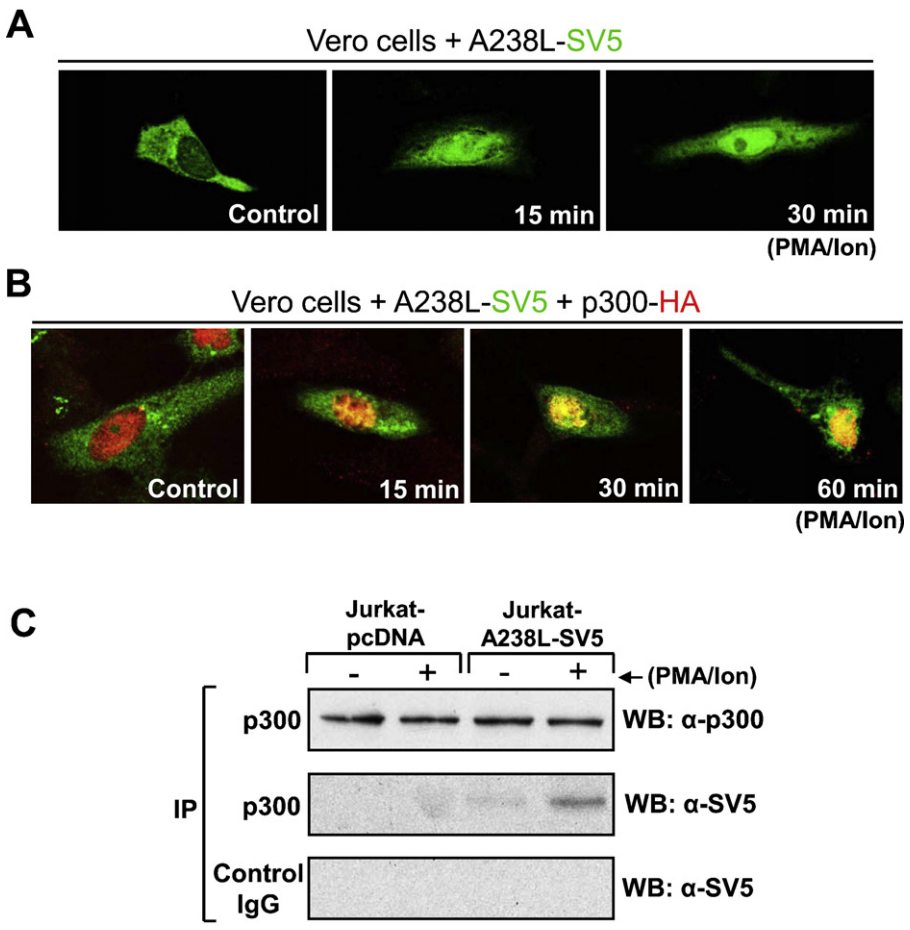

Fig. 4. Subcellular localization of A238L and colocalization with p300. (A) Vero cells were transiently transfected with the pcDNA-A238L-SV5 expression plasmid (kindly gifted by L. Dixon). Twenty-four hours after transfection, cells were unstimulated or stimulated with 15 ng/ml PMA plus 1 μM ion for 15 or 30 min. The cells were labeled with an anti-SV5 Ab and examined by confocal microscopy, showing that after stimulation, A238L is mainly located at the nucleus. (B) Vero cells were transiently transfected with pcDNA-A238L-SV5 and pCMV-p300-HA expression plasmids, and incubated in the absence or presence of 15 ng/ml PMA plus 1 μM ion during 15, 30, or 60 min. Then the cells were labeled with anti-SV5 (green) and with anti-p300 (red) Abs and examined by confocal microscopy. The figure shows images corresponding to the co-localization of p300 and A238L at the nucleus of the cells. (C) Nuclear extracts from 10^7 Jurkat cells transiently transfected with pcDNA3.1 or pcDNA-A238L-SV5, treated or not with PMA/ion for 4 h, were incubated and immunoprecipitated with rabbit polyclonal Ab against p300, or rabbit preimmune normal IgG as a negative control of immunoprecipitation (IgG). Immunoprecipitates were analyzed by Western blot using the same Ab anti p300 to determine levels of this protein in the precipitate, and anti-SV5 to detect levels of A238L-SV5 associated with p300.

We have shown that A238L specifically inhibits the transactivation of transcription factors that require the activity of the amino-terminal TAD (transactivation domain) of p300. In contrast, the transactivation of carboxyl-terminal TAD-dependent transcription factors, such as Sp-1, a p300-independent factor, was not affected by the viral protein. We further demonstrated that A238L modulation involves the autoacetylation activity of p300 that has been shown to be essential to its intrinsic transcriptional activity (Santos-Rosa et al., 2003). Several viruses encode proteins that interact with CBP and/or p300 modulating their activity, such as SV40 T large antigen (Eckner et al., 1996), Adenovirus E1A protein (Felzien et al., 1999) or E6 and E7 proteins from human papillomavirus (Bernat et al., 2003; Patel et al., 1999), and in fact, p300 was described as an E1A-interacting protein (Eckner et al., 1994).

The results further obtained in our lab demonstrated that A238L colocalizes in the nucleus with endogenous p300 in structures compatible with transcription initiation complexes, and associates with p300 (Fig. 4). We found that the viral protein interacts with the amino terminus of p300, but it does not bind the carboxyl-terminal region of the coactivator (Granja et al., 2008). In this regard, it is noteworthy that most of the viral proteins that regulate p300 inhibit the HAT activity and the activation of p300–CH3-interacting transcription factors, such as p53 or E2F. In contrast, in this work we have shown that A238L is inhibiting the amino-terminal TAD without altering carboxyl-terminal activity (Fig. 5). We additionally analyzed whether the interaction of A238L with CH1/KIX domain of p300 interfered with phosphorylation in this domain. We identified a potential PKC site of phosphorylation of p300 at Ser384. Our data demonstrated that this residue is necessary in the activation of the amino-terminal TAD of p300, because mutation of this serine completely abrogated the autoacetylation and the transcriptional activity of the p300 amino terminus. As it had been previously described that PKC-θ activates the signal transduction pathways of NFATc2, p65–NFκB, and AP-1 (Manicassamy et al., 2006) and in order to identify the PKC isotype involved in Ser384 phosphorylation, we achieved experiments to determine that neither PKC-ζ nor PKC-ε phosphorylate the amino terminus of p300. In contrast, we found that PKC-θ efficiently phosphorylated Gal4–p300 fusion protein and in addition, a constitutively active mutant of PKC-θ (pEF-PKC-θ A/E) fully recovered the inhibition induced by A238L, thus enhancing the relevance of PKC-θ in the functional mechanism of the viral protein and enlightening for the first time the importance of PKC-θ in the phosphorylation of this regulatory domain of p300. Therefore, A238L might represent a viral model to find new targets for the control of T cell activation in several pathological processes and immunological diseases.

