African swine fever virus protein p30 interaction with heterogeneous nuclear ribonucleoprotein K (hnRNP-K) during infection

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Received 15 July 2008; revised 6 August 2008; accepted 19 August 2008

Available online 5 September 2008

Edited by Gianni Cesareni

Abstract Heterogeneous nuclear ribonucleoprotein K (hnRNP-K) was identified as interacting cellular protein with the abundant immediate early protein p30 from African swine fever virus (ASFV) in a macrophage cDNA library screening. The interacting regions of hnRNP-K with p30 were established within residues 35–197, which represent KH1 and KH2 domains responsible for RNA binding. Colocalization of hnRNP-K and p30 was observed mainly in the nucleus, but not in the cytoplasm of infected cells and infection modified hnRNP-K subcellular distribution and decreased the incorporation of 5-fluorouridine into nascent RNA. Since similar effects were observed in cells transiently expressing p30, this interaction provides new insights into p30 function and could represent a possible additional mechanism by which ASFV downregulates host cell mRNA translation.

Keywords: African swine fever virus; p30; Ribonucleoproteins; Virus-cell interaction

1. Introduction

African swine fever virus (ASFV), the only member of the Asfarviridae family, is a large, cytoplasmic, double-stranded DNA virus that is responsible of a haemorrhagic and frequently fatal disease of swine [1]. The viral genome comprises more than 150 open reading frames and expression is regulated late depending on their requirement for viral DNA synthesis [2]. Little is known about the regulatory functions of ASFV proteins during infection and early virus proteins are important candidates to play critical roles in the modification of cellular metabolism to take advantage of host cell functions.

ASFV open reading frame CP204L encodes a 30 kDa protein named p30 or p32 [3,4] which represents the most abundantly expressed viral protein early in infection. p30 exhibits a predominantly cytoplasmic location within infected cells and results phosphorylated in serine residues at the N-terminal [4] before its final incorporation to the viral particle. p30 is also one of the most antigenic ASFV proteins [5], eliciting virus neutralizing antibodies in infected animals [6–8]. Previous studies have demonstrated a role for p30 in first stages of infection, since antibodies against p30 are able to inhibit virus internalization into the host cell [6]. Nevertheless, the regulatory function of p30 upon infection remains largely unknown.

To explore the potential targets of p30 during infection, we have used the yeast two-hybrid system to screen a porcine macrophage (the natural viral host cell) cDNA library for cellular proteins that may interact with p30. We have identified heterogeneous nuclear ribonucleoprotein K (hnRNP-K) as the first cellular ligand of p30.

hnRNP-K is a multifunctional protein since it has been described to interact with diverse molecules of cellular [9,10], and viral origin [11–14], being involved in a variety of cellular functions such as regulation of transcription and translation [15], RNA splicing, mRNA stability and transport of premRNA out of nucleus to cytoplasm. It has been demonstrated to interact with different molecules involved in signal transduction such as Src, Fyn and Lyn [16]. Recently, a number of new hnRNP-K partners have been identified by using a proteomic approach [17] and new roles have been suggested for this protein. ASFV p30 was found to interact directly with host hnRNP-K during ASFV infection and we have mapped the interacting regions of both proteins. p30 modifies hnRNP-K subcellular distribution and could contribute to modulate hnRNP-K functions related to processing and export of mRNAs during ASFV infection.

2. Materials and methods

2.1. Plasmids

For the yeast two-hybrid assay, plasmids pGBT9 and pACT2 (BD Sciences) were used as sources of the GAL4 DNA-binding domain (BD) and transcriptional activation domain (AD), respectively. To generate pGβT9-p30, the complete p30 coding sequence was directly amplified from ASFV genome of E70 isolate and inserted in EcoRI and BamHI sites in pGβT9. Unrelated ASFV protein p54 was used as negative control and cloned in pGβT9, as previously published [18]. A cDNA library from porcine macrophage, cloned in XhoI site in pACT2, was kindly provided by Linda Dixon [19]. p30 and
hnRNP-K different mutant truncations used for mapping the regions involved in the interaction were generated by PCR from pGBT9-p30 and pGEX-RNP-K, respectively. The fragments generated were inserted in frame with AD or BD in pGBT9 or pACT2. pACT2-K, containing complete hnRNP-K coding sequence fused to AD, was derived from pGEX-RNP-K. pCMV-p30 was also derived from pGBT9-p30 and used in transfection experiments. For the glutathione S-transferase (GST) pull-down experiments, plasmids pGEX-RNP-K (kindly provided by Dr. Levens) and pGEX-4T (GE Healthcare) were used to express GST-hnRNP-K fusion protein or GST alone in Escherichia coli.

