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Nuclear Export of African Swine Fever Virus p37 Protein Occurs through Two Distinct Pathways and Is Mediated by Three Independent Signals

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Nucleocytoplasmic shuttling activity of the African swine fever virus p37 protein, a major structural protein of this highly complex virus, has been recently reported. The systematic characterization of the nuclear export ability of this protein constituted the major purpose of the present study. We report that both the N- and C-terminal regions of p37 protein are actively exported from the nucleus to the cytoplasm of yeast and mammalian cells. Moreover, experiments using leptomycin B and small interfering RNAs targeting the CRM1 receptor have demonstrated that the export of p37 protein is mediated by both the CRM1-dependent and CRM1-independent nuclear export pathways. Two signals responsible for the CRM1-mediated nuclear export of p37 protein were identified at the N terminus of the protein, and an additional signal was identified at the C-terminal region, which mediates the CRM1-independent nuclear export. Interestingly, site-directed mutagenesis revealed that hydrophobic amino acids are critical to the function of these three nuclear export signals. Overall, our results demonstrate that two distinct pathways contribute to the strong nuclear export of full-length p37 protein, which is mediated by three independent nuclear export signals. The existence of overlapping nuclear export mechanisms, together with our observation that p37 protein is localized in the nucleus at early stages of infection and exclusively in the cytoplasm at later stages, suggests that the nuclear transport ability of this protein may be critical to the African swine fever virus replication cycle.

The control of the subcellular localization of proteins by active nucleocytoplasmic transport mechanisms is essential for the regulation of protein function in various biological processes, such as the cell cycle or the replication cycle of several viruses (42, 47, 49). The translocation of macromolecules larger than 60 kDa into and out of the nucleus is an active, energy-dependent process that is mediated by specific sequence motifs, nuclear localization signals (NLSs), and nuclear export signals (NESs) (5, 18).

Classical examples of NESs include the motifs found in the human immunodeficiency virus type 1 (HIV-1) Rev protein (13) and in protein kinase inhibitor (PKI) (48), which are characterized by clusters of four closely spaced hydrophobic amino acids, such as leucine and isoleucine residues. However, NESs that do not belong to the family of leucine-rich NESs have also been described for various proteins, as exemplified by the atypical NES present in the hepatitis D antigen HDAg-L (27).

Direct interaction of proteins bearing leucine-rich NESs with the nuclear export factor CRM1, also known as exportin-1, is very well documented and essential for the active nuclear export of these proteins (14, 15, 33, 44). Leptomycin B (LMB), a metabolite that disrupts the interaction of NESs with the CRM1 receptor by binding to a cysteine residue localized in the central domain of the receptor, constitutes a useful reagent for studying the CRM1-mediated protein nuclear export (25, 26). Although the CRM1-dependent transport is the best-characterized nuclear export pathway, CRM1-independent pathways have been proposed for various proteins (7, 27, 28).

Recently, we have described the African swine fever virus (ASFV) p37 protein as the first nucleocytoplasmic shuttling protein to be identified in this virus (12). Additionally, we have demonstrated that the nuclear export of the ASFV p37 protein is a very strong process.

African swine fever virus, the only member of the new virus family *Asfarviridae* (9), is the causative agent of a highly lethal hemorrhagic disease that affects domestic pigs (46). The genome of ASFV strain BA71V, a large linear double-stranded DNA molecule, has been completely sequenced, and its analysis has identified 151 open reading frames, which encode approximately 50 structural proteins and several enzymes involved in viral DNA replication, viral gene transcription, and

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protein modification (45, 51). Autoradiography studies of ASFV-infected macrophages, as well as in situ hybridization experiments performed with ASFV-infected macrophages and Vero cells, have demonstrated that viral DNA synthesis is initiated inside the nucleus, followed by a longer replication stage that occurs in the cytoplasm (16, 38). These observations render the identification and characterization of the nucleocytoplasmic transport of ASFV proteins particularly interesting.

p37 protein, as well as p150, p34, and p14 ASFV proteins, is a product of the proteolytic processing of the polyprotein precursor pp220 (41) by a viral protease that shares sequence similarity with proteases of the SUMO-1 family (2). These viral proteins are localized in the viral core shell domain, a matrix-like domain which envelopes the DNA-containing nucleoid (1, 3). Interesting studies on the repression of the expression of pp220 have demonstrated that this polyprotein precursor is essential for viral core assembly and envelopment, as well as for the subsequent steps of core formation, including DNA encapsidation and nucleoid maturation (4).