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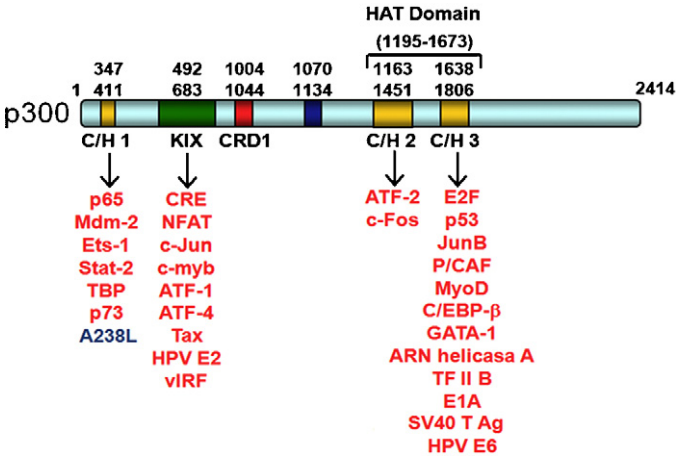

Fig. 5. Map of the p300 coactivator protein showing the functional domains. The amino-terminal region contains the CH1 and KIX functional domains and a bromo domain, and the carboxyl-terminal region contains the CH2 and CH3 domains, which are part of the HAT catalytic domain. Both regulatory regions may act independently and interact simultaneously with the transcriptional machinery and/or with different transcription factors and viral products to build the transcriptional activity mediated by the coactivator. According to our model, ASFV A238L protein interacts with the CH1 domain of p300.

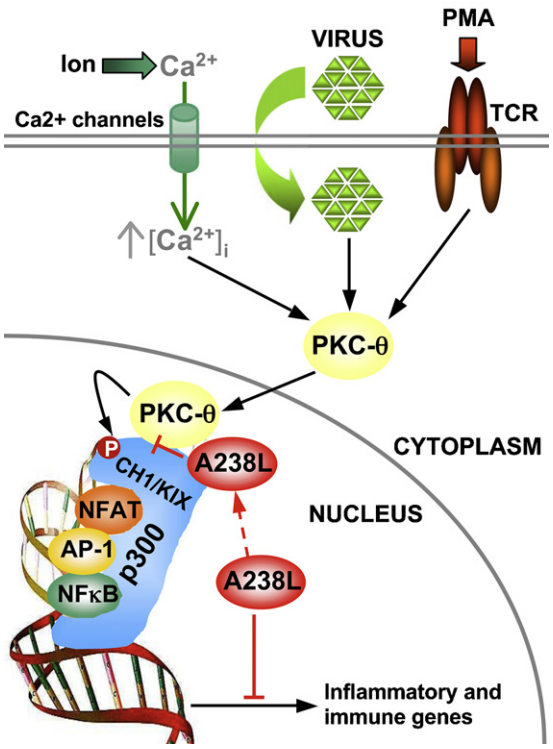

Fig. 6. Model for A238L-mediated inhibition of immune and inflammatory genes transcription. Viral infection and cell activation trigger different signaling pathways which lead to the activation of several kinases. We have found that PKC-θ translocates to the nucleus during ASFV infection where it is able to bind the p300 CH1/KIX domain to activate p300. This event usually up-regulates the transcriptional activity of p300, enhancing the transactivation mediated by NFAT, NFκB and AP-1 transcription factors. According with our results, viral protein A238L impairs the phosphorylation of p300–Ser384 by PKC-θ subsequently inhibiting the p300 activity and the expression of several pro-inflammatory genes.

The results described above using ectopically or recombinant expressed A238L in different cells, prompted us to study the possibility of A238L might also block the transcriptional activity of p300 during ASFV infection. In this regard, we showed that the viral protein regulates the transcriptional transactivation mediated by p300 during the viral infection through the C/H1 and KIX regulatory regions of the coactivator, by using recombinant ASFV lacking of A238L (Granja et al., 2009). We have used the site-directed mutant p300 constructs in which Ser384 was substituted to alanine or aspartic acid, to explore whether the signaling pathway involving this residue was interfered by ASFV. Aspartic acid and alanine are generally accepted as standard substitutions of serine to mimic the phosphorylated and non-phosphorylated state, respectively (Kock et al., 2003). This experimental approach demonstrated that the transcriptional activity of p300 was completely abrogated when Ser384 was substituted to alanine, whereas substitution by aspartic acid resulted in a dramatically increased of p300 activity during the infection. In fact, we demonstrated that the presence of the viral protein impairs the association of PKC-θ and the amino-terminal 192–703 region of p300, thus blocking the amino terminal transactivation activity of p300 in porcine macrophages infected with ASFV E70wt, but not during the infection with E70ΔA238L. We established the relevance of PKC-θ in the activation of the amino-terminal domain of p300 via phosphorylation of the residue Ser384, suggesting that this mechanism is part of a complex signaling network regulating p300 under pathological conditions, such as viral infection. Our model concludes that during ASFV infection the p300 transactivation is efficiently blocked by the viral product A238L to inhibit the synthesis of proinflammatory molecules as a mechanism of virus evasion (Fig. 6).

1.5. Other ASFV proteins involved in host gene transcription regulation

Other ASFV proteins modulating host gene transcription, thus interfering with the function of infected macrophages, have been identified. These proteins include the ASFV inhibitor of apoptosis A224L (Nogal et al., 2001), that has been also reported to be involved in the activation of NFkB (Rodriguez et al., 2002). The mechanism by which A224L activates this transcription factor is likely dependent on the activation of IKK kinases that induce the phosphorylation of NFkB-inhibitor IkB, allowing the translocation of p65–NFkB to the nucleus, where it activates its specific target genes. It is important to note that A224L, also known as IAPv (Nogal et al., 2001), is a late protein in ASFV viral cycle, in contrast with A238L, which is an early protein. Thus, by the expression of these two regulators of the transcription at different times of the infection, ASFV might control the expression of cellular genes to interfere with pathways that could counteract different steps of the viral cycle. Studies from C. Martins lab have also characterized the expression of A224L in porcine macrophages infected with different virulence isolates (Portugal et al., 2009).

Secondly, ASFVj4R protein, which has been described to bind to the host α-NAC protein (Goatley et al., 2002), is also a candidate to regulate host transcription. α-NAC was first reported to play a role in translation by preventing non-specific targeting of proteins lacking signal peptides to the secretory pathway (Wiedmann et al., 1994). Later, the finding that the β subunit of the NAC complex is a yeast transcription factor, BTF3, led to investigate the possible function of α-NAC in transcription, which was finally found to be the regulator of the transcription of c-Jun target-genes (Moreau et al., 1998; Yotov et al., 1998). Thus, it has been speculated that, by binding to α-NAC, ASFVj4R might interfere with the ability of this cellular factor to act as a transcriptional co-activator. Besides, α-NAC has also been shown to interacts with FADD (Fas associated death domain), possibly impairing FADD oligomerization and assembly of the DISC complex in the absence of TNF-α (Stilo et al., 2003). Hypothetical interaction of j4R with α-NAC could regulate apoptosis induced by TNF-α (Dixon et al., 2004).