2.2. Yeast two-hybrid
pGBT9-p30 and unrelated control protein pGBT9-p54 were independently used as baits to screen a pACT2 cDNA library from pig macrophages in S. cerevisiae reporter strain Y190 as previously published [18,20,21]. Yeast were sequentially transformed with bait plasmid and pACT2 library by the lithium acetate method. After auxotrophic and colony size selection, resulting clones were analyzed for expression of GAL4-dependent β-galactosidase. Plasmid DNA from those clones exhibiting β-galactosidase activity was isolated and retransformed into yeast strain Y190 with pGBT9-p30 to eliminate false positives. The sequence of inserts was determined by sequencing using specific primers and compared with the data base of the NCBI using the BLAST program. pGBT9-p30, pGBT9-p54 and pACT2-K were individually transformed in yeast and tested for β-galactosidase activity to exclude activation of gene reporter by itself. The different mutant truncations of p30 were individually transformed with pACT2-K in Y190 and resulting clones tested for expression of GAL4-dependent β-galactosidase. Similarly, different mutant truncations of hnRNPK were transformed with pGBT9-p30 and tested.

2.3. GST pull-down experiments
GST-hnRNP-K and GST proteins were produced in E. coli BL21 cells, previously transformed with vector pGEX-RNP-K or pGEX-4T. Cells were induced with 0.1 mM IPTG for 2 h at 37 °C. Bacteria were harvested and suspended in lysis buffer (PBS, 1% Triton X-100, 1 mM PMSF, 5 mM DTT, and anti-proteases), and sonicated on ice. GST-hnRNP-K and GST alone were purified from cleared lysates by mixing with glutathione-sepharose 4B beads (GE HealthCare), 5 ml of cleared lysate/400 μl of beads, for 1 h at 4 °C. After extensive washing, GST-hnRNP-K or GST beads were incubated in binding buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 0.1% Nonidet P-40 with protease inhibitor mixture (Roche Molecular Biochemical)) at 4 °C for 1 h with insect cell extracts containing either p30 or p54 ASFV proteins overexpressed in a baculovirus system [8]. Equal amounts of GST, GST-hnRNP-K and ASFV proteins p30 and p54 were used as judged by Coomassie Blue staining.

Alternatively, monolayers of Vero cells infected with BA71V and lysed in 50 mM HEPES, pH 7.5, 50 mM NaCl, 0.1% Nonidet P-40 containing protease inhibitor, were similarly mixed with GST-hnRNP-K or GST beads. In all cases, after extensive washing, bound proteins were eluted and analyzed by Western blotting with anti-p30 monoclonal antibody (diluted 1:500) or anti-p54 serum (diluted 1:60).

2.4. Virus infections and immunofluorescence analyses
Vero cells were grown in DMEM 5% onto cover slips, at 50–60% confluence, allowed to attach and then infected or mock infected with 1 pfu/cell of ASFV strain BA71V. Cells were fixed and used from 0 to 24 hpi in cold PBS. Isolation of nuclear and cytoplasmic fractions was performed as described previously [23]. Briefly, cells pellets were suspended in three volumes of hypotonic buffer and homogenization was carried out with a glass Dounce homogenizer. After centrifugation at 3300 × g for 15 min, supernatant was considered as the cytoplasmic fraction while pellets containing nuclei were suspended in half volume of low salt buffer. Extraction of nuclei was performed by adding 1 volume of high salt buffer for 30 min followed by centrifugation at 25000 × g. Resulting supernatant was considered as the nuclear fraction. After dialysis, 20 μg from each cytosolic sample and 13 μg from each nuclear sample were analyzed by Western blot with specific antibodies (anti-p30, anti-hnRNP-K and anti-β-tubulin diluted 1:500, 1:100,000 and 1:500, respectively). Quality of fractionation process was assessed by detection of β-tubulin on both nuclear and cytosolic samples. Bands obtained corresponding to hnRNP-K were densitometrically quantified using an image analyzer with TINA software package (Raytest).

