Small peptide inhibitors disrupt a high affinity interaction between cytoplasmic dynein and a viral cargo protein

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Abbreviations: ASFV, African swine fever virus; hpi, hours post infection; pi, isoelectric point; MW, molecular weight.

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Abstract

Several viruses target the microtubular motor system in early stages of the viral life cycle. African swine fever (ASFV) protein p54 hijacks the microtubular-dependent transport by interaction with a dynein light chain (DYNLL1/DLC8). This was shown to be a high affinity interaction and the residues gradually disappearing were mapped on DLC8 to define a putative p54 binding surface by Nuclear Magnetic Resonance (NMR) spectroscopy. The potential of short peptides targeting the binding domain to disrupt this high affinity protein-protein interaction was assayed and a short peptide sequence was shown to bind and compete with viral protein binding to dynein. Given the complexity and number of proteins involved in cellular transport, prevention of this viral-DLC8 interaction might not be relevant for successful viral infection. Thus, we tested the capacity of these peptides to interfere with viral infection by disrupting dynein interaction with viral p54. Using this approach, we report on short peptides that inhibit viral growth.
Introduction

In order to enter the host cell, a virus must cross several barriers to reach the nucleus. Many viruses hijack the microtubular network in order to be transported along the cytoplasm (7, 18). Dynein is a microtubular motor protein, part of a large macromolecular complex called the microtubular motor complex. Dynein is involved in early stages of the viral life cycle of diverse infections, the first stage being the intracellular transport of the incoming virus along microtubules. Once transported throughout the cytosol, the virus rapidly gains the perinuclear area or the nucleus, where virus replication takes place.

Disruption of microtubules or microtubular motor dynein function impairs the transport of a number of viruses; however, the intrinsic mechanism of this transport is unclear. Neither has it been firmly established whether there is a common mechanism by which these viruses hijack a component of the microtubular motor complex for this purpose (7). A direct interaction between a given viral protein and cytoplasmic dynein for transport has been reported for HIV, herpes simplex, African swine fever virus (ASFV) and rabies virus (4, 14, 22, 25). In adenoviruses, a direct interaction of the viral capsid hexon subunit with cytoplasmic dynein has recently been described (5).

One of these viruses, ASFV, which is a large DNA virus, enters the cell by dynamin- and clathrin-dependent endocytosis (12) and its infectivity is dependent on acidification of the endosome. ASFV protein p54, a major protein of virion membranes, interacts with the light chain dynein of 8 kDa (DLC8), which allows transport of the virus to the perinuclear area (4), in a region called microtubular organizing center (MTOC). In this zone, the virus starts replication in the “viral factory”, a secluded compartment where newly formed virions
assemble (11, 13). By binding DLC8, the virus masters intracellular transport in order to ensure successful infection. However, due to the complexity of the system, the mechanism of this interaction is still elusive.

A variety of names have been used for the subunits of the cytoplasmic dynein complex. A new classification for mammalian cytoplasmic dynein subunit genes based on their phylogenetic relationships has been reported in which DLC8 gene was named DYNLL1 (26).

Light dynein chains are responsible for direct cargo binding in the cell, but how do they select so many different cargos? It is not known whether the mode and site of binding is the same for viral proteins as for physiological cargos. Within these multimeric complexes, there are a number of molecules that could theoretically interact with a given viral protein. However, to date, viral proteins have been described to bind only light or intermediate dynein chains, such as DLC8 and TcTex1 (4, 5, 8). A candidate viral protein would bind one of the DLC binding domains, which in DLC8 are located between the two dimers of the DLC8 molecule (LysXThrThr). Here we analyzed this interaction between a viral protein and DLC8 in an attempt to elucidate its requirements and relevance for viral infection.

In order to determine whether this interaction is crucial for viral replication or whether it is just one of a number of alternatives for the virus-host interplay, we analyzed the capacity of a set of inhibitor peptides targeting a determined binding domain of the DLC8 molecule to interfere with viral infection by disrupting dynein interaction with viral p54.
Materials and methods

Cells and viruses: Vero cells were maintained in Dulbecco’s minimum essential medium (DMEM SC, Lonza). In some cases, DMEM SC was supplemented with 5% inactivated fetal calf serum (Lonza), 4mM glutamine, 200 IU/ml penicillin and 100 IU/ml streptomycin (Invitrogen) (DMEM). The BA71V isolate of the African swine fever virus (ASFV) adapted to grow in the Vero cell line (9) was used in the inhibition tests. When indicated BA71V was purified by ultracentrifugation through a sucrose cushion as previously described (12).