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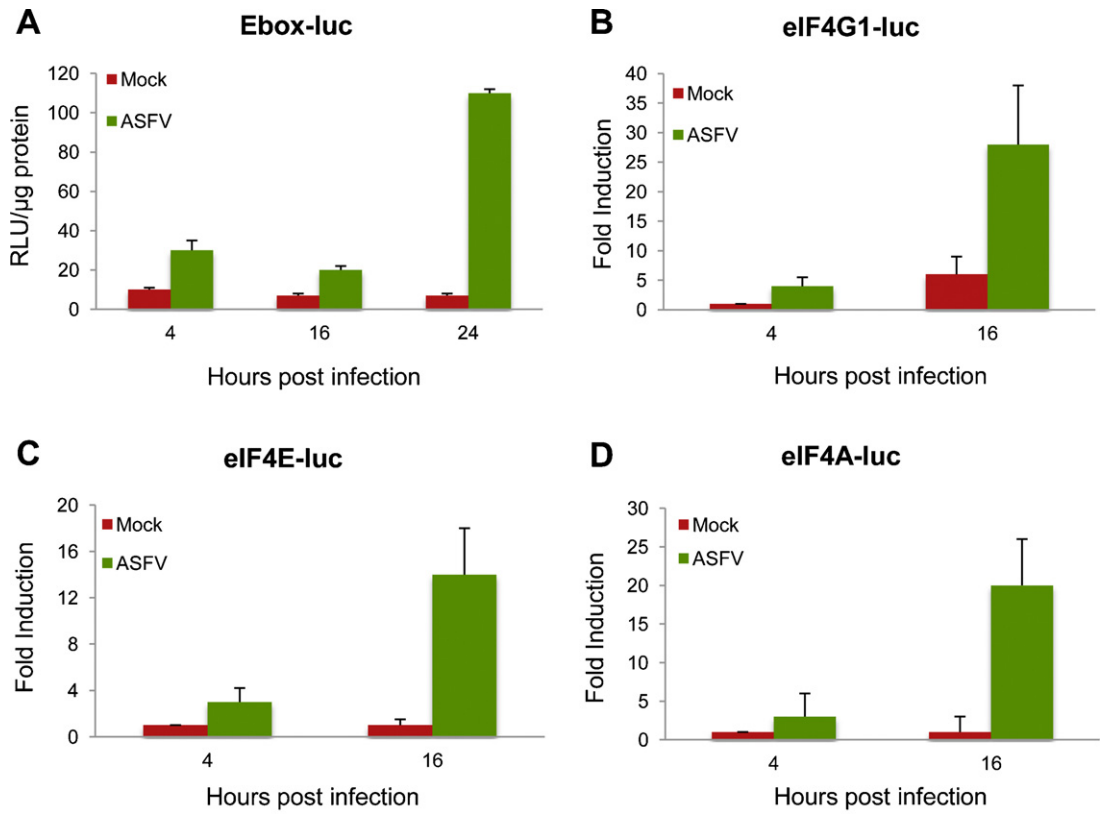

Fig. 7. Effect of ASFV infection in the activation of c-Myc and transcriptional activation of eIF4G1, eIF4E and eIF4A promoters. Vero cells were transfected with p-Ebox-luc (A), pGL3-eIF4G1-luc (B), pGL3-eIF4E-luc (C), and pGL3-eIF4A-luc (D) reporter plasmids (300 ng/10^6 cells). Sixteen hours after transfection, cells were mock-infected or infected with the Vero-adapted isolate Ba71V at a MOI of 5 pfu/cell in 2% FCS medium. Whole extracts were prepared at indicated times post infection and assayed for luciferase activity. Extracts were normalized to Renilla luciferase. Results from triplicate assays are shown in RLU/µg protein or Fold induction relative to mock-infected cells (mean ± SD).

Also, the ASFV ubiquitin conjugating (UBCv) enzyme has been reported as playing a possible role in regulating host gene transcription as it was shown to bind to a host nuclear protein SMCy, which contains an ARID DNA binding domain and is involved in transcription regulation (Bulimo et al., 2000).

1.6. New data about the transcriptional control of eIF4F components by ASFV

The Myc/Max/Mad network of transcriptional regulators controls multiple aspects of cell behavior, including proliferation, apoptosis and differentiation (Grandori et al., 2000). This family of proteins binds DNA to E-box sequence motifs (5′-CACGTG-3′) modulating the transcriptional activity of several genes through chromatin compaction. Both Myc and Mad1 proteins are able to bind Max, forming heterodimers that display different functions. Myc:Max dimers activate the transcriptional activity whereas Mad:Max dimers act as repressors. As a consequence of their antagonic function, Myc activates cell growing and protein synthesis, and Mad1 regulates negatively the cell cycle (Ayer et al., 1993; Rottmann and Luscher, 2006). Therefore, since Myc and Mad1 compete for Max protein, the availability of Max to Myc is profoundly depending on the expression levels of Mad1 and vice versa. Mad proteins are expressed preferentially in non-proliferating cells and Myc proteins are present almost exclusively in proliferating cells.

There are numerous genes regulated by Myc/Max/Mad network such as cdc25, CdK4 and Cyclin D2 (Luscher, 2001). Recently, it has been described that the translation initiation factors eIF4A, eIF4GI and eIF4E are also targets of c-Myc (Coller et al., 2000; Mao et al., 2003; Roeding et al., 2009). It has been also proposed that c-Myc regulates the rate-limiting step of translation initiation and thereby induces eIF4F activity (Lin et al., 2008). Since ASFV stimulates cap-dependent translation to increase the initiation of viral mRNA translation by activating the eIF4F (Castello et al., 2009b), we hypothesized if the virus regulates the transcriptional expression of translational initiation factors of eIF4F complex through c-Myc activation. To confirm this hypothesis, we first studied whether ASFV induces c-Myc trans-activation in infected Vero cells by transfection of a reporter plasmid containing canonical E-box sequence motif. (Plasmids were kindly provided by Dr. J. Pelletier, McGill University, Montreal, Canada). The results show that ASFV strongly induces c-Myc activation from early times after infection (Fig. 7A). Moreover, by using the reporter plasmids containing E-box sites in eIF4G1, eIF4E and eIF4A promoter, we found that the transcriptional activation of these factors was up regulated in infected Vero cells likely as a consequence of ASFV-induced c-Myc activation (Fig. 7B–D). These new results suggest that, further to the recruitment of translational factors within viral factories during ASFV infection (Castello et al., 2009b), the virus is able to control the expression of the components of eIF4F complex at transcriptional level to guarantee the viral protein synthesis. Noteworthy, the ASFV-induced expression of eIF4G1, eIF4E and eIF4A factors is mostly induced at late times after infection, suggesting that the virus might use the cellular factors pool available early in the viral cycle, whereas induce the novo synthesis of these factors at late times of infection.

As mentioned above, Mad1 should be inhibited to allow the expression of c-Myc-regulated genes. It has been described that Mad1 inhibition can be achieved through two different mechanisms. First, phosphorylation-dependent degradation through

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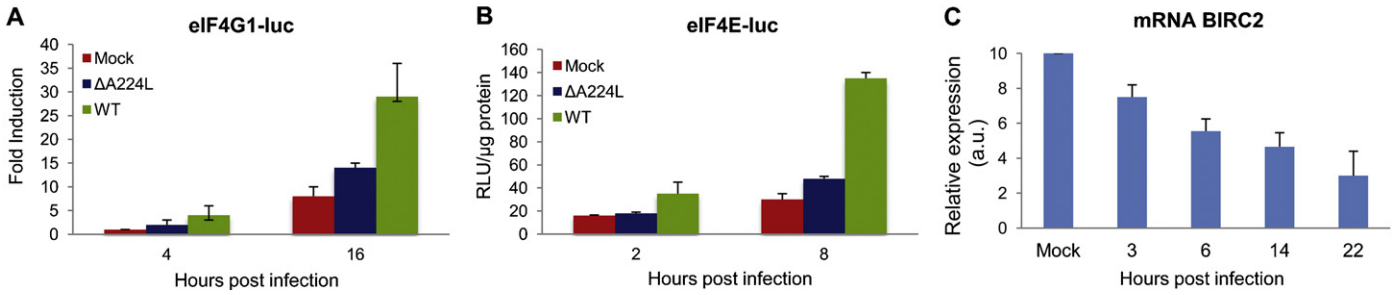

Fig. 8. Role of A224L viral protein in the transcriptional activation of eIF4G1 and eIF4E promoters. Vero cells were transfected with pGL3-eIF4G1-luc (A) and pGL3-eIF4E-luc (B) reporter plasmids (300 ng/10^6 cells). Sixteen hours after transfection, the cells were mock-infected or infected with the Vero-adapted isolate Ba71VWT or Ba71VΔA224L at a MOI of 5 pfu/cell in 2% fetal calf serum medium. Whole extracts were prepared at indicated times post infection and assayed for luciferase activity. Extracts were normalized to Renilla luciferase. Results from triplicate assays are shown in RLU/μg protein or fold induction relative to mock-infected cells (mean ± SD). (C) BIRC2 mRNA expression during ASFV infection. Vero cells were mock-infected or infected with the Vero adapted isolated Ba71V at a MOI of 5 pfu/cell. At different times post infection total RNA was isolated by the TRIzol reagent and was analyzed by quantitative RT-PCR assays (1 μg) with specific primers for BIRC2 mRNA. The levels of mRNA were represented relative to mock expression ($n = 2$; mean ± SD). a.u.: arbitrary units.

