



African swine fever virus p10 protein exhibits nuclear import capacity and accumulates in the nucleus during viral infection

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Abstract

African swine fever virus (ASFV), a large enveloped DNA-containing virus, infects domestic and wild pigs, and multiplies in soft ticks, causing an economically relevant hemorrhagic disease. Evaluation of the nuclear import ability of ASFV p10 protein was the major purpose of the present work. Two approaches were used to determine if p10 protein is imported into the nucleus by an active process: a yeast-based nuclear import assay and the determination of the subcellular localization of p10 protein in mammalian cells by fluorescence microscopy. The results obtained clearly demonstrate that p10 protein is actively imported into the nucleus, both in yeast and mammalian cells. Experiments aiming at identifying the critical residues responsible for the nuclear import of ASFV p10 protein indicate that the amino acids comprised between the positions 71 and 77 are important, although not sufficient, for the protein active nuclear import. In ASFV-infected cells, the p10 protein strongly accumulates in the nucleus at late times post-infection, indicating that p10 protein may accomplish an important function inside the nucleus during the late phase of the viral replication cycle.

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1. Introduction

African swine fever virus (ASFV), the only member of the family *Asfarviridae* has been described as a missing evolutionary link between the families *Poxviridae* and *Iridoviridae*, since ASFV shares the genomic organization and the striking icosahedral symmetry of those families, respectively (Salas et al., 1999). The early descriptions of ASFV infections of domestic pigs were of an acute hemorrhagic fever, which caused approximately 100% mortality. In the last years, an increased number of isolates were identified, where the virus pathogenesis may range from rapidly fatal to subclinical, chronic or no explicit symptoms of disease (Leitão et al., 2001; Dixon et al., 2004).

The virus particle possesses a complex structure composed of several concentric domains with an overall icosahedral shape and an average diameter of 200 nm (Carrascosa et al., 1984; Andrés et al., 1997, 1998). The viral core is composed of a DNA-containing nucleoid surrounded by a thick protein layer, the core shell, and is wrapped by a lipid envelope and an icosahedral capsid (Carrascosa et al., 1984; Andrés et al., 1997). Extracellular ASFV particles usually possess an additional membrane acquired by budding through the host cell plasma membrane (Breese and Pan, 1978). The viral genome is a single molecule of double-stranded DNA ranging in size from 170 to 190 kbp with terminal inverted repetitions and terminal cross-links, which encodes approximately 50 structural proteins (Yáñez et al., 1995). Some of the ASFV proteins have been shown to exhibit DNA-binding activity like the structural p10 protein which is localized in the viral nucleoid (Muñoz et al., 1993; Andrés et al., 2002).

Although ASFV has been considered for many years as a virus that replicates exclusively in the cytoplasm of infected cells, the observation that the virus does not replicate in enucleated Vero cells nor is its DNA synthesized (Ortín and Viñuela, 1977) raised the possibility that the nucleus is involved in viral DNA replication. More recently, it has been reported that the replication of viral DNA is initiated in the nucleus, a stage which is followed by a longer cytoplasmic phase (García-Beato et al., 1992; Rojo et al., 1999). The mechanisms of ASFV viral genome nuclear import and export are still unknown. However,

as described for other viral genomes, this nucleus-cytoplasm transport is most likely mediated by viral proteins associated with the ASFV DNA (Whittaker and Helenius, 1998).

The active and bidirectional transport of proteins across the nuclear pore complex (NPC) is a tightly regulated process that plays a vital role in eukaryotic cells. Small molecules like metabolites, ions and small proteins can diffuse through the 9-nm NPC channel in either direction, between the nucleus and cytoplasm, which sets an upper limit for free diffusion to ~45–60 kDa. However, particles as large as 36 nm in diameter can be transported through the NPC by a selective and energy-dependent mechanism that acts to translocate macromolecules into and out of the nucleus. The active nucleus-cytoplasm transport is mediated by shuttling receptors that interact with localization signals on cargo molecules, RanGTP and proteins of the NPC (Görlich and Kutay, 1999; Macara, 2001).

The active import of proteins across the nuclear envelope requires the presence of specific targeting sequences within the protein, named nuclear localization signals (NLSs), even when the proteins are small enough to diffuse through the NPC (Görlich et al., 1996). There are at least three types of NLSs, which are characteristically rich in the basic amino acids lysine and arginine (Dingwall and Laskey, 1991). The first type, referred as classical NLS, is composed of a basic amino acid stretch $(K/R)_{4-6}$ preceded by a glycine, proline or an acidic amino acid residue, similar to the NLS of simian virus 40 (SV40) large T antigen (Kalderón et al., 1984). The second type is a bipartite sequence composed of two basic amino acid stretches separated by an intervening nonconserved 10–12 amino acid spacer: $(K/R)_2X_{10-12}(K/R)_3$, an example of this being the *Xenopus Laevis* nucleoplasmin (Dingwall et al., 1988). The third type is a less well-conserved sequence with few basic residues such as that of the adenovirus E1A protein (KRPRP) (Lyons et al., 1987). However, many other NLSs have been identified that differ from these sequences with respect to size and/or the content in basic amino acids (Makkerh et al., 1996; Michael et al., 1997). In addition to linear NLSs, discontinuous epitopes that come together upon folding into tertiary structure have been described to contribute to the nuclear import of histone proteins (Baake et al., 2001). Therefore, it is

difficult to identify an NLS without experimental evidence.

ASFV p10 structural protein is encoded by the K78R gene and its DNA binding capacity has been described for both double- and single-stranded DNA (Muñoz et al., 1993). This protein is an extremely hydrophilic polypeptide with a relatively high content of basic residues (23%), namely lysine residues (Muñoz et al., 1993). Motivated by such characteristics, we decided to investigate whether ASFV p10 protein exhibits nuclear transport activity.