Expression and purification of the recombinant proteins: To obtain the DLC8 spectra by NMR, we used $^{15}$N-labeled DLC8 produced in E. coli BL21 DE3 E. coli strain, previously transformed with vector pET23a-DLC8 (20) was grown in minimal medium including 1% $^{15}$NH$_4$Cl (Cambridge Isotope Laboratories) and induced with 1 mM IPTG at 30°C for 16 h. The cell pellet was then resuspended in lysis buffer (50 mM Na$_2$HPO$_4$, pH 7.0, 300 mM NaCl, 10% glycerol and anti proteases (Roche)). After disruption by pulse sonication and incubation with lysozyme 1 mg/ml, histidine-tagged DLC8 was purified by binding to the TALON metal affinity agarose (Clontech) after clearance of cell debris by ultracentrifugation. To prevent precipitation, labeled DLC8 was finally eluted from the column with 200 mM imidazole and dialyzed three times against 1.5 litres of 20 mM ammonium bicarbonate, pH 7.8, and 100 mM NaCl to prevent precipitation. In the case of p54, the corresponding coding sequence from BA71V isolate lacking the transmembrane domain was cloned into XhoI site in pET19b (Novagen) to generate pET-p54ΔTM. BL21 DE3 cells transformed with pET-p54ΔTM were grown in LB medium and protein
expression was induced with 1 mM IPTG for 3 h at 37ºC. The pellet was
resuspended in lysis buffer and disrupted by sonication and lysozyme
treatment. His-tagged p54 was purified by binding to TALON, as indicated, and
finally eluted in 200 mM imidazole. Protein was dialyzed three times against
PBS overnight at 4ºC.

**NMR spectroscopy:** All NMR spectra were recorded on a Bruker spectrometer
operating at 600 MHz, equipped with a 5-mm inverse triple-resonance
cryogenic probe. Sweep widths of 12 ppm (\(^1\)H) and 30 ppm (\(^{15}\)N), 48 scans, 128
indirect and 2048 direct data points and a recycle delay of 1 s were used. A
total volume of 160 µl of 0.2 mM DLC8 in 3-mm NMR tubes was used. All DLC8
samples were prepared in 20 mM ammonium bicarbonate and 100 mM NaCl
pH 7.8, in (H\(_2\)O/D\(_2\)O 9:1). To prevent precipitation, p54 was concentrated to 1.5
mM in the same buffer used for its purification and was then added to DLC8
sample. Peptides were dissolved to 25 mM with acetonitrile:H\(_2\)O (1:1) and
added to DLC8 sample. All spectra were recorded at 298 K. Spectra processing
was performed using TOPSPIN 2.0 software (Bruker).

**Peptide compounds:** The sequence of p54 in field and laboratory isolates of
ASFV deposited in Genebank (NCBI) was plotted into the BLAST database and
compared with the Local Alignment Search Tool. The sequence coding for p54
(E183L gene) is included in the complete sequence of the BA71V isolate, which
is deposited in the NCBI database with accession number U18466 (Table 1).

Peptide compound INTSTP1 (interactionstop1) was designed to contain the
DLC8 binding domain found in the ASFV p54 protein. From the sequences
flanking the dynein binding domain in different virus isolates we selected those
that were more favourable for chemical synthesis. Peptide characteristics are described in Table 2. For this peptide to be used in vivo testing, an arginine tail (8R) was added at the N-terminal end for internalization into cells (peptide DNBLK1, dynblock1) (21). Another series of peptides containing an irrelevant aa sequence were synthesized to be used as negative controls (INTCT1 and INTCT2 peptides). INTCT2 was the control sequence, INTCT1 plus an octa-arginine tail to be useful as control for in vivo testing. DNBLK1 was the original sequence of the dynein binding domain plus “HPAEP” flanking sequence similar to different virus isolates (Pretoriuskop, Namibia, Lillie-148, Warmbaths WB87, Tengani isolates; Tables 1, 2). The following modifications were included: DNBLK2, conjugation to fluorescein at the N-terminal end of DNBLK1 for its direct visualization with fluorescence microscopy. DNBLK3, DNBLK4 were modified to include conservative artificial point mutations in the binding domain that, not being the original sequence, still retained ability to bind dynein in the 2-hybrid system as described before (4). DNBLK3 included two point mutations and DNBLK4 just one point mutation (Table 2). DNBLK3 and 4 included another flanking sequence “HPTES”, similar to another set of virus isolates and conjugation to fluorescein at the N-terminal end for direct visualization with fluorescence microscopy. All the peptides designed were synthesized by Sigma-Genosys. Purification was carried out by HPLC to a degree of purity over 90%. Once synthesized and purified, lyophilized peptides were received in the laboratory. Depending on their molecular weight (see Table 2), the peptides were resuspended in a volume of sterile H$_2$O with a corresponding degree of purity mQ to obtain a stock solution at a concentration of 5 mM. Special efforts were made to prevent turbidity in the solution and filter tips were used to
prevent cross contaminations. Aliquots of 20 µl were made and then conserved at -80º C until use. The working solutions with the peptides were made from the stock solutions in DMEM SC medium in the 0-100 µM range of concentrations prior to addition to the cell culture.