3. Results

3.1. Identification of hnRNP-K as cellular interacting protein with ASFV p30
To identify cellular proteins interacting with ASFV early protein p30, yeast two-hybrid system was used to screen a porcine macrophage cDNA library. After selection from a total of 5 × 10^6 transformants screened, two potential positive clones were obtained in the reporter gene assay. DNA sequence analysis showed that cDNA contained in these clones, identical in size and composition, matched the cDNA sequence encoding hnRNP-K. cDNA sequences from positive clones represent nucleotides from 169 to 870 of the hnRNP-K cDNA sequence (GeneBank™ accession number 241477), with 98% nucleotide identity, corresponding to amino acid residues 13–246 of hnRNP-K protein. To confirm this result, the plasmid contained in these two clones was isolated and retransformed together with pGBT9-p30 detecting β-galactosidase activity. Conversely, clones obtained screening the library with pGBT9-p54 never encoded a similar sequence. Moreover, no reporter gene activity was detected in clones transformed with pACT2-K alone, excluding activation of gene reporter by itself.

3.2. p30 binds directly to hnRNP-K in vitro
The interaction was further confirmed by in vitro binding assays using a GST-hnRNP-K fusion protein bound to glutathione-sepharose 4B beads. GST pull-down experiments were carried out followed by Western blot with specific antibodies.
First, p30 and another unrelated ASFV protein (in this case, p54 as negative control) were used to bind GST fusion protein or GST alone. Protein p30, and not p54, was retained in the presence of GST-hnRNP K, showing a band of appropriate size (30 kDa) for p30 in Western blotting. This band did not appear in the presence of GST alone, indicating specific interaction of p30 with hnRNP K and not with GST (Fig. 1A). Identical results were obtained in subsequent experiments using BA71V infected or mock-infected cells extracts instead of baculovirus infected cell extracts in the pull-down assay (Fig. 1B).

3.3. Mapping the interacting domains of p30 and hnRNP-K

To examine the regions of both proteins involved in this interaction, different truncations of p30 fused to GAL4-BD domain and different truncations of hnRNP-K fused to GAL4-AD domain were tested for interaction (Fig. 2). As p30 sequence does not contain any previously characterized functional domain, we analyzed independently p30 amino terminus, central region and carboxy terminus fragments (1–70, 1–140 and 140–204 aa residues) for hnRNP-K interaction. In addition, diverse truncations of hnRNP-K were performed attending to previously well characterized functional domains [10] and tested for interaction using the yeast two-hybrid system. The results showed that none of the three different p30 truncations interacted with hnRNP-K (Fig. 2A). On the other hand, we could determine that the hnRNP-K fragment from amino acid residue 35–197 contained the interacting region with p30 (Fig. 2B). This region harbours KH1 and KH2 domains, identical each other, which have been previously identified as functional elements responsible for RNA binding.

3.4. Partial colocalization of protein p30 and hnRNP-K in ASFV infected cells

To ascertain the time in infection and the subcellular compartment at which the interaction occurs, ASFV infected cells were examined by confocal laser scanning microscopy at different times post infection (at least 30 infected cells were analyzed for each time point at 0, 6, 8 and 12 hpi). Newly synthesized viral protein p30 could not be detected in the nucleus or cytoplasm of examined cells before 8 hpi. Since that time point, p30 and hnRNP-K were found to colocalize in discrete areas within the nucleus of infected cells. Statistical analysis of colocalization showed that the totality of protein p30 present in the nucleus was detected colocalizing with hnRNP-K since 12 hpi (Fig. 3B). In contrast, most hnRNP-K was detected also in other parts of the nucleus exhibiting no colocalization with p30, indicating a partial colocalization of hnRNP-K with p30. Interestingly, in the cytoplasmic region of those infected cells hnRNP-K was barely detected and colocalization percentages were not significant (data not shown).