2. Materials and methods

2.1. Plasmid constructs

pNIA, pNIAE2, pNIA + E2, pNIAD2(ant) and pNIAGFP plasmids were described previously (Rhee et al., 2000; Eulálio et al., 2004).

pNIAGFPp10 and GFPp10 were generated by cloning p10 cDNA, downstream of the green fluorescent protein (GFP). The cDNA encoding p10 was amplified by PCR with ASFV Lisbon 60 strain DNA as a template and using specific primers. The primers introduced BamHI and PstI recognition sites, respectively, at the 5' and 3' ends of the amplified DNA sequence. The DNA fragment was then cloned into the BamHI/PstI restriction sites of the plasmid for nuclear import (pNIA) containing the GFP (pNIAGFP).

GFPβGUS was generated by cloning β-glucuronidase protein (βGUS) cDNA downstream of GFP. The cDNA encoding βGUS was amplified by PCR using specific primers that introduced BamHI and BglII recognition sites, respectively, at the 5' and 3' ends of the amplified DNA sequence. The DNA fragment was then cloned into the BglII restriction site of pEGFP-C2 (Clontech).

ASFV p10 protein and the various fragments used for mapping the NLS of p10 protein were amplified by PCR, using specific primers that introduced BamHI or BglII and PstI recognition sites, respectively, at the 5' and 3' ends of the amplified DNA sequence. The DNA fragments were then cloned into the BglII/PstI restriction sites of GFPβGUS.

Proper framing and accuracy of the sequences of all DNA constructs were confirmed by DNA sequencing.

All primers used in this study and construction details are available on request.

Purification of plasmid DNA for sequencing and transfection experiments was performed using Plasmid Midi Kit (Qiagen) or Wizard[®] Plus SV Minipreps DNA Purification System (Promega).

2.2. Yeast nuclear import assay

The yeast nuclear import assay was performed as described previously (Rhee et al., 2000). Briefly, the pNIA-derived constructs were transformed into *Saccharomyces cerevisiae* strain L40, which contains the two LexA-inducible genes *HIS3* and *lacZ* (Hollenberg et al., 1995), by the lithium acetate method (Kaiser et al., 1994). The transformed yeasts were then plated on selective medium without tryptophan. Since pNIA contain the *TRP1* gene, only transformed yeasts can grow on this medium.

After growth, a few colonies were spread on minimal medium without tryptophan and in parallel were plated on minimal medium lacking both tryptophan and histidine, and supplemented with 10 mM 3-amino-1,2,4-triazole (3AT; Sigma), a repressor of yeast endogenous production. Yeast growth in the absence of histidine was evaluated.

Additionally, yeast cells plated on tryptophan-deficient medium were transferred to nitrocellulose filters and assayed for β-galactosidase activity (Breedon and Nasmyth, 1985). Briefly, after disruption of yeast cell membrane by incubation at -70°C for 30 min, the yeast lysate was incubated with β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-GAL; Sigma), and blue color development was evaluated.

For quantitative determination of β-galactosidase activity, an enzymatic assay was performed in liquid cultures (Stachel et al., 1985). After growth in liquid medium without tryptophan, yeast cells were disrupted, and the β-galactosidase chromogenic substrate *o*-nitrophenyl-D-galactopyranoside (ONPG; Sigma) was added in excess. After incubation at 30°C , the reaction was stopped by raising the pH to 11, which inactivates β-galactosidase activity. The β-galactosidase activity was calculated according to the following equation (Miller, 1972): β-galactosidase units = $1000 \times V \times \text{OD}_{600} \times \text{OD}_{420}/t$, where *V* is the volume of the sample used in the assay (in ml), OD_{600}

is the optical density at 600 nm of the yeast cell culture at the start of the assay, OD_{420} is the optical density at 420 nm of the sample measured after the incubation of yeast cell lysate with ONPG and t is the time of incubation (in min).

2.3. Cell culture and viruses

For transfection experiments, Vero cells (ATCC CCL81) were grown in VP-SFM (GIBCO) supplemented with L-Glutamine (GIBCO) and 1% fetal calf serum (FCS) or in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% fetal calf serum (FCS). For viral infection, Vero cells were grown in DMEM (GIBCO) supplemented with 10% FCS, which was reduced to 2% during viral infection. Highly purified extracellular ASFV BA71V strain, adapted to grow in Vero cells, was obtained by Percoll equilibrium centrifugation (Carrascosa et al., 1985) and used in the infections.

2.4. Transfection

Transfection experiments were performed using LipofectamineTM 2000 (Invitrogen), according to manufacturer's instructions. Vero cells were incubated 36 h to allow gene expression.

2.5. Fluorescence microscopy

For fluorescence analysis of GFP β GUS fusion proteins, Vero cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 30 min at room temperature, and rinsed with PBS. Then, coverslips were mounted with Mowiol 4–88 (Fluka) on glass slides and the preparations were examined with a Zeiss Axioskop2 plus fluorescence microscope, at a 600 \times magnification. Images were processed using Adobe Photoshop software.