**Infectivity assays:** 9 x 10^4 Vero cells were cultured in 24-well plates overnight. The next morning cells were washed in DMEM and medium was replaced with 300 µl of the solutions containing the peptides at a range of concentrations. Cells were incubated with the peptides for 1 or 3 h at 37 ºC and 5 % CO2. The medium was then removed and cells were infected with 1 pfu/cell of ASFV strain BA71V. After 2 h at 37ºC, residual virus was removed by washing twice with DMEM and finally cells were left in 300 µl of fresh DMEM containing the corresponding peptide concentration. Infection was allowed to proceed at 37º C for the desired time in each experiment, depending on the parameter of the infection to be analyzed.

**Indirect immunofluorescence:** Detection of cells infected by ASFV in cells previously exposed to the peptides was performed at 6 hpi. Cells were washed with PBS before being fixed with a 3.8% PBS-paraformaldehyde solution at RT for 10 min. After 3 washes with PBS, cells were permeabilized using 0.2% PBS-Triton X-100 15 min at RT. After another 3 washes with PBS, cells were incubated in blocking solution 3% PBS-BSA at 37º C for 45 min. Detection of the early protein of ASFV p30 (1), the viral antigen chosen for detection, was performed with the anti-p30 antibody 1:200 incubated for 1 h at 37ºC. After 3 washes in PBS, cells were incubated for 30 min at RT with 1:300 mouse anti-IgG antibody, and nuclei were detected after staining with TO-PRO 3.
Detection of ASF virions was performed by incubation with mouse monoclonal antibody anti-p72 clone 1BC11 (Ingenasa) diluted 1:1000. Coverslips were mounted with Prolong and observed in a conventional fluorescence microscope (Leica) to count the number of positive cells for the viral antigen p30. Microtubules were detected by staining with anti α-tubulin monoclonal antibody (Sigma) diluted 1:1000. A specific rabbit serum against DLC8 was raised after immunization with non-labeled DLC8 produced in E. coli, as described above. This serum was diluted 1:100 in PBS to detect DLC8 cellular distribution. Anti-mouse IgG or anti-rabbit IgG Alexa 594 conjugated antibodies (Molecular Probes), diluted 1:200 in PBS were used as secondary antibodies.

**Analysis of ASFV proteins by Western blot:** 20 µg of total soluble protein extracts from Vero cells exposed or not to the peptides and further infected or not with ASFV during 16 h, were separated by electrophoresis in 15% acrylamide:bis-acrylamide gels and separated proteins were transferred to a nitrocellulose membrane. As primary antibodies the anti-p30 monoclonal antibody diluted 1:100 in PBS and the anti-p72 monoclonal antibody clone 18BG3 (Ingenasa) diluted 1:2000 in PBS and rabbit anti-actin (Sigma) diluted 1:500 were used in independent membranes. ASFV p30 protein is expressed during the initial phases of the infection (1) and p72 protein (also called p73) during the late phase of this process (6, 34). As secondary antibody, anti mouse IgG (GE Healthcare) or anti-rabbit IgG (Biorad) conjugated to horseradish peroxidase were used diluted 1:5000. Finally, bands obtained after development with ECL reagent (GE Healthcare) and corresponding to p30, p72 and β-actin were densitometrically quantified and data were normalized to
control values using a Chemidoc XRS imaging system (Biorad) with Image Lab 2.01 software.

**Detection and quantitation of the ASFV genome:** Detection and quantitation of the ASFV genome was achieved by *quantitative real time PCR* using specific oligonucleotides and a TaqMan probe (16). DNA from infected or mock-infected cells with BA71V 0.5 pfu/cel was extracted and purified with DNeasy blood and tissue kit (Qiagen) at 16 hpi. DNA concentration and purity was estimated by measuring absorbance at 260nm ($A_{260}$). Amplification mixture was prepared on ice as follows: 3µl template DNA (1 µg), 1 µl oligonucleotide OE3F 50 pmol, 1 µl oligonucleotide OE3R 50 pmol, 10 µl Quantimix Easy Probes (Biotools) 2X, 1 µl TaqMan™ probe SE2 5 pmol, and 4 µl H$_2$O.

The amplification reaction was performed in a Rotor-Gene RG3000™ (Corbett Research), as indicated: 1 cycle at 94°C for 10 min, 45 cycles at 94°C for 15 sec and 45 cycles at 58°C for 1 min. Positive amplification controls (DNA purified from ASFV virions) and negative amplification controls (DNA from mock-infected cells) were included in the assay and duplicates from each sample were analyzed.

