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Short communication

Characterization of African swine fever virus IAP homologue expression in porcine macrophages infected with different virulence isolates

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ABSTRACT

Genes modulating apoptosis are encoded by many viruses and have an important role in viral evasion mechanisms. Our objective was to characterize the expression of the IAP homologue gene of African swine fever virus (ASFV), *4-CL*, during *in vitro* infection of porcine macrophages, the preferential target cell for viral replication. Expression was compared along parallel infections by two naturally occurring ASFV isolates of different virulence: highly virulent ASFV/L60 (L60) and low virulent non-hemadsorbing ASFV/NH/P68 (NHV). Efficiency of macrophage infection by both isolates was similar, as observed both by the percentage of infected cells in culture and by virus progeny production. Our results showed that transcription of *4-CL* initiates very early after infection with both isolates, since specific mRNAs were observed and quantified at 1.5 h post-infection (p.i.). However, the protein was produced later, from 4 to 8 h p.i., around the same time when viral DNA replication is reported to occur. 4-CL protein was more abundant in L60 than NHV infected cells, at both 8 and 16 h p.i. The mRNA levels, however, did not correlate with those of protein expression. Overall our results suggest the existence of a post-transcriptional step in the regulation of *4-CL* gene expression.

1. Introduction

African swine fever virus (ASFV) is a large double stranded DNA virus with 170–190 kb, classified as a sole member of *Asfarviridae* family (Dixon et al., 2005). It is responsible for a highly contagious and fatal disease of domestic pigs, representing a serious threat to swine industry worldwide.

In many viral infections, host cells trigger the apoptotic cell death program as a defence mechanism, in order to abort viral replication and limit viral dissemination in the organism. Thus, along evolution, especially large and complex animal viruses with long replication cycles, acquired tools to prevent premature death of the host cells, namely genes intervening in the apoptotic signalling cascades (Benedict et al., 2002). Similarly, ASFV possesses in its genome at least three genes implicated in apoptosis inhibition: a *bcl-2* homologue (Afonso et al., 1996; Galindo et al., 2008), a lectin homologue (Hurtado et al., 2004), and an IAP homologue (Nogal et al., 2001), the last being subject of this study. IAPs are key inhibitors of apoptosis through their capacity to interfere with caspases, the proteolytic enzymes acting at the heart of the apoptotic process, ultimately responsible for cell demise (Shi, 2004). Current knowledge on the mode of action of IAPs indicates that their inhibitory effect on caspases is exerted by direct

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interaction with the processed active enzymes, either by blocking the catalytic part of the enzymes via their BIR domains, or through E3 ubiquitin ligase activity from RING domains, targeting caspases for rapid degradation via proteasome (Vaux and Silke, 2005). The ASFV IAP, denominated 4-CL in the naturally occurring virulent isolate Malawi Lil-20/1 (Neilan et al., 1997) and A224L in the non-virulent Vero cell line adapted isolate Ba71V (Yanez et al., 1995), contains a single N-terminal BIR-like motif, and instead of a C-terminal RING finger, possesses a putative zinc finger motif of the C_4 type (Chacon et al., 1995). On a Vero cell line adapted infection model, *A224L* deletion leads to higher caspase-3 activity and cell death comparatively to infection with Ba71V parental virus (Nogal et al., 2001). Also, over-expression of A224L inhibited caspase-3 activity in cells treated for apoptosis induction, seemingly by a direct interaction, since the viral protein was co-precipitated with active caspase-3, suggesting a mode of action similar to other IAPs (Nogal et al., 2001). It was previously reported that A224L protein is produced after the onset of viral replication, hence at a late phase of infection (Chacon et al., 1995). However, viral DNA replication starts around 6 h post-infection (p.i.), and morphogenesis and viral cycle completion take several more hours, being largely concluded at around 18 h p.i. (Brookes et al., 1996). Thus, it would be important to assess more accurately when during the infection cycle the viral IAP homologue is expressed.