2.6. Immunofluorescence microscopy

Pre-confluent Vero cells grown on coverslips were infected with ASFV at 10 pfu per cell. At different times post-infection, the cells were fixed with 2% paraformaldehyde for 1 h at room temperature or with 2% paraformaldehyde, under the same conditions, and then with methanol at -20°C for 5 min. In the case

where no methanol was used, cells were permeabilized with PBS/0.2% Triton X-100 for 5 min at room temperature. After being washed with PBS, the cells were blocked for 30 min with 1% cold fish skin gelatin (Sigma) and 10% normal goat serum (NGS) (Jackson Immunoresearch) in PBS with 0.1% Triton X-100. Vero cells were then incubated for 1 h with rabbit polyclonal serum anti-p10 (Andrés et al., 2002) and mouse monoclonal antibody 17LD3 anti-p72 (Sanz et al., 1985) diluted 1/1000 and 1/200, respectively, in blocking solution without NGS. After extensive washing with PBS/0.1% Triton X-100, cells were blocked for 30 min with 1% cold fish skin gelatin and 10% of NGS in PBS with 0.1% Triton X-100 and then incubated for 1 h with Alexa 488 goat anti-rabbit immunoglobulin G (IgG) and Alexa 594 goat anti-mouse IgG (Molecular Probes) diluted 1/500 in blocking solution without NGS. Finally, coverslips were washed with PBS/0.1% Triton X-100 and mounted with Mowiol 4–88 on glass slides. Preparations were examined using a Bio-Rad Microradiance confocal laser connected to a Zeiss Axioskop2 plus fluorescence microscope or using a Zeiss Axioskop2 plus fluorescence microscope, at a 600 \times magnification. Images were processed using Adobe Photoshop software.

2.7. Nucleotide sequence accession number

The sequence containing p10 gene from ASFV Lisbon 60 strain was deposited in GenBank under the accession number DQ275352.

3. Results

3.1. ASFV p10 protein is imported into the nucleus of yeast cells

To assess the nuclear import ability of ASFV p10 protein, a yeast-based nuclear import assay was used (Rhee et al., 2000). The underlying principle of this assay is the expression in yeast cells of a triple fusion protein encoded by pNIA, comprising mLexA (bacterial lexA modified to inactivate its internal NLS), Gal4AD (yeast Gal4p activation domain), and the protein to be tested, subcloned in-frame downstream of Gal4AD. The expression of the two reporter

genes *HIS3* and *lacZ* present in the L40 *S. cerevisiae* strain under the control of LexA (Hollenberg et al., 1995) depends on the intracellular localization of the tested fusion protein. If the tested protein contains a functional NLS, the fusion product is localized in the yeast cell nucleus. Yeast cells harboring this construct possess β -galactosidase activity and are able to grow in the absence of histidine as a consequence of *lacZ* and *HIS3* gene expression, respectively. Conversely, if the protein lacks a functional NLS, the fusion product is retained in the yeast cytoplasm, at least partly abolishing the β -galactosidase activity and impeding growth on histidine-deficient medium. The green fluorescent protein (GFP) encoding DNA was cloned upstream of p10 protein to ensure that the molecular weight of the fusion protein would be higher than 60 kDa, which is the diffusion limit of the nuclear pore complex (Görlich and Kutay, 1999), therefore excluding the possibility of any nucleus-cytoplasm diffusion.

As shown in Fig. 1a, yeast cells expressing the fusion product containing the ASFV p10 protein (pNIAGFPp10) were able to grow on histidine and tryptophan deficient medium, indicating that expression of the reporter *HIS3* gene was induced. In addition, this construct induced β -galactosidase activity (Fig. 1a). pNIA + E2 was used as a positive control for the nuclear import assay. This plasmid encodes the protein VirE2, an *Agrobacterium* protein shown to lack a NLS functional in animal cells (Guralnick et al., 1996), fused to the NLS of SV40 large T antigen which is actively imported into the nucleus, thus leading to maximal expression of the two reporter genes. No yeast growth was observed for pNIAE2 and pNIAD2 antisense (pNIAD2(ant)), which were used as negative controls. pNIAE2 expresses VirE2, and pNIAD2(ant) contains the cDNA sequence of VirD2, an *Agrobacterium* protein that carries a functional NLS (Citovsky et al., 1994), subcloned in the antisense orientation (Rhee et al., 2000).

To validate the qualitative results of β -galactosidase activity obtained by the filter lift assay, the liquid culture assay was used to quantify the activity of this enzyme in yeasts expressing the different constructs. Yeast cells harboring pNIAGFPp10 showed significantly higher levels of β -galactosidase activity than those observed for the negative controls (pNIAE2 and

pNIAD2(ant)) (Fig. 1b). pNIAGFP which encodes the fusion protein mLexA-Gal4AD-GFP, was used as a negative control. As expected, the presence of GFP did not have any effect on the levels of β -galactosidase activity (Fig. 1b).

Together, these results strongly suggest that p10 protein contains a functional NLS.

3.2. ASFV p10 protein is imported into the nucleus of mammalian cells

To confirm the results obtained using the yeast-based nuclear import assay in an ASFV native system, we performed studies on the subcellular localization of p10 protein in ASFV host mammalian cells (Vero cells). For this purpose, we generated a plasmid coding for a fusion protein that contains GFP and β -glucuronidase (GFP β GUS), thus being larger (~94 kDa) than the size limit for diffusion through the NPC (Görlich and Kutay, 1999), and p10 protein was then subcloned at the C-terminus of β GUS. Fusion proteins were expressed in Vero cells by transient transfection, and protein intracellular localization was visualized by fluorescence microscopy.

As shown in Fig. 2, localization of the expressed GFP β GUS protein was exclusively cytoplasmic, in contrast to what was observed with GFP alone, which is distributed throughout the cell as a consequence of its molecular weight that allows its passive diffusion within the cell. However, it is interesting to observe that the fusion protein containing GFP, β GUS and p10 protein (GFP β GUSp10) accumulated strongly in cell nuclei (Fig. 2), providing clear evidence that ASFV p10 protein contains at least one NLS that is capable of mediating active protein transport from the cytoplasm to the nucleus in mammalian cells.

3.3. Subcellular localization of different fragments of the ASFV p10 protein in mammalian cells

To identify the localization of the functional nuclear import signal(s) within ASFV p10 protein, the subcellular localization of several GFP-tagged p10 fragments was analyzed in Vero cells by fluorescence microscopy (Fig. 3).