PI3K/Akt/mTOR and MAPK activation pathways (Zhu et al., 2008), and second, via ubiquitination by c-IAP1 that triggers degradation by 26S proteasome pathway (Xu et al., 2007). Since the viral protein A224L has been described to be an IAP homolog that inhibits caspase activation and promotes cell survival (Nogal et al., 2001), we speculated whether this viral protein could be involved in the transcriptional control of eIF4G1 and eIF4E. To analyze this, Vero cells were transfected with the specific reporter plasmids containing the promoters regions of eIF4G1 and eIF4E and infected either with Ba71V-WT or with the deletion mutant Ba71V-ΔA224L. As Fig. 8 shows, the transcriptional activation of both eIF4G1 (A) and eIF4E (B) induced by ASFV was strongly reduced when cells were infected with Ba71V-ΔA224L, indicating that this viral protein has an important role on the expression of these genes. Further experiments are needed to clarify the molecular mechanisms by which A224L cooperates with c-Myc to stimulate the expression of eIF4G1 and eIF4E, thus promoting the viral protein synthesis. It can be speculated that A224L could promote the degradation of Mad1 to achieve the transcriptional activation of these translational factors, as it has been described for cellular IAPs (Xu et al., 2007). On the other hand, as shown in Fig. 8A and B, when the infection is carried out with the A224L deletion mutant virus, the eIF4G1 and eIF4E transcriptional activation is still higher than in mock-infected cells. This result suggests that other factors, apart from A224L, could be involved in the control of these factors. In this regard we hypothesized whether ASFV might induce the expression of cellular IAPs which could complement the A224L function during viral infection. Thus, we have investigated the mRNA expression level of BIRC2, a member of cellular IAPs family. As shown in Fig. 8C, the transcriptional level of this gene diminished in response to the virus, making difficult to establish any conclusion about our hypothesis. Other possible candidates will be study in the next future to explore this interesting possibility.

2. Translational regulation by ASFV

2.1. Introduction

Viruses have developed mechanisms to monopolize the cellular translation activity in order to synthesize their own proteins. Most of these strategies are based on switching on/off the activity of key initiation factors essentials for host protein synthesis. African swine fever virus, in analogy to other DNA virus, hijacks the translation machinery by affecting not only the activity of the translation initiation factors, but also their localization. Furthermore, ASFV impacts on RNA metabolism promoting the degradation of cellular RNAs, process in which a putative viral decapping enzyme could play an important role. The ASFV-specific mechanisms to overcome cellular function will be dissected below.

2.2. The cellular protein synthesis machinery: hijacking cellular sources by viruses

The initiation of translation consists in the recruitment of the ribosome to the messenger RNA (mRNA) and is one of the most regulated steps in gene expression. The eukaryotic initiation factors (eIFs) play a central role in this process and are usually targets for fine tune regulation. Due to their key role in protein synthesis, many viruses target these factors during the infection. Frequently, viral infection modulates two important events in initiation of translation: (i) the phosphorylation of eIF2 and (ii) the recruitment of the ribosome to the mRNA by targeting eIF4F and 4E-BPs. The phosphorylation of eIF2 represents one of the most important steps in the regulation of the cellular translation since it allows rapid and reversible modulation of translation initiation. eIF2 is a heterotrimeric initiation factor composed by 3 subunits: α is involved in regulation, while β and γ are implicated in tRNA and GTP binding. The phosphorylation of the α subunit inhibits eIF2 activity and is performed by several cellular protein kinases (Proud, 2005; Wek et al., 2006), and among them, the double stranded RNA-activated kinase (PKR), the PKR-like endoplasmic reticulum (ER) kinase (PERK) and general control non-derepressible-2 (GCN2) play a major role in response to viral infections (Barber et al., 1993; Berlanga et al., 2006; Cheng et al., 2005; Garcia et al., 2007). eIF2 binds Met-tRNA and GTP to form a ternary complex necessary for the formation of the pre-initiation complex. Following the recognition of the AUG start codon and the joining of the ribosomal subunit 60S, eIF2–GDP releases the Initiation Complex and GDP is exchanged to GTP by eIF2B. The phosphorylated eIF2α (P-eIF2α) has greater affinity than its non-phosphorylated counterpart for eIF2B and inhibits eIF2B GDP–GTP exchange activity, causing a decreased in the eIF2–GTP pools and inhibiting translation (Van Der Kelen et al., 2009).

eIF2α phosphorylation is one of the most important host defense mechanisms against viral infections. For this reason, several viruses have developed mechanisms to evade the activation of PKR and the phosphorylation of this factor. Some viruses, as adenovirus (AdV) and Epstein–Barr virus (EBV), encodes double stranded (ds) RNAs that bind PKR but do not trigger the activation of the kinase (Schneider and Mohr, 2003; Walsh and Mohr, 2011). Herpes simplex virus-1 (HSV-1), vaccinia virus (VV), reovirus and influenza virus encodes dsRNA binding proteins that mask or sequester dsRNA and prevent activation of PKR (Beattie et al., 1995; Khoo et al., 2002; Lloyd and Shatkin, 1992; Mulvey et al., 1999;

Salvatore et al., 2002). In the case of poliovirus, PKR is degraded during the infection (Gale et al., 2000). Often, viruses employ more than one mechanism to assure the inhibition of this pathway. One example is HSV-1, which in addition to the previous mechanism encodes for proteins that avoid the accumulation of P-eIF2α by targeting the phosphatases involved in the regulation of this factor (Mulvey et al., 2003). Moreover, HSV-1 and VV also possess viral proteins (glycoprotein B and K3L, respectively) that are able to prevent the phosphorylation of eIF2α by PERK (Mulvey et al., 2003; Sood et al., 2000).

On the other hand, not all the virus avoids phosphorylation of eIF2α. Some viruses as hepatitis C virus (HCV), Sindbis virus, pestivirus, poliovirus, cricket paralysis virus and Semilinki Forest virus induce eIF2α phosphorylation as a mechanism to impair cellular protein synthesis, being able to translate its own mRNA in an eIF2-independent manner (Beckham and Parker, 2008; Garaigorta and Chisari, 2009; Garrey et al., 2010; Jordan et al., 2002; O'Neill and Racaniello, 1989; Ventoso et al., 2006).