**Effect of peptide treatment on ASFV progeny:** 9 x 10^4 Vero cells were seeded in 24-well plates the night before the experiment. One hour before infection, cells were incubated in 300 µl DMEM containing distinct concentrations of DNBLK1 and control INTCT2 peptides. Cells were then infected or mock-infected with 0.5 pfu/cell of ASFV strain BA71V. At 36 hpi, 100 µl of media was collected from wells and stored at -80°C until analysis of extracellular virus progeny. Infected cells were also collected in 100 µl of fresh
DMEM. Cells were frozen and thawed three times to allow solubilization of intracellular virus progeny and then stored at -80°C until use. Virus titers from intracellular or extracellular samples were analyzed by plaque assay as previously described (13).

**Cytotoxicity analysis:** Cell viability and proliferation assays. To evaluate cell viability, Vero cells seeded in 24-well plates were incubated in DMEM containing inhibitor DNBLK1 or negative control INTCT2 at concentrations ranging from 0 to 100 µM. After incubation with peptides for 24 h, cells were harvested and the number of viable cells in the suspensions was determined by Tripblue (Sigma) dye exclusion assay. Briefly, 20 µl of PBS with Tripblue 0.08 % was added to an equal volume of cell suspension and mixed. After 2 min blue cells (dead cells) were counted using a hematocytometer and a conventional light microscope.

To evaluate cell proliferation, 3x10⁴ Vero cells seeded in 96-well plates were incubated in 50 µl DMEM containing inhibitor peptide DNBLK1 or negative control INTCT2 at concentrations ranging from 0 to 100 µM. After 36 h of incubation, cell proliferation was determined using CellTiter 96 Aqueous™ (Promega) assay, following the manufacturer’s instructions.

**Results**

**Sequence analysis comparison of p54 from several viral isolates and peptide design**

The minimal region in p54 protein from ASFV isolate BA71V, which maintained DLC8 binding properties, was within a 13-amino acid fragment between residues 149-161 (4). In order to study the two players in this viral -cellular
protein interaction, we started with a comparison analysis of this interacting region in the sequences from several ASFV isolates. This analysis revealed that the DLC8 binding domain in p54 (TVTTQNTASQT) is highly conserved independently of geographic distribution (East and West Africa, Europe or America), year of isolation, host or virulence (Table 1). This protein sequence has been extensively studied because its relevance as universal diagnostic reagent (3). It was described the coexistence of heterogeneous virus populations in culture adapted isolates with short aminoacid repetitions in p54 without any impact in virus growth, while no modifications were found in the dynein binding domain (2, 29).

On the basis of this analysis, we designed a set of peptides representing the dynein binding domain and selecting the most favorable flanking sequences with some variations among isolates. Peptide compound INTSTP1 (interactionstop1) was designed to contain the DLC8 binding domain found in the ASFV p54 protein. From the sequences flanking the dynein binding domain in different virus isolates we selected those that were more favourable for chemical synthesis. Peptide characteristics are described in Table 2. For this peptide to be used in vivo testing, an arginine tail (8R) was added at the N-terminal end for internalization into cells (DNBLK1, dynblock1) (21). Another series of peptides containing an irrelevant aminoacid sequence were synthesized to be used as negative controls (INTCT1 and INTCT2 peptides). INTCT2 was the control sequence, INTCT1 plus an octa-arginine tail to be useful as control for testing in cells. DNBLK1 was the original sequence of the dynein binding domain plus “HPAEP” flanking sequence similar to different virus isolates (Pretorisuskop, Namibia, Lillie-148, Warmbaths WB87, Tengani
isolates; Tables 1, 2). The following modifications were included: DNBLK2 was conjugated to fluorescein at the N-terminal end of DNBLK1 for its direct visualization with fluorescence microscopy. DNBLK3, DNBLK4 were modified to include conservative artificial point mutations in the binding domain that, not being the original sequence, retained ability to bind dynein in the 2-hybrid system as described before (4). DNBLK3 included two point mutations and DNBLK4 just one point mutation in the critical aminoacid sequence (Table 2). DNBLK3 and 4 also included another flanking sequence “HPTES”, similar to another set of virus isolates and conjugation to fluorescein at the N-terminal end for direct visualization with fluorescence microscopy.