A detailed analysis of the amino acid sequence of ASFV p10 protein of the Lisbon 60 strain (GenBank

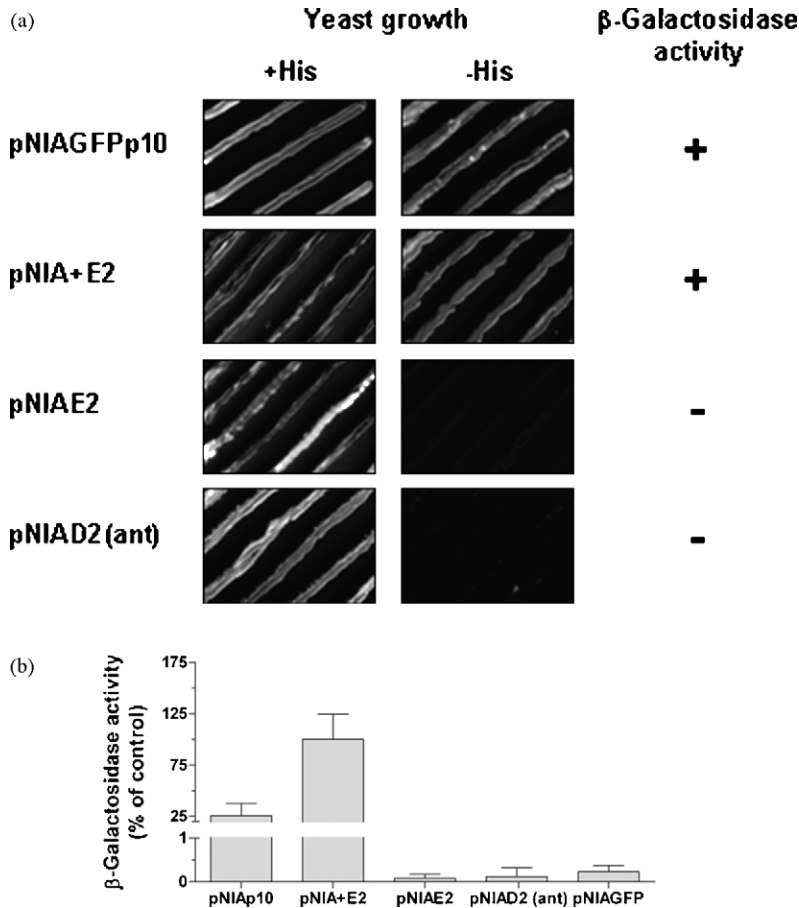


Fig. 1. Evaluation of the nuclear import ability of ASFV p10 protein in yeast cells. (a) Evaluation of yeast cell growth and β -galactosidase activity. The growth of L40 yeasts transformed with the indicated constructs was analyzed both on selective medium without tryptophan (+His) and on medium without tryptophan and histidine (–His). The β -galactosidase activity was evaluated using the colony-lift filter assay. (b) Quantification of β -galactosidase activity in liquid cultures. The data are expressed as a percentage of maximal enzymatic activity obtained for pNIA + E2. Standard deviations are shown based on triplicates of at least three independent experiments.

accession number DQ275352) led to the identification of a lysine and arginine rich sequence between amino acids 71 and 77 (KKIKRSK), this constituting, therefore, a potential candidate to mediate the nuclear import of p10 protein. This sequence is also present in ASFV p10 protein of BA71V strain (Muñoz et al., 1993) (Fig. 4), and resembles the most commonly known classical NLSs which are composed of 4–6 K/R amino acids, similar to the NLS of SV40 large T antigen (Kalderón et al., 1984). In contrast to what was observed for the full-length protein (GFP β GUSp10), the fragment GFP β GUS (1–70) lost the exclusivity of nuclear localization (compare Figs. 2 and 3), indicating that although not alone, the sequence

between amino acids 71 and 77 may be involved in the active nuclear import of p10 protein, as predicted by the amino acid sequence analysis. Unexpectedly, the fragments GFP β GUS (31–77) and GFP β GUS (54–77) were not imported into the nucleus of transfected cells (Fig. 3), clearly indicating that the presence of the sequence between amino acids 71 and 77 is not sufficient to mediate the active nuclear import of p10 protein.

Analysis of the nuclear import ability of several other p10 fragments (GFP β GUS(1–30), GFP β GUS(1–16), GFP β GUS(9–70), GFP β GUS(31–70), GFP β GUS(30–53), GFP β GUS(30–40) and GFP β GUS(40–53)) (Fig. 3), did not allow the

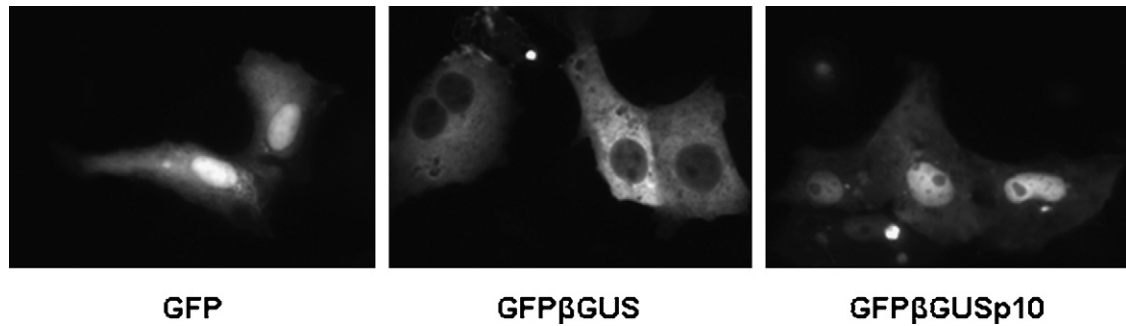


Fig. 2. Evaluation of the nuclear import ability of ASFV p10 protein in mammalian cells. Subconfluent cultures of Vero cells were transiently transfected with the plasmids encoding GFP, GFPβGUS or GFPβGUSp10. Thirty-six hours after transfection, the cells were fixed and the subcellular localization of the different proteins was analyzed by fluorescence microscopy (magnification 600×). Experiments were performed at least two times and representative images are shown.