In the present study, we provide evidence that the nuclear export of the ASFV p37 protein is mediated both by the CRM1-dependent and CRM1-independent nuclear export pathways. Furthermore, we demonstrate that when considered independently, these nuclear export pathways have comparable strengths. Additionally, we have identified and characterized two NESs at the N terminus of p37 protein, which are responsible for its nuclear export via the CRM1 pathway, as well as an additional NES at the C terminus, which mediates the CRM1-independent nuclear export. Finally, experiments on the localization of p37 protein in ASFV-infected cells demonstrate that at early stages of infection, the p37 protein is localized in distinct nuclear regions and that at later stages, the protein is localized exclusively in the cytoplasm. Overall, these findings reinforce the importance of p37 protein nuclear transport to the ASFV replication cycle.

MATERIALS AND METHODS

Plasmid constructs. GFP-expressing plasmids pNEAp37, pNEARev, GFPp37, pRev(1.4)-GFP, and pRev(1.4)-NES were described previously (12, 19, 35).

The various fragments used for mapping the NESs of p37 were amplified by PCR, using specific primers that introduced BamHI and NsiI recognition sites, respectively, at the 5′ and 3′ ends of the amplified DNA sequences. Each DNA fragment was cloned into the BamHI/PstI restriction sites of the plasmid for the yeast nuclear export assay pNEA (35) and/or into pEGFP-C1 (Clontech).

Full-length p37 and the p37 fragments (amino acids 1 to 160, 158 to 234, 231 to 372) were amplified by PCR using specific primers, which introduced a 5′ BamHI restriction site and a 3′ AgeI restriction site, to facilitate cloning into pRev(1.4)-GFP.

All the point and deletion mutants used throughout this study were generated by PCR-based mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions.

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

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

Purification of plasmid DNA for sequencing and transfection experiments was performed using a plasmid midi kit (QIAGEN) according to the manufacturer's instructions.

Yeast nuclear export assay. The yeast nuclear export assay has been previously described by Rhee et al. (35). Briefly, pNEA-derived constructs were transformed into *Saccharomyces cerevisiae* strain L40, which contains the two LexA-inducible genes *HIS3* and *lacZ* (21), by the lithium acetate method (22). The transformed yeast cells were then plated on selective medium without tryptophan. Since pNEA contains the *TRP1* gene, only transformed yeast cells can grow on this medium. After growth, a few colonies were spread on minimal medium without tryptophan and in parallel plated on minimal medium deficient in both tryptophan and histidine and supplemented with 10 mM 3-amino-1,2,4-triazole (Sigma), a repressor of yeast endogenous histidine production (35). For pNEARev, the minimal medium deficient in both tryptophan and histidine was supplemented with 100 mM 3-amino-1,2,4-triazole (35). 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 (6). Briefly, after disruption of yeast cell membrane by incubation at −70°C for 30 minutes, yeast lysates were incubated with the β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-β-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 (43). After growth in liquid medium without tryptophan, yeast cells were disrupted, and the β-galactosidase chromogenic substrate *o*-nitrophenyl-β-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. β-galactosidase activity was calculated as described previously (30).

Cell culture. Vero and COS-7 cells were grown and maintained in Dulbecco's modified Eagle's medium-high glucose (DMEM-HG; Sigma) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; Biochrom KG) and with 100 U of penicillin and 100 μg of streptomycin (Sigma) per ml in a 5% CO_2 humidified atmosphere at 37°C.

For the transfection and ASFV infection experiments, 7.5×10^4 Vero or COS-7 cells were seeded onto glass coverslips on a 12-well plate.

Transfection, energy depletion, and leptomycin B treatment. Transfection experiments were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, using 1 μg of plasmid DNA per well. The cells were incubated for 48 hours to allow gene expression.

For energy depletion studies, prior to fixation, the cells were incubated in energy depletion medium composed of glucose-free DMEM (Invitrogen), 6 mM 2-deoxy-D-glucose (Sigma), and 10 mM sodium azide (Sigma) and supplemented with 10% FBS (40) for 3 hours at 37°C.

Where indicated, the cells were incubated with 20 ng/ml leptomycin B (Sigma) in DMEM-HG supplemented with 10% FBS for 3 hours prior to fixation.