**Analysis of the interaction between ASFV protein p54 and dynein light chain by NMR**

Nuclear magnetic resonance (NMR) has evolved into a powerful tool for characterizing protein-ligand interactions in solution under near physiological conditions. Since the natural abundance of one of the most common NMR observable isotopes, $^{15}$N (0.37%), is too low for NMR experiments, the protein to be studied must be isotopically labeled with $^{15}$N through expression in *E. coli*. Therefore, all backbone amides as well as the nitrogen containing side chains are labeled with this magnetically active nucleus. Then, heteronuclear $^1$H-$^{15}$N correlation NMR experiments can be recorded which generate spectra containing at least one signal for each amino acid, except proline. Additional signals arise from amides in the side chains. When signal assignment is available, a chemical shift perturbation (CSP) experiment enables mapping of changes in the protein’s backbone amides that are induced by binding of a ligand. (31).
Chemical shift perturbation (CSP) was used to further characterize the p54-DLC8 interaction. First, we obtained a [$^1$H,$^{15}$N]-HSQC spectrum corresponding to free $^{15}$N-labeled DLC8. This spectrum was similar to that previously reported by (19) (Fig. 1A). This allowed us to assign most of the signals. We then titrated $^{15}$N-labeled DLC8 with a range of concentrations of unlabeled p54 (Fig. 1D). DLC8 resonances progressively disappeared as p54 concentration increased, thereby suggesting the formation of a higher molecular weight complex. Most of the DLC8 resonances were not observable when 2 eq. of p54 were added (Fig. 1B). This observation is indicative of a loss of sensitivity as a result of relaxation-dependent line broadening that occurs upon the formation of high molecular weight species. As it is well known, in large proteins the magnetization relaxes faster and as a result the peaks become broader and weaker, and eventually disappear (35). The first signals to disappear after addition of 0.1 eq. of p54 corresponded to residues W54, I57, K9, Y75, V58, N61, E15 and H68. These residues were mapped on the DLC8 structure (PDB accession number 1PWJ) in order to define a putative p54 binding surface (Fig. 2).

We then studied the interaction of DLC8 with the peptide INTSTP1, which contains the DLC8 binding domain, and an irrelevant aa sequence INTCT1, as a control (Table 2). Increasing amounts of both peptides were added to a $^{15}$N-labeled DLC8 and [$^1$H,$^{15}$N]-HSQC spectra were recorded. Substantial changes in DLC8 resonances were detected when 5 eq. of INTSTP1 were added (Fig. 1F) while no significant effects were observed with the same amount of INTCT1 (Fig. 1D). Thus this observation indicates that INTSTP1 bound to DLC8.
Finally, we performed a competition experiment where \(^{15}\text{N}\)-labeled DLC8 was incubated with \textit{INTSTP1} before the addition of p54 (Fig. 1C). The binding of p54 to DLC8 was prevented by 5 eq. of \textit{INTSTP1}, as indicated by the observation that the higher molecular weight complex did not form.

These results confirm the p54-DLC8 interaction and demonstrate that a short peptide containing the minimal DLC8 interaction domain of p54 binds DLC8 \textit{in vitro} and prevents its interaction with p54.

**\textit{DNBLK} peptide is efficiently internalized in Vero cells with no apparent effect on cell proliferation**

For this peptide to be used in cells, it must reach the intracellular environment with null or very low toxicity in living cells, then a guanidinium-rich tail was used to increase cellular uptake. Then, an arginine tail (8R) was added at the N-terminal end of \textit{INTSTP1} peptide for internalization (\textit{DNBLK1}, dynblock1; Table 2). In order to test the intracellular delivery of peptides, we used fluorescein-labeled peptides at the N-terminal end and fluorescence confocal microscopy analysis. \textit{DNBLK2} peptide incorporating the transporter tail was efficiently internalized after 1- and 3-h incubations when peptide concentration was higher than 25 \(\mu\text{M}\), almost all the cells present in the culture incorporated the fluorescent peptide (Fig. 3A) In addition to peptide internalization; we analyzed the integrity of cytoskeletal components. Microtubules were not modified in the presence of any peptide concentration. Also, the DLC8 distribution pattern on microtubules was not modified by the peptide treatment and DLC8 colocalized to the characteristic condensed perinuclear area corresponding to the MTOC (Fig. 3A).
Dynein function is crucial during mitosis, playing an essential role in the formation of the mitotic spindle and the migration of chromosomes. We then studied whether mitosis spindle formation was modified in response to peptide treatment. We found that cell division was not altered (Fig. 3C). Furthermore, the proliferative capacity of the cells was analyzed, measured as the rate of reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrasodium bromide (MTT) tetrazolium salt (Fig. 3B). This parameter was not impaired with increasing concentrations of DNBLK1 when compared to the same concentrations of the control peptide INTCT2 (INTCT1 plus the intracellular transporter tail, Table 2). In both cases, the proliferation rates were similar to those obtained for healthy control cells, which was usually 0.4 – 0.5 units. Moreover, no morphological changes indicating cytotoxicity, such as rounding or detaching, were observed in cells incubated with the peptide concentrations for the times assayed.