The other key point involves the recruitment of ribosomes and eIF4F formation. eIF4F, as central component of the cap-dependent translation machinery, is fine regulated in response to extracellular stimuli, stress and viral infections. eIF4F is a complex composed by three proteins: eIF4A, eIF4E and eIF4G (Prevot et al., 2003). eIF4A is a RNA helicase implicated in unwinding the secondary structure of the 5′-end of the mRNA together with eIF4B; eIF4E binds the cap structure at the mRNA 5′-terminus, and eIF4G is a scaffolding protein that forms a molecular bridge between the mRNA and the small ribosomal subunit 40S. eIF4G coordinate the initiation of translation via protein–protein interactions: (i) the N-terminus domain is involved in the recruitment of the mRNA by its interaction with the cap-binding factor eIF4E and the poly (A) binding protein (PABP); (ii) simultaneously, the C-terminal domain recruits the small ribosomal subunit by means of its interaction with eIF3 (Jackson et al., 2010). Furthermore, eIF4G is also interacting with other viral and cellular proteins implicated in the regulation of the translation machinery, as the mitogen activated kinase 1 (Mnk-1, which phosphorylates eIF4E), the non-structural protein 1 of influenza or the 100 kDa protein of adenovirus (Gingras et al., 1999; Prevot et al., 2003).

Some RNA viruses, such as retroviruses, calciviruses and picornaviruses, encodes for viral proteases that cleavage eIF4G (Alvarez et al., 2003; Castello et al., 2011; Lloyd, 2006; Ventoso et al., 2001). eIF4G cleavage by picornavirus proteases hydrolyze this factor in two moieties, which decouples the capacity of this host factor to recruit the mRNA (by interaction with eIF4E and PABP in the N-terminus) and the ribosome (via eIF3 interaction in the C-terminus) (Castello et al., 2011). However, viral RNAs can drive translation by non-canonical mechanisms. During the last two decades many laboratories worldwide joined efforts to better understand the mechanisms that viruses display to initiate translation, and one of the most important discoveries was the existence of the internal ribosome entry sites (IRES) in picornavirus RNAs (Jang et al., 1990). These RNA elements, which were later discovered in many virus families, drive translation initiation in the absence of key initiation factors such as eIF4E or when eIF4G is cleaved, allowing viral translation under conditions where host protein synthesis is inhibited (Hellen, 2009). Other virus such as AdV, influenza virus or vesicular stomatitis virus (VSV) (Burgui et al., 2007; Connor and Lyles, 2002; Cuesta et al., 2000; Welnowska et al., 2009) promote the dephosphorylaton of 4E-BP and eIF4E to repress the host mRNA translation. Some of these viruses transcribe capped mRNAs, which cannot be distinguished among cellular mRNAs. Nevertheless, proteins of such viruses can be synthesized in spite of the inactivation of eIF4E and the subsequent cellular shutoff. How these "cellular-like" viral RNAs drive translation under these conditions is still unclear.

Complex DNA viruses constitute one of the most intriguing cases. Conversely to most of the viruses studied so far, they enhance the assembly of eIF4F but still are able to inhibit cellular protein synthesis (Walsh et al., 2008; Walsh and Mohr, 2004; Walsh et al., 2005). This review will try to shed light in this "abnormality" in the virus kingdom, with special focus on the molecular mechanisms displayed by ASFV to regulate the cellular machinery of protein synthesis.

2.3. Regulation of eukaryotic initiation factors by African swine fever virus

ASFV mRNAs are structurally similar to the cellular mRNAs. In vitro transcribed ASFV early mRNAs possess a cap structure in its 5′-UTR and a poly (A) tail of 33 nucleotides in average (Salas et al., 1981). The cap structure is predominantly the type m7G (5′) pppA^{m}, which suggests that an enzymatic activity is required for RNA capping to occur. In this regard, ASFV encodes a guanylyltransferase (NP868R) able to exert triphosphatase and guanylyltransferase activities (Pena et al., 1993; Yanez et al., 1995). The fact that ASFV mRNAs are capped indicates that they drive translation by a canonical cap-dependent mechanism, as happens with most of cellular mRNAs.

We have recently described that, similarly to VV infection, P-eIF2α levels decrease at early times post infection, and remain undetectable throughout the infection (Castello et al., 2009b), suggesting a viral mechanism to ensure eIF2 availability for viral protein synthesis. In this regard, ASFV–Ba71V genome encodes a protein, DP71L, which possesses a characteristic binding Protein Phosphatase 1 motif (VxF) (Cohen, 2002). Furthermore, it shares an N-terminal sequence of basic residues and is similar in its C-terminal domain to the Herpes simplex virus-encoded neurovirulence factor ICP34.5 (Goatley et al., 1999). Indeed, DP71L is able to interact with PP1 in vitro (Rivera et al., 2007) and in vivo (Zhang et al., 2010) (Fig. 9A). Individual expression of DP71L induces a decrease of phosphorylated eIF2α and enhances the expression of co-transfected reporters, suggesting that DP71L plays a role keeping the translation machinery active to allow viral protein synthesis (Zhang et al., 2010). Furthermore, it has been predicted that DP71L, like ICP34.5, could act as a regulatory subunit of PP1 and targets it to dephosphorylate-specific substrates in the nucleus of infected cells altering their function. ASFV–Ba71V DP71L gene is named l14L in ASFV E70 and 23NL in Malawi Lil 20/1 isolates, respectively. Deletion of the gene 114L from the genome of E70 reduced virulence in domestic pigs (Zsak et al., 1996) whereas deletion of the gene 23NL from the virulent Malawi Lil 20/1 isolate did not reduce virulence, suggesting that Malawi isolate may encode another additional gene, with a similar function (Afonso et al., 1998). Moreover, the depletion of DP71L in the viral strains Malawi Lil 20/1 and E70 not leads to an increase in the levels of P-eIF2α, suggesting that as VV, ASFV possess multiple mechanisms to avoid eIF2α phosphorylation (Zhang et al., 2010).

Interestingly, new preliminary data from our lab reveal that PKR is found phosphorylated in murine Raw cells at 6 h after virus addition inducing eIF2αs phosphorylation (Fig. 9B), in contrast to that was observed in productive infection in Vero cells. It is important to realize that Raw cell is a murine macrophage line, which only allows the expression of early ASFV proteins, such as p32. Neither late viral protein (p72) (Fig. 9B), nor viral production could be detected, even though more than 80% of the murine macrophages were shown to express ASFV p32 protein, as found by confocal experiments (data not shown). Thus, the sustained eIF2α phosphorylation observed in this murine system could be involved in the impairment of eIF2α binding to the translational initiation complex, causing the blockage of the infection in these cells and partially explaining the ASFV tropism for swine macrophages. Moreover, the mRNA levels of PKR