CRM1 silencing experiments. The short interfering RNA (siRNA) duplex used for depletion of the CRM1 receptor (CRM1 siRNA) [sense, UGUGGUG AAUUGCUUAUACd(TT); antisense, GUAUAAGCAAUUCACCACAd(TT)] (29), targeted to residues 90 to 108 of the human CRM1 receptor, and a nonsilencing control siRNA were obtained from QIAGEN. Vero cells were transfected at a confluence of 40 to 50% either on 12-well plates with 16-mm coverslips (fluorescence microscopy) or on 35-mm dishes (Western blotting). Cell transfection with siRNAs and simultaneous transfection with the green fluorescent protein (GFP) fusion plasmids and siRNAs were performed using Lipofectamine 2000 (Invitrogen) (10). The cells were harvested 48 hours post-transfection, and analysis of the subcellular localization of the different p37 constructs was performed.

Efficient depletion of the CRM1 receptor by the CRM1 siRNA was confirmed by Western blot analysis using the nonsilencing siRNA as a control.

Western blot analysis. Forty-eight hours posttransfection, Vero cells were washed with phosphate-buffered saline (PBS) and lysed in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue). After brief sonication, the samples were heated to 95°C for 10 minutes. Total cell extracts were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the resolved proteins were transferred onto nitrocellulose membranes (Amersham Biosciences). The blot was blocked with 5% (wt/vol) dried milk in Tris-buffered saline plus Tween and then incubated overnight at 4°C with the rabbit polyclonal anti-CRM1 antibody (Santa Cruz) or mouse monoclonal anti-α-tubulin antibody (Sigma). After being washed three times with Tris-buffered saline plus Tween, the membrane was incubated for 1 hour with alkaline phosphatase-conjugated secondary antibodies. The blots were revealed by chemifluorescence (ECF; Amersham).

Rev(1.4)-GFP nuclear export assay. To assess the strength of the nuclear export activity of different fragments of p37 protein, the Rev(1.4)-GFP nuclear export assay, described previously by Henderson and Eleftheriou (19), was used. This assay is based on the ability of functional nuclear export sequences to promote the nuclear export activity of the Rev(1.4)-GFP fusion protein, which is composed of a NES-deficient mutant of the HIV-1 Rev protein and GFP.

The assay was performed in COS-7 cells as described previously (19). Briefly, the cells were transfected with pRev(1.4)-GFP (negative control) or its derivative

plasmids containing either the NES of the HIV-1 Rev protein (positive control) or each of the sequences to be tested. Forty-eight hours posttransfection, all cell samples were treated with 10 μg/ml cycloheximide to ensure that any cytoplasmic GFP fluorescence resulted only from the nuclear export of GFP fusion proteins and not from de novo protein synthesis. Simultaneously, part of the cell samples were treated for 3 hours with 5 μg/ml actinomycin D (ActD), which is known to specifically block the nuclear import of Rev protein by a mechanism not yet elucidated. This treatment allows the detection of weak NESs, which would not be detected if Rev protein nuclear import was occurring. The remaining cell samples were treated with 20 ng/ml of LMB for 3 hours.

The nuclear export activity of the various tested sequences was determined according to a scoring system, which is based on the proportion of cells showing exclusively nuclear, both nuclear and cytoplasmic, or exclusively cytoplasmic GFP fluorescence under the different experimental conditions (19). For this purpose, the subcellular localization of each GFP fusion protein was determined in at least 200 cells per experimental condition from three independent experiments.

Immunofluorescence studies of ASFV-infected cells. The antibody against ASFV p37 protein used for the immunofluorescence studies was obtained from rabbits immunized with the synthetic peptide NLTHNKQEFQSYEENY, which corresponds to amino acids 39 to 54 of this protein. The antibody was purified by affinity chromatography (Eurogentec), and the specificity of the purified antibody was characterized by Western blot and immunofluorescence analyses in ASFV-infected Vero cells (data not shown).

Vero cells were infected with ASFV strain BA71V (11) at a multiplicity of infection of 40 and 10 PFU/cell for immunofluorescence analysis at 6 (hours postinfection) hpi and 14 hpi, respectively. Adsorption of ASFV to the cells was performed for 2 hours at 4°C in DMEM-HG supplemented with 2% (vol/vol) FBS and 25 mM HEPES (pH 7.4) with gentle stirring. Following adsorption, the cells were washed thoroughly with DMEM-HG supplemented with 2% (vol/vol) FBS, and the infections were allowed to progress at 37°C for the indicated times.