3.5. ASFV infection modifies hnRNP-K distribution

Subcellular distribution of p30 and hnRNP-K were analyzed by immunofluorescence microscopy in ASFV or mock infected cells. ASFV infection resulted in an intensification of nuclear, but not cytoplasmic, staining of hnRNP-K when compared to uninfected cells. When nuclei of infected cells were examined in detail, nuclear hnRNP-K was detected in characteristic granular structures coincident with areas completely devoid of nucleic acid staining. Interestingly, these kind of hnRNP-K accumulation was also observed in cells transiently expressing p30 (pCMV-p30), but not in mock-infected cells, suggesting...
the involvement of p30 on these changes (Fig. 4) during ASFV infection.

To confirm the retention of hnRNP-K in the nucleus of infected cells observed by immunofluorescence, the levels of hnRNP-K were assessed by Western blot at different times post infection using total, cytoplasmic and nuclear protein cell extracts. Total levels of hnRNP-K (Fig. 5A) were determined as infection proceeded (monitored by detection of p30) in order to discard that these changes could be due to changes in newly synthesized hnRNP-K or protein degradation. While no changes in total hnRNP-K levels were observed, quantitative analysis revealed a reduction of hnRNP-K levels in cytoplasm protein extracts from 12 hpi until the end of the infectious cycle (Fig. 5B). This reduction was in agreement with the observed increase of nuclear hnRNP-K levels, as judged by Western blot analysis. These results are consistent with the immunofluorescence analysis and indicate nuclear accumulation of hnRNP-K during ASFV infection.

These changes in hnRNP-K distribution might disrupt the normal behaviour of this ribonucleoprotein which is involved in diverse biological processes including regulation of transcription and translation. To examine the transcriptional activity in ASFV infected cells we performed immunodetection of nascent RNA after FU pulse labelling (Fig. 5C). When compared with mock-infected cells, the incorporation of FU into nascent RNA was almost completely abolished in infected cells from 12 hpi, coincident with the time point at which hnRNP-K redistribution was observed.

4. Discussion

Infection of eukaryotic cells with large DNA viruses often results in extensive interactions of viral gene products with macromolecular pathways of the host cell. By using the yeast two-hybrid system, we identified cellular hnRNP-K as an interacting protein with ASFV early protein p30. This interaction was further confirmed by an in vitro GST-fusion pull-down assay, using either p30 obtained from baculovirus system or ASFV infected cell extracts.

hnRNP-K is closely coupled to gene expression [15,24] and signal transduction pathways [16,25]. One feature which distinguishes hnRNP-K from other hnRNPs is the presence of three repeated K homology motives (KH) which are responsible for RNA-binding [26–28]. Interestingly, the p30 interacting region within hnRNP-K contains KH1 and KH2 motives, suggesting that the interaction described might modify hnRNK function such as processing and export of the cellular mRNAs than on signal transduction pathways.

hnRNP-K has been previously described to interact with several proteins from diverse viruses. Core protein from Hepatitis C virus (HCV) and Dengue virus (DEN), are proposed to relieve the repressive effect of hnRNP-K on transcription regulation of different human genes in vivo, disrupting the multiple functions of hnRNK and contributing to virus infection.
pathogenesis [11,12]. Studies with phosphoprotein IE63 (also named ICP27) from Herpes simplex virus (HSV) suggest that an IE63-mediated phosphorylation of hnRNP-K by casein kinase 2, results in inhibition of binding to RNA, affecting transport of cellular mRNAs and altering the subcellular location of hnRNP-K [13]. Nevertheless, no homology sequence in nucleotides or amino acids has been found when comparing these virus proteins interacting with hnRNP-K, including p30, indicating that this common interaction could occur in multiple and diverse ways.