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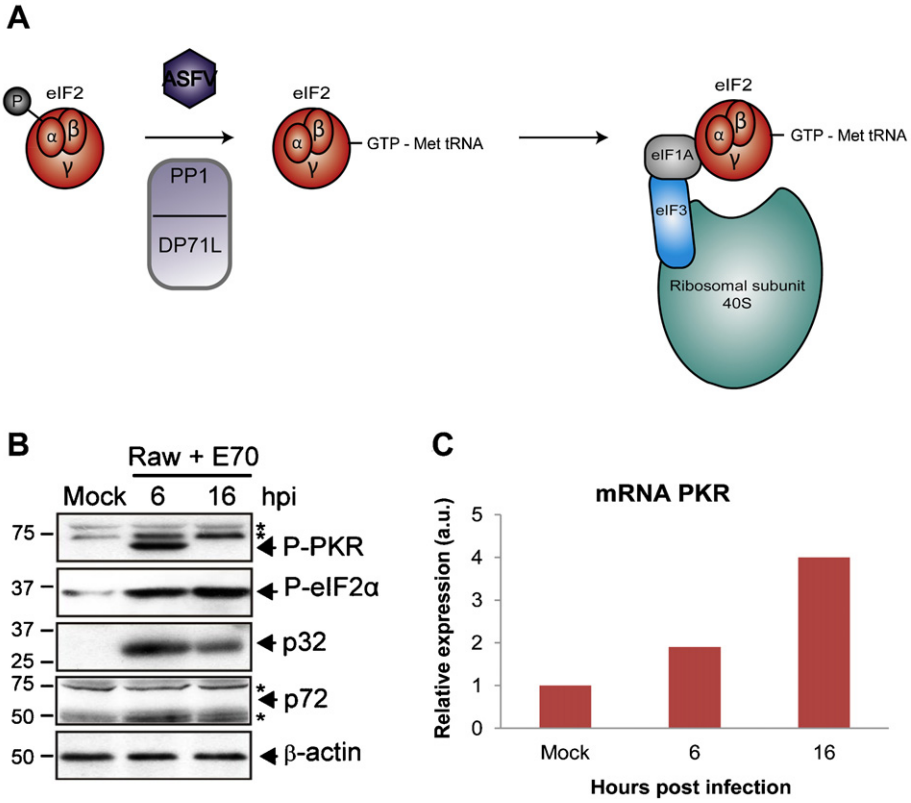

Fig. 9. Regulation of eIF2α and PKR phosphorylation by ASFV. (A) ASFV DP71L protein has been shown to bind the catalytic subunit of protein phosphatase 1 (PP1), leading to PP1 activation and, consequently, eIF2a dephosphorylation (Rivera et al., 2007; Zhang et al., 2010), therefore allowing the participation of this factor in the initiation of translation. (B and C) Raw cells were treated with the virulent isolate E70 at a MOI of 5 pfu/cell for 6 and 16 h. (B) Cellular extracts (50 μg) were lysed with RIPA modified buffer, subjected to SDS-PAGE and phospho-PKR, phospho-eIF2α, p32, p72 and β-actin proteins were detected by immunoblotting with specific antibodies. (C) At indicated times post virus addition total RNA was isolated by the TRIzol reagent and was analyzed by quantitative RT-PCR assays (1 μg) with specific primers for PKR mRNA. The levels of mRNA were represented relative to mock expression. Representative experiment is shown. a.u.: arbitrary units.

were found to increase from early times after virus addition in Raw cells (6 h) until 16 h (Fig. 9C), whereas the phosphorylation of the kinase strongly decreased at late times of the infection. Further experiments will be developed to better understand and to complete these data.

ASFV induces apoptotic response at late times post infection, and consequently, caspase-3 activation (Granja et al., 2004a; Ramiro-Ibanez et al., 1996). eIF4G has been reported to be a substrate for caspase-3 (Bushell et al., 1999; Prevot et al., 2003), being its proteolytic cleavage a potential cause of the shut off during apoptosis (Marissen and Lloyd, 1998). Surprisingly, eIF4G was refractory to caspase-3 cleavage in ASFV-infected cells (Castello et al., 2009b), indicating that (i) the cleavage sites are not accessible to the protease (perhaps protected by protein–protein interactions); (ii) eIF4G is not at the same location than caspase-3; or (iii) caspase-3 activity is abrogated. Regarding the last possibility, ASFV encodes for an inhibitor of the apoptosis (IAP)-like protein (A224L), which has been reported to be an inhibitor of caspase-3 (Nogal et al., 2001). The possibility that this factor protects translation machinery from caspase-3-mediated degradation should be explored in the future. Conversely, ASFV induces a rapid mTOR-mediated phosphorylation of eIF4G at Ser1108 (Castello et al., 2009b), which is has been associated to "translational activation" (Kimball et al., 2000; Raught et al., 2000) (Fig. 10).

In parallel, we showed that ASFV triggers the phosphorylation of eIF4E at Ser209 by Mnk-1. Although the biological relevance of the eIF4E phosphorylation is still controversial (Morley and Naegele, 2002; Richter and Sonenberg, 2005), its importance for some viral infections has been demonstrated. For instance, during Influenza virus, VSV and AdV infection, the dephosphorylation of eIF4E correlates with the inhibition of the cellular protein synthesis (Connor and Lyles, 2002; Feigenblum and Schneider, 1993; Xi et al., 2004). During ASFV infection, as occurs with HSV-1, HCMV and VV, eIF4E phosphorylation is associated to an enhancement of the viral replication and protein synthesis (Buchkovich et al., 2008; Castello et al., 2009b). eIF4E phosphorylation takes place after 8 h post ASFV infection (hpi) and reaches its maximum levels at 14–18 hpi. This phosphorylation in infected cells is avoided in the presence of the Mnk-1 inhibitor CGP57380, and correlates with the phosphorylation of this kinase, suggesting that it relies on Mnk1 activation as reported before (Pyronnet, 2000; Pyronnet et al., 1999).

The eIF4E-binding proteins (4E-BPs) are well-known negative regulators of the cap-dependent translation (Sonenberg and Hinnebusch, 2009). In its hypo-phosphorylated state, 4E-BPs are able to associate with eIF4E and compete the interaction of the cap-binding factor with eIF4G, impairing eIF4F assembly. Conversely, 4E-BPs are inactivated by mTOR-mediated hyperphosphorylation, allowing cap-dependent translation (Bhandari et al., 2001; Richter and Sonenberg, 2005). Similarly to VV and other DNA viruses (Buchkovich et al., 2008), ASFV infection promotes 4E-BP1-phosphorylation at early times post infection, but in this case it is progressively hypo-phosphorylated at later times (from 14 hpi). eIF4G, eIF4E and 4E-BP1 phosphorylations are concomitant with a boost of eIF4F assembly (Castello et al., 2009b). The hypo-phosphorylation observed at late times post infection may be due to a viral mechanism to stop the viral protein synthesis when late morphogenesis stage is taking place (Fig. 10).

Nevertheless, we have shown that phosphorylation of eIF4G, eIF4E and 4E-BP1 by specific kinases, is important but not essential to the ASFV infection in cultured Vero cells, since the presence