Following the incubation period, ASFV-infected cells were washed with PBS and fixed with 4% paraformaldehyde for 15 minutes, followed by 5 minutes of incubation in methanol at −20°C. The cells were then permeabilized with 0.2% Triton X-100 for 5 minutes at room temperature, blocked with 3% bovine serum albumin in PBS at room temperature for 30 minutes, and incubated for 1 hour at room temperature with anti-p37 antibody diluted 1:500 and anti-α-tubulin monoclonal antibody (Sigma) diluted 1:250 in blocking solution. After being washed extensively with PBS, the cells were incubated for 1 hour at room temperature with Alexa Fluor 488 goat anti-rabbit immunoglobulin G and Alexa Fluor 594 goat anti-mouse immunoglobulin G antibodies (Molecular Probes) diluted 1:500 in blocking solution. The cells were washed further, and coverslips were mounted with Mowiol (Calbiochem) on glass slides.

Confocal fluorescence microscopy. For fluorescence microscopy analysis of GFP fusion proteins, Vero or COS-7 cells were washed with PBS, fixed with 4% paraformaldehyde for 15 minutes, and rinsed with PBS. The coverslips were then inverted and mounted on glass slides with Vectashield mounting medium (Vector Laboratories).

Fluorescence observations were performed using either a Bio-Rad MRC 600 or a Bio-Rad Radiance 2000 fluorescence confocal microscope equipped with an argon/krypton laser at a magnification of ×600.

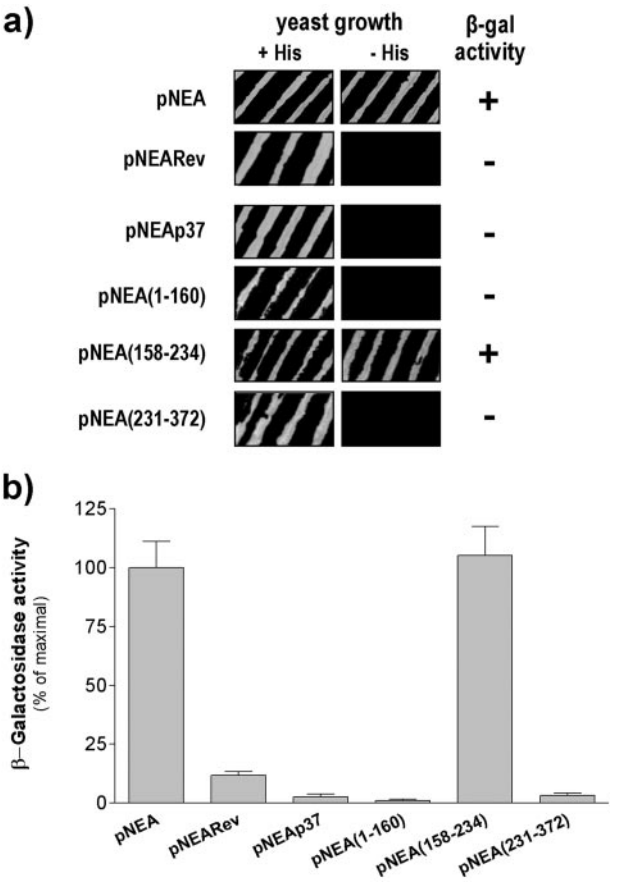

FIG. 1. Both the N and C termini of the ASFV p37 protein are exported from the nucleus to the cytoplasm of yeast cells. (a) Growth of L40 yeast transformed with the indicated constructs was analyzed both on selective medium without tryptophan (+His) and on medium without tryptophan and histidine (−His) as described in Materials and Methods. β-Galactosidase (β-gal) activity was evaluated using the colony-lift filter assay. (b) Quantitative β-galactosidase activity was determined following cell growth in minimal medium deficient in tryptophan as described in Materials and Methods. Data are expressed as percentages of maximal enzymatic activity obtained for pNEA alone (negative control); standard deviations are shown based on quintuplicates for at least three independent experiments.