identification of any other stretch of amino acids responsible for the nuclear import of p10 protein, since all these fragments exhibited an essentially cytoplasmic localization. On the other hand, the p10 protein fragments that contain the N-terminal of the protein (GFPβGUS(1–51), GFPβGUS(1–62) and GFPβGUS(1–70)) exhibited partial nuclear localization (Fig. 3). Curiously, the fragment GFPβGUS(9–77) was the only one that exhibited a nuclear localization similar to that observed for the full-length p10 protein (Fig. 3).

Taken together, these results strongly suggest that the functional NLS of ASFV p10 protein is not simply composed of a linear (either continuous or bipartite) stretch of amino acids. Instead, it seems to be dependent on a specific and complex three-dimensional protein folding pattern.

3.4. ASFV p10 protein accumulates in the cell nucleus during viral infection

To investigate whether p10 protein is actively imported into the nucleus of ASFV-infected cells, immunofluorescence experiments were performed in virus-infected Vero cells, which were fixed at different times post-infection. As a control we also monitored the intracellular distribution of ASFV p72, which is the major ASFV capsid protein and whose expression occurs during the late phase of the infection cycle (Cistué and Tabarés, 1992).

Fig. 5 shows the images obtained by confocal fluorescence microscopy from experiments involving a double labeling with the rabbit polyclonal serum

against p10 protein (upper panels) and the mouse monoclonal antibody against protein p72 (lower panels). During the first 8 h post-infection (hpi), p10 protein although poorly detected, was confined to the incoming virus particles, similarly to what was observed for p72 protein. On the other hand, at 10 hpi and at later times p10 protein was localized almost exclusively in the nucleus of the infected cells, although some labeling was also visualized in the viral factories. Anti-p72 antibodies strongly labeled the virus factories, as well as virus particles scattered throughout the cytoplasm at late times post-infection, as observed previously (Andrés et al., 2001). Since p10 is a structural protein, it would be expected to detect it more strongly at the viral factories where viral assembly takes place, as observed for protein p72.

It should be emphasized that in the present study we fixed the cells with paraformaldehyde and methanol. Methanol fixation has been reported to cause precipitation of cellular proteins and thus should overcome the problem of protein redistribution due to insufficient crosslinking. Moreover, methanol fixation washes out soluble cellular proteins (Brock et al., 1999), disrupts the cytoskeleton and leads to extraction of antigen and membrane lipids (Vekemans et al., 2004). On the other hand, paraformaldehyde provides good stabilization of permeabilized membranes, preventing the linkage of intracellular proteins with extracellular molecules and increases the indirect fluorescence of intranuclear antigens (Pollice et al., 1992). To combine the advantages of both procedures, methanol fixation preceded by paraformaldehyde crosslinking, as performed in our study, has been