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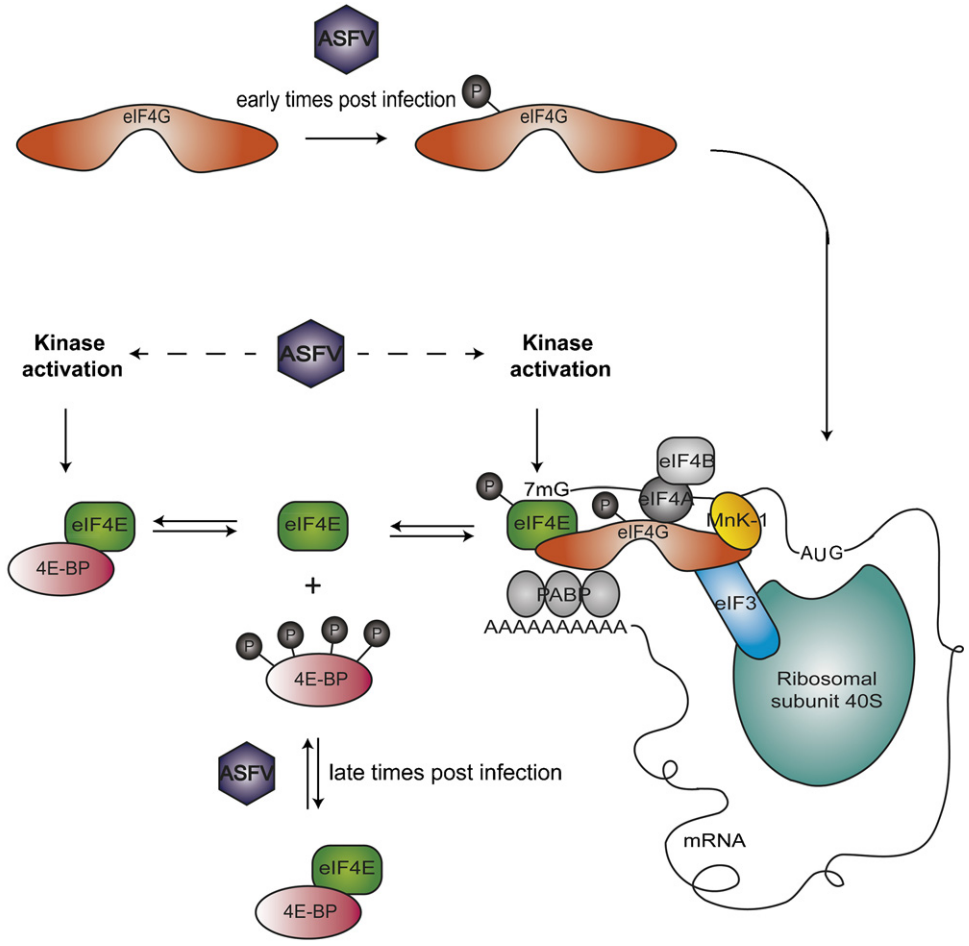

Fig. 10. ASFV infection promotes cap-dependent translation. Activation of eIF4E and eIF4G during ASFV infection. ASFV promotes phosphorylation and of eIF4E, eIF4G and the repressor of eIF4E, 4E-BP, which is inactivated by hyperphosphorylation. However, at late times post infection, 4E-BP is hypophosphorylated. eIF, eukaryotic translation initiation factor; 4E-BP, eIF4E binding protein, Mnk-1, mitogen activated kinase 1; ASFV, African Swine Fever Virus.

of inhibitors of Mnk-1 (CGP57380) and mTOR (rapamycin) affects only moderately to the viral protein synthesis and virus production (Castello et al., 2009b). In contrast, the viral protein synthesis was found to be abrogated by inhibitors of mTOR in other DNA virus infections (Moorman and Shenk, 2010). Interestingly, the activities of eIF4G and eIF4E themselves are essential for ASFV infection as depletion of those factors by specific siRNAs, strongly abrogates viral protein synthesis, viral factory formation and virus production (Castello et al., 2009b).

2.4. Monopolizing the sources: ASFV recruits eIFs, ribosomes and mitochondria to viral factories

Re-localization of eIFs during viral infection has been described for some viruses as tobacco mosaic virus, poliovirus, Sindbis virus, and poxvirus (Katsafanas and Moss, 2007; Sanz et al., 2009; Thivierge et al., 2008; Walsh et al., 2008). Interestingly, it seems like only factors that are required are recruited to viral factories (Sanz et al., 2009; Katsafanas and Moss, 2007).

Data from our lab showed that during the ASFV infection, all the components of the translation machinery examined (eIF4G, eIF4E, eIF2, eIF3b and the eukaryotic elongation factor 2 [eEF2]) are relocated from a diffused distribution throughout the cytoplasm of infected cells to the viral factories, were viral replication and virus morphogenesis take place (Fig. 11A). At 8 hpi, eIF4E and eIF4G are clustered together to the viral DNA and in proximity but not overlapping with the ASFV protein p72; suggesting that "active translation" and morphogenesis areas are in near but independent environments (Castello et al., 2009b). However, both translation factors are found at the periphery of the factories at 16 and 24 hpi, correlating with a clear accumulation of DNA in the central area of the replication foci. These facts point to the idea that ASFV activates and recruits eIF4F to areas where active viral translation takes place. Mobilization of eIFs to ASFV replication foci relies on late viral proteins since treatment with AraC prevents all these effects; although the viral proteins involved in this process are still unknown. During ASFV infection, not only eIFs are recruited to the viral factories but also ribosomes, which strongly support the coupling of viral replication and translation by attracting the protein synthesis machinery to ASFV replication sites. Also viral RNAs localize at the periphery of the viral factories, supporting the idea that active translation is limited to these foci in infected cells. Finally, the mitochondria network are mobilized together with ribosomes to the viral factories, showing that ATP production, translation and viral replication are in proximity (Castello et al., 2009b) (Fig. 11B).

The more obvious role of the virus-mediated redistribution of all these key host machineries may be to increase the availability of the host sources needed for the viral biological cycle in the locations where they are required, i.e. viral factories. Simultaneously, accumulation of the protein synthesis machinery at the viral replication foci may induce its depletion in the cytoplasm, which may result in the shut off of the host mRNA translation. In the future, this attractive hypothesis should be explored.

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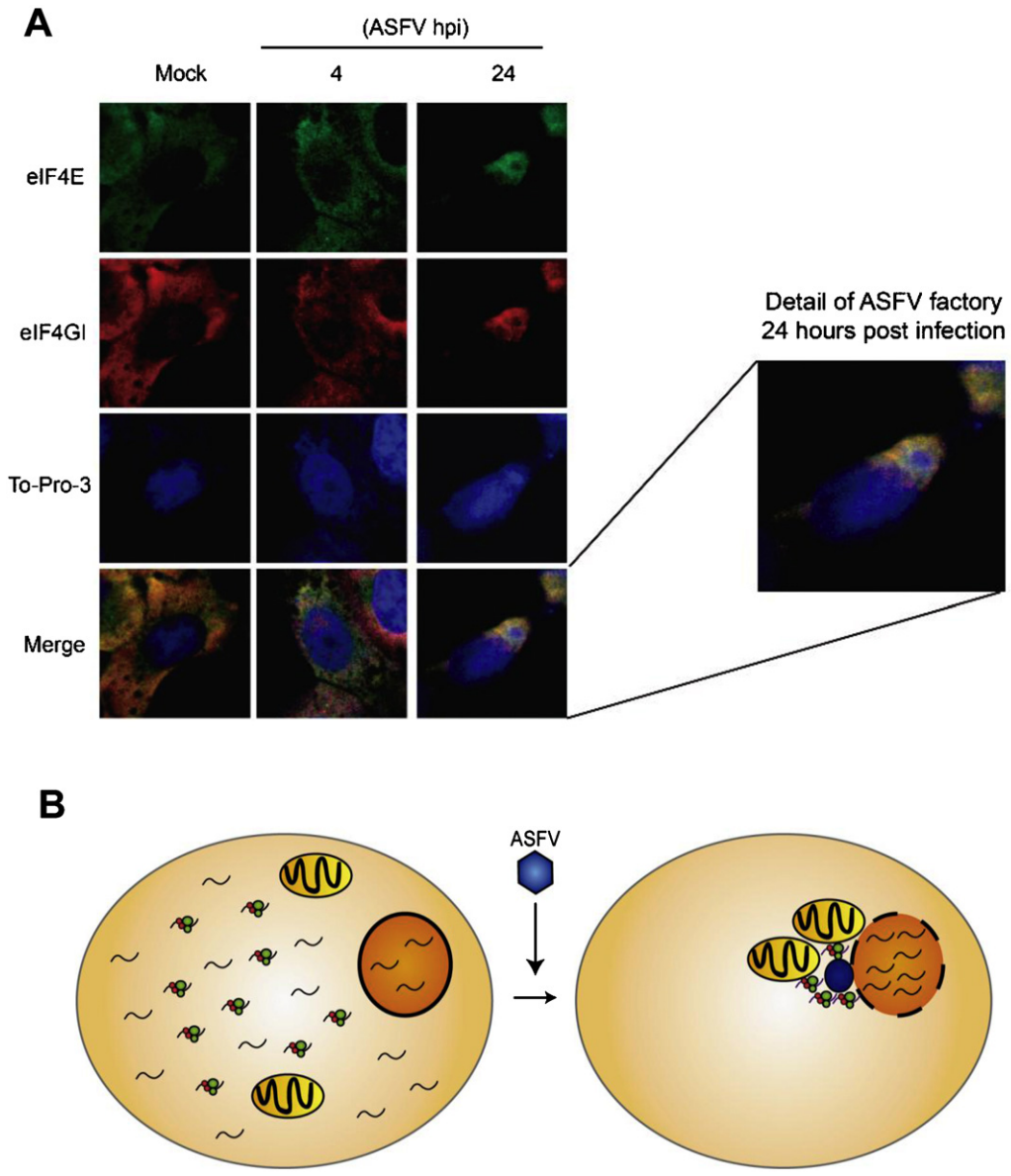