RESULTS

The N- and C-terminal fragments of p37 protein are exported from the nucleus to the cytoplasm of yeast and mammalian cells. As a first approach to identify the localization of a functional nuclear export signal(s) within the ASFV p37 protein, the ability of three p37 fragments (amino acids 1 to 160, 158 to 234, and 231 to 372) to undergo nuclear export was evaluated using a yeast-based nuclear export assay (35). This assay is based on the activation of two reporter genes (*HIS3* and *lacZ*) present in *Saccharomyces cerevisiae* strain L40 by a fusion protein encoded by pNEA, comprising mLexA (bacterial LexA modified to inactivate its internal NLS), simian virus 40 (SV40) NLS (SV40 large T-antigen NLS), Gal4AD (yeast Gal4p activation domain), and the protein sequence to be tested.

Figure 1a presents the results obtained for pNEA and pNEARev, which were used as negative and positive controls for the nuclear export assay, respectively. pNEA encodes the mLexA-SV40NLS–Gal4AD fusion protein that is actively imported into the nucleus but not exported, thus leading to maximal expression of the two reporter genes. pNEARev encodes the fusion protein containing the HIV-1 Rev protein (35), which is known to be exported from the nucleus due to the presence of a strong leucine-rich NES. When expressed in yeast cells, the fusion protein encoded by pNEARev was actively exported from the nucleus of yeast cells, thus preventing the expression of the reporter genes. As a consequence, yeast cells were not able to grow on histidine-deficient medium and did not exhibit β-galactosidase activity.

In a pattern identical to that observed for the positive control (pNEARev) and for full-length p37 protein (Fig. 1a) (12), cells expressing pNEA encompassing amino acids 1 to 160 [pNEA(1-160)] and pNEA(231-372) did not grow on histidine-deficient medium and expressed residual levels of β-galactosi-