ASFV isolates of different virulence circulate in nature and lead to different outcomes of infection, ranging from acute and fatal to clinically unapparent forms of disease. Relevant to viral pathogenesis, ASFV replicates preferentially in porcine macrophages, known to play central roles for the development of immune responses. Our studies have been focused on the characterization of viral–macrophage interactions during infection with ASFV isolates of different virulence. Such studies aim at identifying the still unknown viral or host factors subjacent to the different immune responses and outcomes of infection. Given the important role that genes modulating apoptosis may have for pathogenesis and viral evasion of protective mechanisms, our objective was to further characterize the expression of one of these genes, the IAP homologue denominated *4-CL*, during *in vitro* infection of porcine macrophages by two naturally occurring ASFV isolates of different virulence: highly virulent ASFV/L60 (L60) and low virulent non-hemadsorbing ASFV/NH/P68 (NHV) (Leitao et al., 2001).

2. Materials and methods

2.1. Cells

Heparinized blood samples from crossbred Large White × Landrace pigs (6-month old) were obtained in aseptic conditions during bleeding at the abattoir. Blood samples were incubated at 37 °C for 15 min with 10% v/v of a 5% v/v Dextran T500 solution in Hank's balanced saline solution. Supernatants were collected, diluted 1:1 in culture medium (RPMI 1640 with 100 IU/ml penicillin, 100 μg/ml streptomycin, 20 mM HEPES), and seeded (100 ml/flask) in T175 tissue culture (TC) flasks (Nunc 156502). Cultures were incubated at 37 °C for 48 h, and non-adherent cells were removed by extensive washing in pre-warmed phosphate buffered saline (PBS). Adherent macrophages were harvested by treatment with cold 0.8 mM EDTA in PBS, washed in PBS, and viability of cells was determined by Tripan blue dye exclusion. Macrophages were plated at 1.5×10^5 viable cells/cm^2 for each assay, allowed to adhere for 3 h, and washed again to remove non-adherent cells. Culture medium was RPMI 1640 with 100 IU/ml penicillin, 100 μg/ml streptomycin, 20 mM HEPES, supplemented with 10% foetal calf serum.

2.2. Virus and viral infections

ASFV isolates used were the low virulent NHV and the highly virulent L60 (Leitao et al., 2001). These were added to parallel macrophage cultures at MOI 3, unless otherwise stated, and after 1 h adsorption cells were washed twice, new medium was added, and incubation proceeded for the indicated times. Similarly treated mock-infected cultures were kept in parallel as a control. At each independent assay, infections/mock-infections were performed with cell cultures obtained from the same pig donor.

2.3. RNA extraction

Total RNA was extracted from macrophage cultures on 6-well TC plates, at the indicated times post-infection (p.i.), through TRIzol Reagent (Invitrogen) following manufacturer instructions. Contaminating DNA was removed through "Turbo DNA-*free*" system (Ambion).

2.4. Real time PCR

RNA (0.6 μg/reaction) was used for cDNA synthesis through Superscript II Reverse Trancriptase (Invitrogen) and oligo pdT_{12-18} for priming. 2 μl of 1:10 diluted cDNA was used in each amplification reaction. Primers for *4-CL* sequence were (5′–3′) GCTCCATAGATGCACGAAATC and AAGCCGCAGTTTCTACTCCA, amplifying a 193 bp sequence. Primers for house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were designed according to the known mRNA sequence from *Sus scrofa* (GenBank accession AF017079). Intron locations were inferred from existing information for *Homo sapiens GAPDH* (GenBank accession NM_002046). Chosen primers were (5′–3′) GTCGGAGTGAACGGATTTG (on the first exon–intron junction) and CTTGACTGTGCCGTGGAA (on the third exon), amplifying a 168 bp sequence in cDNA. Quantification of target sequences on cDNA was obtained through standard curve construction (absolute quantification) (Whelan et al., 2003). The above primers were used to obtain DNA of known concentration by PCR amplification on cDNA, as previously described (Granfar et al., 2005). 0.6 μM of each primer, 1.5 mM $MgCl_2$, 200 μM each dNTP and 59 °C annealing temperature permitted to obtain specific amplification products, as confirmed in 2.5% agarose gel electrophoresis. Amplification products were purified through "High Pure PCR Product Purification Kit"