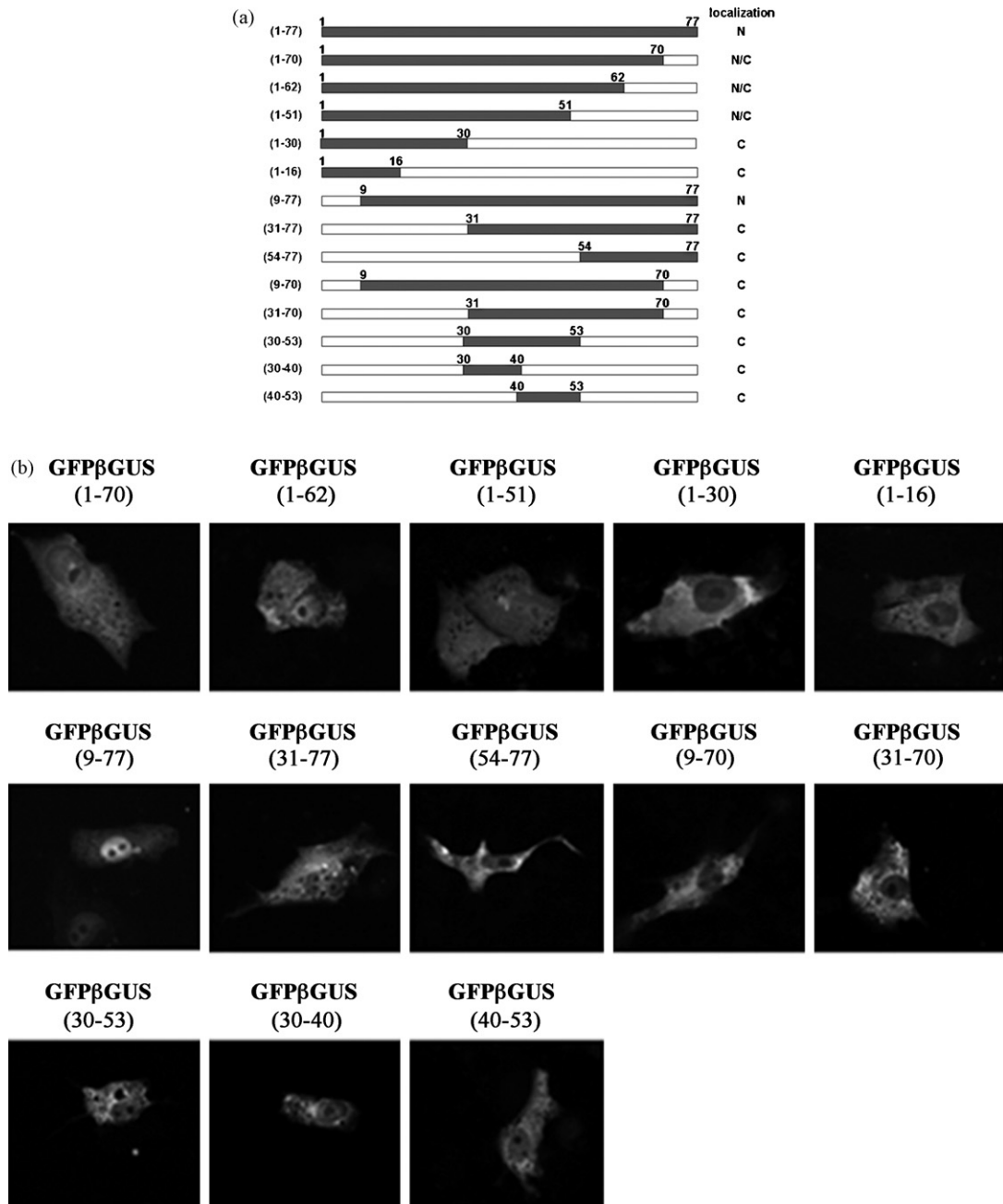


Fig. 3. Subcellular localization of different fragments of the ASFV p10 protein. (a) Schematic diagram of the different fragments of ASFV p10 protein and their subcellular localization in Vero cells: C, C/N and N indicate that, in the majority of the transfected cells, the fusion protein being tested presents an exclusively cytoplasmic, both cytoplasmic and nuclear, or nuclear localization, respectively. (b) Subcellular distribution of the different fragments of p10 protein evaluated by fluorescence microscopy (magnification 600 \times). Vero cells were fixed 36 h following transfection with the indicated constructs. Experiments were performed at least two times and representative images are shown.

Lisbon 60:		ATGCCTACAAAAGCTGGCACAAAAGTACCGCAAATAAAAAACGATCAAGGGGCCTTCC
Lisbon 60:	1	M P T K A G T K S T A N K K T I K G P S
BA71V:	1	M P T K A G T K S T A N K K T T K G S S
Lisbon 60:		AAGTCTGGTTCTGCCAAAAGGCCACACCGGCCAAAACCCATGCTACGGCCCTGCATCAA---
Lisbon 60:	21	K S G S A K G H T G K T H A T A L H Q -
BA71V:	21	K S G S S R G H T G K T H A S S S M H S
Lisbon 60:		GGGATGCTCTATAAAGATATGGTAAACATTGCTAAATCTAAAGGTATTCCGATTACCAG
Lisbon 60:	40	G M L Y K D M V N I A K S K G I P I Y Q
BA71V:	41	G M L Y K D M V N I A R S R G I P I Y Q
Lisbon 60:		AATGGATCACGTCTTACTAAAAGTGAATTGGAGAAAAAATTAAACGATCAAAAATGA
Lisbon 60:	60	N G S R L T K S E L E K K I K R S K -
BA71V:	61	N G S R L T K S E L E K K I K R S K

Fig. 4. Nucleotide sequence that codes for ASFV p10 protein in Lisbon 60 strain, and its respective amino acid sequence. The amino acid sequence of ASFV p10 protein of BA71V strain is represented for comparison. Common amino acids to both strains are highlighted in grey. Amino acids comprised between positions 71 and 77 of ASFV p10 protein in Lisbon 60 strain, are inserted in a rectangle.

described as a good option for the fixation of transmembrane and soluble cytoplasmic proteins (Pollice et al., 1992; Brock et al., 1999; Hoetelmans et al., 2001). Nevertheless, to investigate whether the low detection of p10 protein in the viral factories was due to the use of methanol, we determined p10 localization in the ASFV-infected cells fixed both solely with paraformaldehyde and with paraformaldehyde and methanol, using fluorescence microscopy. As observed in Fig. 6, no significant differences were detected using both procedures. However, the presence of p10 in the viral factories was more evident in the images obtained by fluorescence microscopy than in those by confocal fluorescence microscopy (compare Figs. 5 and 6), confirming the presence of this structural protein in the viral assembly sites, as it would be expected.

4. Discussion

The gene encoding p10 protein of ASFV strain BA71V was first described by Muñoz et al. (1993). This small protein (~10 kDa) is a structural protein that exhibits a strong DNA binding activity with similar affinity for both double- and single-stranded DNA. Moreover, it contains a large number of basic amino acids (Muñoz et al., 1993), is localized at the virus nucleoid (Andrés et al., 2002) and might be involved in DNA packaging. In face of these characteristics, we hypothesized that this viral protein

may assist in the transport of ASFV DNA into the host cell nucleus during the viral infection cycle.

The active nuclear import ability of p10 protein was assessed by using the described yeast-based nuclear import assay (Rhee et al., 2000). Using this approach we were able to demonstrate that p10 protein is actively imported into the nucleus of yeast cells, as illustrated by the results showing that p10 protein is capable of directing nuclear accumulation of a large fusion protein, comprising mLexA, Gal4AD and GFP.

These results were validated in a more relevant biological system by determining the intracellular localization of p10 protein in mammalian cells by fluorescence microscopy. It should be noted that the fusion protein containing p10 protein (GFPβGUSp10) has a molecular weight above the limit for diffusion (~60 kDa) through the nuclear pore complex (Görlich and Kutay, 1999). In agreement with the results obtained in yeasts, Vero cells transfected with GFPβGUSp10 exhibited fluorescence almost exclusively inside the cell nucleus. It is interesting to note that this observation is in contrast to what was obtained in cells transfected with GFPβGUS, where the fluorescence is detected exclusively in the cell cytoplasm. These results show that, even being smaller than the diffusion limit of the NPC, ASFV p10 protein is able to exploit cellular mechanisms for active nuclear import.