Effect of DNBLK peptides on ASFV infection

To confirm previous data indicating that microtubules and DLC8 are essential for productive ASFV infection (4), we first analyzed virion distribution by confocal microscopy during the first stages of infection. As expected, we found association of virions with DLC8 along microtubules (Fig. 4A) immediately after internalization. Figures 4B and C illustrate early transport of viral particles to the MTOC area from 45 min after infection. ASFV BA71V was used to infect untreated Vero cells or cells treated with DNBLK1 (Fig. 4C) or with control peptide INTCT2 (Fig. 4B). At early time points (20-45 min after infection) incoming virions were rapidly internalized to the perinuclear area both in untreated and control peptide treated cells. In presence of the peptide DNBLK1,
viral particles remained dispersed in the cytoplasm or in the periphery at the same times.

To evaluate the effect of our set of synthesized peptides on ASFV infection, Vero cells grown to 70 % confluence were incubated for 30 min in the absence of FBS with increasing concentrations (from 0.5 to 100 µM) of peptides and then infected with approximately 1 pfu/cell of ASFV strain BA71V. Cytopathic effect (cpe), consisting of progressive cell rounding and detachment from the plate surface, is common during ASFV infection. Cells were then examined for this effect at 24 hpi. The cpe was dramatically reduced in cells previously incubated with peptides containing the DLC8 binding domain of p54 and also the internalization sequence signal (8xR) (Fig. 4D). However, incubation with the same peptide lacking the internalization signal did not result in cpe abolition, and control peptides (INTCT2) yielded the same level of cpe as non-treated cells.

Then, we analyzed the infectivity of ASFV after incubation with the peptide containing the DLC8 binding domain and internalization signal (DNBLK1) by detecting the number of infected cells (Fig. 4E, F). Results showed that the percentage of infected cells detected by indirect immunofluorescence (IFI) decreased in a dose-dependent manner after incubation with DNBLK1 but not with control peptides (INTCT2). From these experiments we calculated EC50, the minimal peptide concentration at which infectivity was inhibited by 50 %, of approximately 20 µM.

Also, the synthesis of early (p30) and late (p72) ASFV proteins was analyzed by Western blot using specific antibodies. The synthesis of early and late viral
proteins also decreased in a dose-dependent manner when ASFV infection proceeded in the presence of DNBLK1 (Fig. 5A), thereby indicating that inhibition mediated by DNBLK1 occurs early during the ASFV infection cycle.

Similar experiments by qPCR and infectious virus progeny by plaque assay were performed to analyze ASFV replication, as previously described. Levels of ASFV DNA were reduced in a dose-dependent manner during infection after incubation with peptides containing the DLC8 binding domain and internalization signal (Fig. 5B). Using this quantitative assay, peptide DNBLK1 inhibitory effect was compared with two other peptides incorporating point mutations within the dynein binding domain. Those are previously described artificial conservative point mutations that retain ability to bind dynein assayed by 2-hybrid system (4). DNBLK3 included two point mutations and DNBLK4 just one point mutation (underlined in Table 2). Peptide DNBLK1 showed a greater inhibitory effect than DNBLK3 and DNBLK4, and was therefore selected as the most effective sequence. Nevertheless, control INTCT2 did not affect normal virus replication. Finally, and to evaluate a possible effect of inhibitory peptides on viral egress, we analyzed intracellular and extracellular virus titers obtained at 36 hpi in the presence of inhibitory and control peptides (Fig. 5C and D). Results showed that both intra- and extra-cellular virus titers decreased in a dose-dependent way after incubation with DNBLK1 but not with INTCT2.

Discussion

Dynein is a microtubular motor protein responsible for the intracellular transport linked to microtubules. This protein is crucial for the endosomal pathway and organelle intracellular trafficking. In coordination with a number of regulatory molecules, dynein drives motion to regular cargos in the cell (30).
Viruses use dynein for their internalization and intracellular transport, as demonstrated for a number of viral models, such as HIV, rabies virus, ASFV, poliovirus, herpes simplex virus and adenovirus (4, 5, 22, 25, 27). Also, many questions remain unanswered about the intrinsic mechanism of this mode of intracellular transport.

We have analyzed one of the first described direct interactions between a viral protein and molecular motor dynein for intracellular transport (4). The ASFV protein p54 interacts with DLC8 and this interaction is essential for the intracellular transport of the incoming virus prior to viral protein synthesis and replication in the viral factory, which is located in the perinuclear area in the MTOC, where the new virions are assembled. The p54-dynein interaction was found using the double hybrid system in yeast, searching for interacting proteins with the viral p54 protein in a swine macrophage cDNA library (4). The clones obtained and identified as positive were sequenced to discover that they contained the complete coding sequence of the light chain dynein of 8 kDa, named DLC8, LC8, DLC1 or DYNLL1 (26).