Fig. 11. (A) eIF4E and eIF4GI redistribution during ASFV time-infection steps. Vero cells were cultured on glass coverslips and mock infected or infected with ASFVBa71V. Cells were permeabilized and fixed at 4 and 24 hpi. Cellular factors eIF4E and eIF4GI were detected by indirect immunofluorescence. Cell nuclei and ASFV factories were stained with To-Pro-3. Cells were visualized by confocal microscopy and the cell outline was defined by phase contrast microscopy. Images were obtained under restricted conditions and processed with Huygens 3.0 software. (B) Mobilization of the cellular translation machinery and mitochondria to viral factories. During ASFV infection, eukaryotic initiation factors (eIFs), ribosomes and mitochondria are recruited to the periphery of the viral factories and cytoplasmic poly (A) RNAs are degraded. Simultaneously, poly (A) RNAs accumulates in the nucleus of infected cells.

2.5. Regulation of RNA metabolism by ASFV

Several viruses strongly alter RNA metabolism (Castello et al., 2009b; Covarrubias et al., 2011; Cheng et al., 2005; Park et al., 2008; Parrish and Moss, 2007; Parrish et al., 2007; Richner et al., 2011; Sandri-Goldin, 2011; Satterly et al., 2007; von Kobbe et al., 2000). Due the genetic complexity of ASFV, it is not surprising that it displays multiple mechanisms to alter transcription and, most probably, nuclear RNA export and RNA stability.

During infection viruses can alter the distribution and quantity of cellular mRNAs, decreasing the competence for the translational machinery. This can be achieved by altering cellular RNA metabolism functions. In the case of HSV-1, the splicing machinery is inhibited leading to an accumulation of cellular mRNAs at the nucleus of infected cells (Sandri-Goldin, 2011), while in the case of AdV (Yatherajam et al., 2011), VSV (von Kobbe et al., 2000), poliovirus (Castello et al., 2009a; Park et al., 2008) and influenza virus (Satterly et al., 2007) host mRNAs export from the nucleus to the cytoplasm is impaired by targeting the nuclear pore complex. In other cases, viruses stimulate mRNA degradation, as occur in HSV-1 (Cheng et al., 2005) and gammaherpesvirus (Covarrubias et al., 2011; Richner et al., 2011) that encode for ribonucleases. Vaccinia virus also induced RNA degradation and, even though the mechanism is still unclear, it is proposed that viral-encoded decapping enzymes could trigger RNA degradation (Parrish and Moss, 2007; Parrish et al., 2007).

A progressive decrease in the amount of cytoplasmic polyadenylated mRNAs, together with an increment of the pool of nuclear RNAs was observed during ASFV infection (Castello et al., 2009a), suggesting that poly (A) mRNA stability and nuclear RNA export could be altered. Nuclear organization is damaged as a consequence of ASFV infection, which includes nucleoli and nuclear envelope

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disorganization (Ballester et al., 2011). At early times post infection, laminin A/C is phosphorylated and the lamina network is disassembled at the proximity of the viral factories, showing lamin A/C in the cytoplasm of infected cells at late times post infection. Similarly, both nucleoporin p62, a marker of the nuclear envelope; and B-23, a marker of the nucleolus, are found in the cytoplasm of ASFV infected cells at late times post infection, suggesting an important damage in nuclear functions. Although lamin A/C localizes in the cytoplasm, a pool of this protein have been found to co-localize with the viral protein p54 (a marker of the viral factories). Nevertheless, no direct interaction between lamin A/C and viral proteins was found (Ballester et al., 2011). In addition to the cytoplasmic distribution, the lamin A/C that remains in the nucleus is reorganized into nucleoplasmic sites together with other transcriptionally related markers as SC35 at early times post-infection. Simultaneously, RNApol II is early dephosphorylated and then degraded at late times post infection. This suggests the existence of a viral mechanism to inactivate global cellular transcription (Ballester et al., 2011). Collectively, all these data support that ASFV is altering important RNA metabolism steps within the nucleus that should impact in host gene expression.

ASFV genome encodes for a protein called g5R that harbors a Nudix motif, which is comparable to other Nudix hydrolases such as the host decapping enzyme 2 (Dcp2) or the viral proteins D9 and D10 of VV (McLennan, 2007). g5R exerts in vitro decapping activity (Parrish et al., 2009), although it preferentially degrades diphosphoinositol polyphosphates under this conditions (Cartwright et al., 2002). Nevertheless, it is still unknown the role of this protein in the viral biological cycle, but its high homology (at least for the Nudix motif) with Dcp2 suggests its involvement in mRNA decapping. Therefore, this viral enzyme represents a candidate to be a viral factor involved in the degradation of the cellular mRNAs, which may contribute in the cellular shut off.

Unpublished data from our lab shows the predicted structure of g5R and its structural homology with the cellular decaping enzyme Dcp2 from *Schizosaccharomyces pombe*, but further studies have been planned to demonstrate the function of this ASFV protein in vivo.

2.6. Future perspectives

During the last decades, numerous studies focused on how viruses manipulate and control the host translational machinery. However, the molecular mechanism that complex DNA viruses such as ASFV develop to monopolize host cell translation is still unclear and requires extensive research.

Even though it has been described the state and distribution of the eIFs in ASFV-infected cells (Castello et al., 2009b), the exact mechanism by which these events are accomplished remain elusive. Future studies should face this biological problem at the molecular level, i.e. (i) identification of the signaling pathways triggered by ASFV and their activation mechanism; (ii) determination of the viral mechanisms that promote the redistribution of cellular sources (eIFs, ribosomes, mitochondria and other host factors) to the viral factories; and (iii) decipher the role of these events in host and viral gene expression.

Understanding how ASFV mRNA translation takes place, will give further insights into new strategies for antiviral treatments. A recent study has reported the atlas of RNA-binding proteins of proliferating HeLa cells, adding hundreds of novel RBPs (Castello et al., 2012). This represents a useful tool for in silico identification of new RNA-binding proteins and domains in the ASFV proteome. g5R represents a viral-encoded putative RBP and this discovery raises several questions: (i) Is g5R a bona fide RBP? (ii) Does it harbor decapping activity in vivo? (iii) What is the role of this protein in viral and host gene expression? and (iv) Could it be an alternative target for antiviral therapy?

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