Interestingly, the highly basic region comprised between amino acids 71 and 77 of p10 protein (KKIKRSK), which resembles other described NLSs

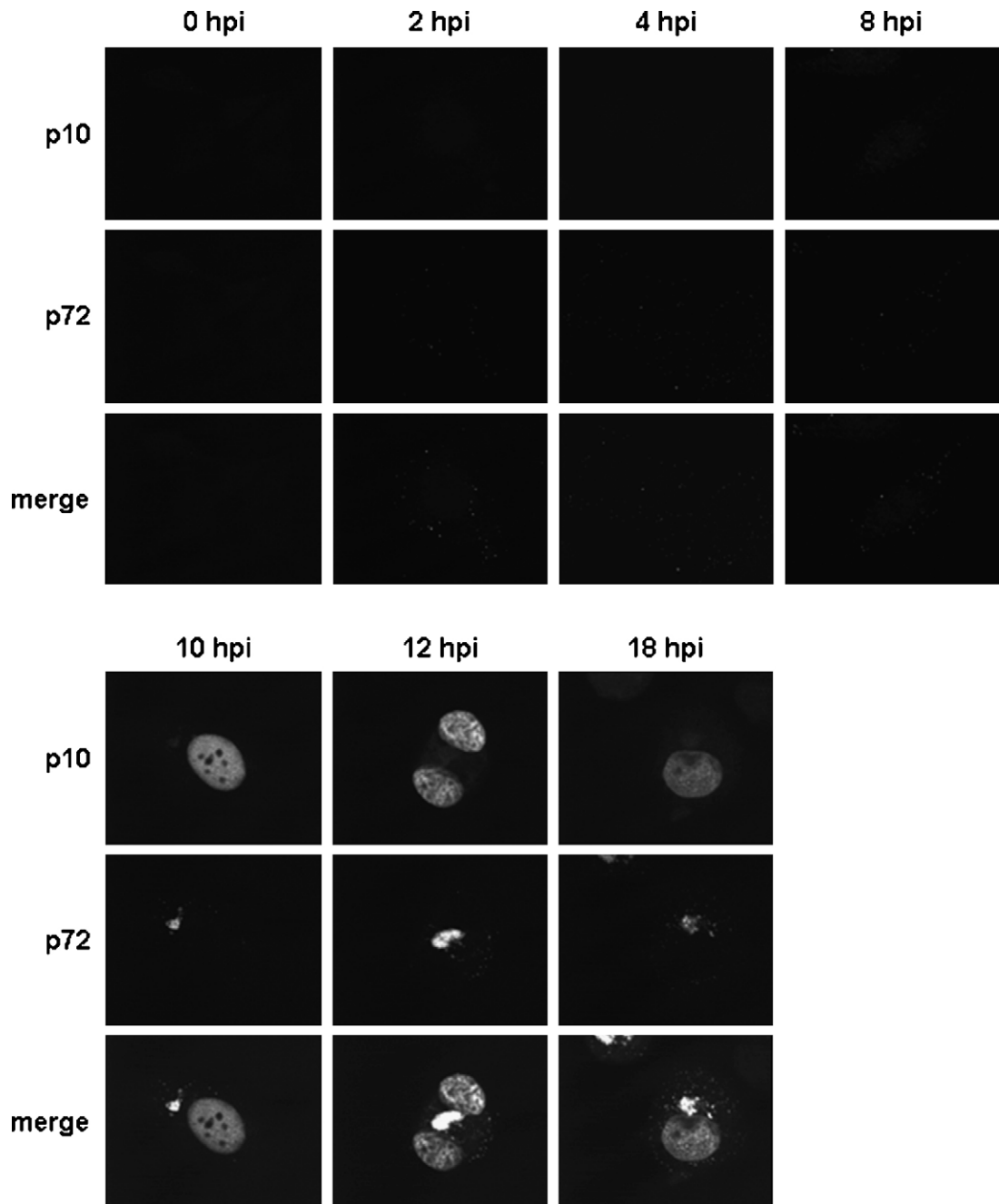


Fig. 5. Immunofluorescence microscopy of ASFV-infected cells. Vero cells infected with ASFV BA71V strain were fixed with 2% paraformaldehyde for 1 h at room temperature and then with methanol at -20°C for 5 min and processed for immunofluorescence analysis at the indicated times post-infection. Samples were double-labeled with rabbit serum anti-p10 and mouse monoclonal antibody 17LD3 anti-p72, followed by incubation with secondary antibodies conjugated to Alexa 488 and 594, respectively. Confocal laser scanning images acquired are presented both separately and merged. Experiments were performed at least two times and representative images are shown.