Cytoplasmic dynein is a large multimeric complex composed of two heavy chains, a stalk of intermediate chains, and several light chains. Light chains are responsible for a direct interaction with physiological cargos to be transported. Intermediate chains associate with a multi-subunit complex called dynactin, through its projecting arm, named p150-glued. The backbone of this complex is formed by the actin-related protein Arp1 (32). Dynactin is critical in most dynein functions and works as an adaptor, connecting to cargo (15). Dynein-driven motion is minus-end directed, but it has recently been shown to direct bidirectional motion to cargos towards both the plus- and minus-ends of
microtubules in diffusive and processive runs (28, 30). The ability of dynein to
switch directions increases the flexibility of the system and the capacity to
progress around obstacles in a crowded cellular environment.

Several families of dynein light chains have been described, among these
DLC8. There are up to six light chains belonging to three protein families
(DYNLL/DLC8, DYNLT/tctex, and DYNLRB/LC7/roadblock), two light
intermediate chains (DLIC), and two intermediate chains (DIC), all of which
have been implicated in cargo binding (33, 36). DLC8 has a highly conserved
nucleotide amino acid sequence between evolutionary distant species (10, 17).

Regarding the viral interacting protein, the minimal binding domain was
identified at the carboxy-terminal end of the protein by expressing several
truncated fragments of the p54 protein in the yeast system in 13 amino acids
comprised between Tyr149-Thr161

\[\text{TyrThrThrThrValThrThrGlnAsnThrAlaSerGlnThr} \] (4). Sequence analysis
comparison between distinct viral isolates demonstrated that this is a highly
conserved sequence among isolates, irrespective of their geographic origin,
host or virulence. This observation could be attributable to the critical function of
this sequence in early infection stages.

The identification of the amino acid sequences involved in the binding
between a given viral protein and DLC8 when these sequences are on primary
structure does not necessarily mean that these linear sequences have the
capacity to bind or even compete for binding of the viral protein to DLC8. NMR
spectroscopy is a powerful analytical tool to study protein-ligand and protein-
protein interaction systems (24, 31). We studied the DLC8- p54 interaction by
means of NMR and designed a peptide sequence suitable to target the main 
residues involved in this binding. CSP analysis showed that DLC8-p54 bound in 
vitro to form a stable molecular weight complex. It was found that it was a high 
affinity interaction and the residues gradually disappearing were mapped on 
DLC8 to define a putative p54 binding surface. A short peptide sequence, 
containing the DLC8 binding domain, also bound DLC8 and the competition 
experiments demonstrated that it prevented the binding of the viral protein to 
DLC8 in vitro. This short peptide was then used as a tool to compete with the 
viral protein binding to dynein in cells to provide an insight into the result of 
preventing this single interaction throughout the infection.

In the living cell, however, dynein adaptors and regulators, such as 
dynactin, help to link dynein to its cargo and it has been shown that Arp1 and 
βIII spectrin interaction is sufficient to produce a dynein cargo link (23). In fact, 
inhibition of the dynein-dynactin complex by overexpression of p50 dynamitin 
blocks ASFV transport in infected cells (4). The p150 subunit also links dynein 
to cargos, notably through interactions of p150 with regulatory GTPases. Given 
the complexity and number of proteins involved in cellular transport, blockage of 
a determined viral-DLC8 interaction might not suffice to prevent a virus from 
using the microtubular transport system. Thus we studied the relevance of this 
binding for viral infection and the potential of peptides targeting the binding site 
was assayed in cells in order to determine the impact on the viral infection 
outcome. At micromolar concentrations, a peptide sequence shown to displace 
and compete with the binding of the viral protein to DLC8 by NMR, was also 
efficient at inhibiting viral infection in susceptible cells. We observed reductions
in infectivity, virus replication and viral production yields. These results highlight
the significance of this single interaction and the protein domains involved.

The findings reported here contribute to our understanding of the
specificity and relevance of viral strategies evolved to take advantage of cellular
transport. In fact, several virus models use dynein for microtubular transport;
some of them binding the same DLC8 domain (27). These, and other viruses
reported to bind dynein for transport are potential candidates for infection
inhibition with peptides targeting a crucial step common to many viral infections.
Thus DNBLK peptides might be the first clue for the development of an effective
treatment against ASFV and other viruses that share the same transport
mechanism. Given that viruses are dependent on host cell functions for
replication, transient interference with selected interactions of the virus with the
cell in which it reproduces may be useful to retard viral replication or spread,
and in turn spare the host from morbidity or mortality.
Acknowledgments

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References


Table and Figure Legends

Table 1. Sequence analysis comparison of p54 dynein binding domain from distinct viral isolates.