During ASFV infection, hnRNP-K normal function could be affected since changes related to hnRNP-K subcellular distribution are observed. First, accumulation of hnRNP-K within the nucleus of ASFV infected cells was evident and subsequently the levels of cytoplasmic hnRNP-K decreased while nuclear levels were increased, as infection proceeded, supporting the idea that accumulation of hnRNP-K within nucleus is most likely due to relocation from cytoplasm. Changes in hnRNP-K subcellular distribution have been reported previously in poliovirus infection where virus infection affects the normal nucleo-cytoplasmic trafficking of the host cell [29]. Second, this hnRNP-K accumulation is coincident with the appearance of granular structures in the nucleus where the ribonucleoprotein is detected. Since no nucleic acids can be detected in these areas, these structures could represent inactive sites within the nucleus where hnRNP-K remains sequestered as consequence of infection, as postulated for HSV1 [13]. Similar structures have been previously reported during HCV infection. In this case, HCV core protein and hnRNP-K also colocalized in granules in the nucleus [12]. The presence of p30 in nucleus colocalizing with hnRNP-K suggests an involvement of this interaction in the appearance of these characteristic structures. Moreover, the identification of these structures also in cells transiently expressing p30 supports this idea.

Interestingly, neither conventional nuclear localization nor classical nuclear import signal have been previously found within ASFV protein p30 sequence, since it exhibits a predominantly cytoplasmic distribution. In this report we have demonstrated that small amounts of p30 are also present in the nucleus of the infected cell, so it can not be excluded that interaction with shuttling protein in cytoplasm could facilitate p30 traffic to the cell nucleus. Nevertheless, colocalization of both proteins in the cytoplasm during infection was not detectable by immunofluorescence, probably because of the low relative abundance of hnRNP-K in cytoplasm and its shuttling properties which difficult its visualization by immunofluorescence microscopy.

In conclusion, protein p30 could have further regulatory actions associated to its specific binding to hnRNP-K protein in the nucleus of infected cells. It is well known that viruses have evolved different mechanisms to alter host cell transcription and translation to promote transcription of its own DNA [30]. Here, we confirmed that all changes observed in hnRNP-K distribution during ASFV infection take place simultaneously with an extraordinary reduction in 5-fluouridine (FU) incorporation into nascent RNA, which represents an alteration in the cellular transcriptional activity. One way to inhibit cellular transcription is the recruitment of host cell RNA polymerase II. Nevertheless this is not a requirement for ASFV, since ρ-amanitin does not inhibit the production of infectious virus [31]. However, in cell systems previously studied (macrophages and Vero cells), ASFV infection produces a general shut-off of protein synthesis that affects up to 65% of the cellular proteins [2,32,33]. This inhibition of protein synthesis is detectable very early after ASFV infection, so it

Fig. 5. Analysis of hnRNP-K levels during ASFV infection by Western blot. Vero cells were infected with 5 pfu/cell of BA71V strain and analyzed by WB with specific antibodies at different times after infection. (A) p30 and hnRNPK were detected in total protein cell extracts with specific antibodies (see Section 2). β-Tubulin was detected to ensure that same amount of total protein was loaded. (B) Levels of hnRNP-K were analyzed independently from nuclear and cytoplasmic protein infected cell extracts. One of three representative experiments is shown including the relative intensities of quantified hnRNP-K (rel. int.). β-Tubulin detection was included as quality control of the cellular fractionation. (C) Vero cells were infected with BA71V (0.5 pfu/cell) and analyzed at 12 hpi for incorporation of FU into nascent RNA after FU pulse (see Section 2) to evaluate transcriptional activity. Arrows indicate the position of infected cells identified by p30 detection with specific antibody. Note the increase of nuclear hnRNP-K staining of infected cells when compared to the non infected cells. Bar, 30 µm.
will be interesting to determine whether the interaction here described is contributing to the general host cell shut-off or to a concrete cellular pathway.

Acknowledgements: We thank Dr. Linda Dixon for providing the pACT2 cDNA library from pig macrophages. We thank Dr. Levens for vector pGST-RNP-K and Dr. Bomsztyk for antibody anti-hnRNP-K serum. This work was supported by Grants Wellcome Trust 075813, Consolider-Ingenio 2010 CSD2006-00007-2, AGL2007-66441-C03-03, BIO2005-0651 and PET2006_0785.

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