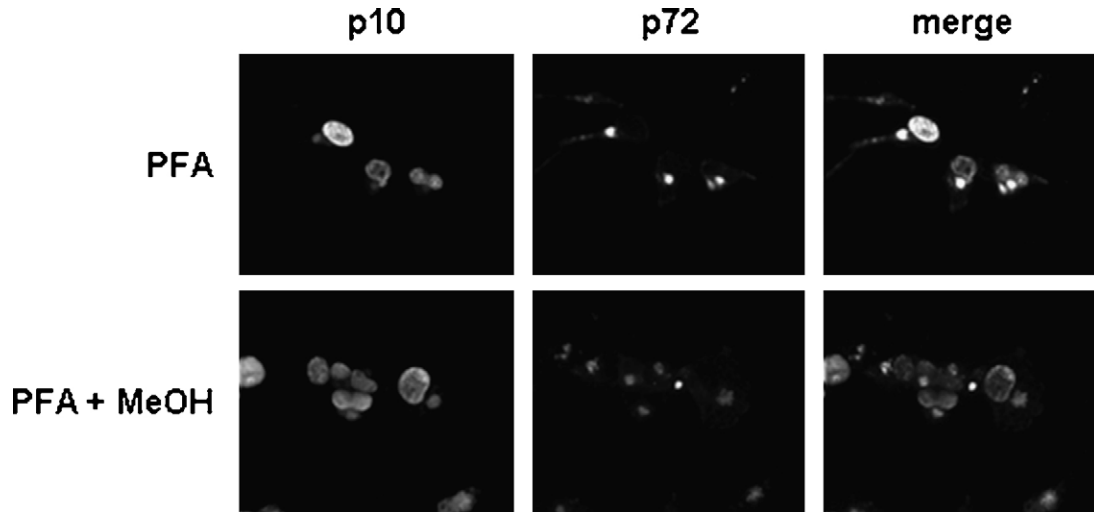


Fig. 6. p10 protein localization in ASFV-infected cells using different fixation conditions. Vero cells infected with ASFV BA71V strain were fixed with 2% paraformaldehyde for 1 h at room temperature (PFA) or with 2% paraformaldehyde for 1 h at room temperature and then with methanol at -20°C for 5 min (PFA + MeOH). In both cases, the cells were processed for immunofluorescence analysis at 18 hpi. Samples were double-labeled with rabbit serum anti-p10 and mouse monoclonal antibody 17LD3 anti-p72, followed by incubation with secondary antibodies conjugated to Alexa 488 and 594, respectively. Fluorescence microscopy images (magnification $600\times$) acquired are presented both separately and merged. Experiments were performed at least two times and representative images are shown.

like the classical NLS of SV40 large T antigen (PKKKRKV) (Kalderón et al., 1984) and the yeast histone 2B (GKKRSKA) (Moreland et al., 1987), was required for exclusive nuclear localization, as inferred from the observation that the fragment GFP β GUS(1–70) lost the exclusivity of nuclear localization. However, the presence of this sequence was not sufficient to induce protein nuclear import since the constructs GFP β GUS(31–77) and GFP β GUS(54–77) exhibited exclusively cytoplasmic fluorescence. The constructs GFP β GUS(1–70), GFP β GUS(1–62) and GFP β GUS(1–51) that do not contain the stretch of amino acids 71–77 exhibited fluorescence both in the cytoplasm and in the nucleus, indicating that although not sufficient to mediate an exclusive nuclear localization, the first 51 amino acids of ASFV p10 protein are also involved in the active protein nuclear import. However, since the constructs GFP β GUS(1–16) and GFP β GUS(1–30) exhibited exclusively cytoplasmic fluorescence, it was expected that amino acids comprised between positions 31 and 51 could promote at least partial protein nuclear localization. Nevertheless, this hypothesis was not confirmed since the constructs GFP β GUS(30–40), GFP β GUS(40–53), GFP β GUS(30–53), GFP β GUS(31–70)

and GFP β GUS(9–70) exhibited exclusively cytoplasmic fluorescence. We observed that solely the constructs GFP β GUSp10 and GFP β GUS(9–77), both containing the stretch of amino acids from position 71 to 77, exhibited exclusive nuclear localization.

To summarize, the results of the present work provide evidence that the amino acids comprised between positions 71 and 77 are important, although not sufficient, for the active nuclear import of ASFV p10 protein. It is possible that the role of this sequence depends also on the presence of discontinuous epitopes that may come together to form a specific three-dimensional arrangement of amino acids on the protein surface upon its folding, as observed for other proteins (Baake et al., 2001). It should be noted that the three-dimensional structure of a protein might be severely affected when the protein is expressed as a fusion protein, as is the case in our study. Further studies, namely the determination of the three-dimensional structure, may help to identify putative amino acids responsible for the active nuclear import of ASFV p10 protein.

The results from immunofluorescence studies in ASFV-infected Vero cells (strain BA71V) demonstrate that p10 protein is localized inside the nucleus at

10 hpi and also at later times (12 and 18 hpi). These results validate those obtained from studies involving transfection of the cells with p10 protein, confirming that this ASFV protein contains a functional NLS. In this context, it is relevant to point out that the amino acid sequences of ASFV p10 protein from the BA71V (GenPept accession number S29971) and Lisbon 60 strains, deposited in GenBank under the accession number DQ275352, Exhibit 85% amino acid identity, and that the sequence containing the last seven amino acids, identified as a potential NLS, display 100% identity in both strains. To our knowledge, the sequence of ASFV p10 protein of other strains has not yet been described.

It has been described that ASFV viral DNA synthesis is initiated inside the nucleus, a stage that is followed by a longer replication phase that occurs in the cytoplasm. The nuclear phase takes place during the early times post-infection, namely, during the first 8 h (García-Beato et al., 1992; Rojo et al., 1999). The fact that we were unable to detect p10 inside the nucleus of infected cells, by immunofluorescence, during the first 8 h post-infection does not support our hypothesis that ASFV p10 protein would be involved in the active transport of viral DNA into the host cell nucleus during the viral infection cycle. Further studies need to be addressed in order to understand the role of p10 protein during the replication cycle of ASFV. However, the observation that p10 strongly accumulates inside the nucleus of infected cells and that this protein has a functional NLS, which may induce a more rapid and efficient nuclear localization of the protein than that achieved via simple diffusion, raises the interesting possibility that ASFV p10 protein may play a role inside the nuclear compartment during the late phase of the viral infection cycle.

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