Table 2. Short peptide sequences designed to compete for p54-cytoplasmic dynein interaction. Mutations in critical binding domain underlined.

Figure 1. NMR spectra of free dynein, dynein with the viral protein and with competitor peptides. NMR spectra of free cytoplasmic dynein compared with the protein incubated with increasing concentrations of the viral protein, with or without a competitor peptide. $^1$H-$^5$N HSQC spectra of $^{15}$N-labeled DLC8 at several titration points. In all the experiments the observable protein is the $^{15}$N-labeled DLC8 as $^{15}$N is the NMR observable isotope. Therefore, we are recording changes in the $^{15}$N-labeled DLC8 NMR spectrum after addition of p54 or the competitor peptides. The p54 or the peptides are not observable in these kind of experiments. The spectra in different colors represent the $^{15}$N-labeled DLC8 in different experimental conditions. A, Free DLC8; B, free DLC8 (black spectrum) and with 2 eq. of unlabeled p54 (red spectrum); C, free DLC8 (black spectrum) and with 5 eq. of peptide INTSTP1 and 2 eq. of unlabeled p54 (green spectrum); D, Black, free DLC8 (black spectrum), with 0.1 eq. of unlabeled p54 (red spectrum), with 0.3 eq. of unlabeled p54 (green spectrum) and with 0.6 eq. of unlabeled p54 (blue spectrum); E, free DLC8 (black spectrum) and with 5 eq. of peptide INTCT1 (red spectrum); F, free DLC8 (black spectrum) and with 5 eq. of peptide INTSTP1 (red spectrum).
Figure 2. Mapping of the residues interacting with p54 on the DLC8 surface. (DLC8, PDB accession number 1PWJ). Interacting residues are shown in green.

Figure 3. Cell internalization capacity and cytotoxicity evaluation of selected peptide sequences. (A) Vero cells treated with increasing concentrations of DNBLK2 peptide: competitor peptide sequence linked to an arginine-rich molecular transporter and labeled with fluorescein. Green fluorescence is observed in cells in which the peptide was successfully internalized. Left panel: Cells with conserved microtubular cytoskeleton architecture stained with anti-tubulin antibody (red). Note the perinuclear localization of the MTOC. Line (a) 5 µM DNBLK2 peptide, Line (b) 25 µM DNBLK2 peptide, (c) 50 µM DNBLK2 peptide. (B) Proliferative index of cells after a 36-h treatment with control INTCT2 and DNBLK1 peptides. In both cases, the proliferation rates were similar to those obtained for healthy control cells, 0.4 – 0.5 units. (C) Tubulin fibers forming the mitotic spindle during cell division at different stages in control Vero cells treated with INTCT2 (a) and (b) in cells treated with DNBLK1 peptide. Mitosis morphology and migration along microtubules were not modified by peptide treatment.

Figure 4. Decrease of infectivity in cells treated with inhibitor peptide DNBLK1. (A) ASF virions labeled in red are transported bound to microtubules by means of p54- microtubular motor dynein interaction. Bar 2 µm. (B, C) 0.1 µm optical sections of Vero cells, 45 min after infection in the presence of 50 µM INTCT2 or DNBLK1 peptides, show ASF virions distribution at early infection stages using a monoclonal antibody against p72 labeled in red. Nuclei labeled with TO-PRO 3 and cell contour are shown. Bar 12 µm. (D) Reduction of ASFV infectivity in cells treated with control peptide INTCT2 and peptide DNBLK1.
cytopathic effect in the presence of increasing concentrations of short peptide sequences designed to compete for p54-cytoplasmic dynein interaction. Those peptides without an internalization sequence (arginine octapeptide) lack an inhibitory effect. (E) Vero cells incubated with increasing concentrations of inhibitor and control peptides labeled with an antibody against ASFV p30 in red. (F) The figure shows percentages of infected cells at 6 hpi.

**Figure 5.** Effect of peptide pretreatment on viral proteins production, viral replication counts and total virus production. (A) Western Blot analysis of early and late protein synthesis with a range of concentrations of inhibitor and control peptides. Quantification of the intensity of each band is listed under each band. (B) ASFV DNA replication at 16 hpi after treatment with increasing concentrations of inhibitor peptides DNBLK 1, 3 and 4 in comparison with cells treated with control peptide INTCT2. (C) Effect on extracellular and (D) intracellular virus titers recovered after 36 hpi with increasing concentrations of inhibitor peptide DNBLK1 in comparison with control peptide INTCT2.
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A) Image showing a cell with green fluorescence and a white arrowhead.

B) Image showing a cell with blue and red fluorescence.

C) Image showing a cell with blue fluorescence.

D) Table with data.

E) Images showing different cell conditions.

F) Bar graph showing percentage of infected cells at different peptide concentrations.