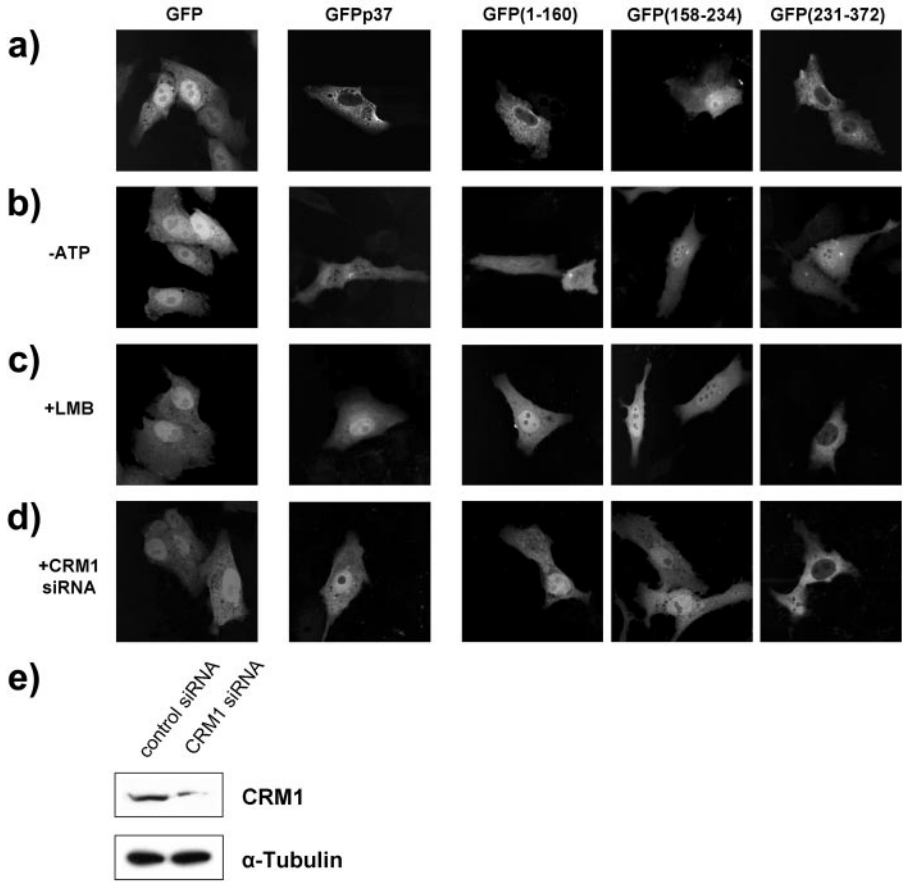

FIG. 2. The nuclear export of p37 protein is mediated by both the CRM1-dependent and CRM1-independent pathways. Subconfluent cultures of Vero cells were transiently transfected with the plasmids encoding GFP, GFPp37, GFP(1-160), GFP(158-234), or GFP(231-372). Forty-eight hours posttransfection, the subcellular localization of the different fusion proteins was visualized by fluorescence confocal microscopy (magnification, ×600) under different experimental conditions: (a) untreated cells; (b) cells incubated in energy depletion medium for 3 hours (−ATP); (c) cells treated with leptomycin B (20 ng/ml) for 3 hours (+LMB); and (d) cells simultaneously transfected with siRNA for CRM1 depletion and plasmids encoding the different GFP fusion proteins (+CRM1 siRNA). Experiments were performed at least three times, and representative images are shown. (e) Western blot analysis of CRM1 expression in Vero cells following transfection with siRNA for depletion of CRM1 (CRM1 siRNA) or with a nonsilencing control siRNA. Forty-eight hours after transfection, the cells were harvested, and Western blot analysis was performed using anti-CRM1 and anti-α-tubulin antibodies as described in Materials and Methods.

dase (Fig. 1a). In contrast, yeast cells expressing pNEA(158-234) showed both cell growth on histidine-deficient medium and β-galactosidase activity, indicating efficient activation of the reporter *HIS3* and *lacZ* genes (Fig. 1a).

Overall, these results demonstrate that both the N- and C-terminal fragments of p37 protein are actively exported from the nucleus to the cytoplasm of yeast cells and that the p37(158-234) fragment is not exported.

A quantitative evaluation of β-galactosidase activity in yeast expressing the different constructs showed that yeast cells harboring pNEA(1-160) and pNEA(231-372), similar to those harboring pNEARev and pNEAp37, presented significantly lower levels of β-galactosidase activity than those observed for the negative control (pNEA) (Fig. 1b), further supporting the ability of these p37 fragments to undergo nuclear export in yeast cells.

To investigate their nuclear export activity in mammalian cells, the subcellular localization of these three fragments fused with GFP was analyzed in Vero cells by confocal fluorescence microscopy.

Similar to what was observed for full-length p37 protein (12), the GFP(1-160) and GFP(231-372) fusion proteins were localized exclusively in the cytoplasm of transfected cells (Fig. 2a), strongly suggesting that these two p37 fragments are exported from the nucleus to the cytoplasm of mammalian cells. Conversely, GFP(158-234) was present in both the cell cytoplasm and nucleus (Fig. 2a), indicating that this p37 fragment is not actively exported from the nucleus to the cytoplasm of mammalian cells. GFP alone was distributed throughout the cell as a consequence of its low molecular weight, which allows its passive diffusion within the cell (Fig. 2a).

To confirm that the exclusively cytoplasmic fluorescence observed in cells expressing GFP(1-160) and GFP(231-372) was due to active nuclear export rather than to cytoplasmic retention of the fusion proteins, their subcellular localization was evaluated in energy-depleted cells. Under these experimental conditions, the active Ran-dependent nucleocytoplasmic transport processes are blocked whereas diffusion remains unaffected (40).

As can be observed in Fig. 2b, energy depletion of cells

transfected with GFPp37, GFP(1-160), and GFP(231-372) resulted in a significant increase of fluorescence inside the nucleus, thus demonstrating that these fusion proteins are able to diffuse between the cytoplasm and the nucleus and thereby providing unequivocal evidence that the cytoplasmic localization of the GFP(1-160) and GFP(231-372) fusion proteins in nontreated cells is a consequence of active nuclear export.

Overall, the observations in mammalian cells are consistent with the results obtained using the yeast-based nuclear export assay, which collectively demonstrate that both the N- and C-terminal regions of p37 protein are exported from the nucleus to the cytoplasm. In addition, these findings clearly indicate that p37 protein contains at least two independently functional NESs.

The nuclear export of p37 protein is mediated by two distinct pathways, the CRM1-dependent and CRM1-independent nuclear export pathways. We had previously observed that the nuclear export of full-length p37 protein is inhibited by leptomycin B (12), a metabolite that potently and specifically blocks the CRM1-dependent nuclear export pathway by binding directly to a cysteine residue of the CRM1 receptor (25, 26), thus demonstrating that the CRM1 pathway is involved in the nuclear export of p37 protein.

To investigate the contribution of the CRM1 pathway to the nuclear export of the p37(1-160) and p37(231-372) fragments, the effect of LMB on the subcellular localization of fusions of these p37 fragments with GFP was analyzed. Treatment of Vero cells with LMB had no effect on the subcellular distribution of GFP alone, whereas in cells expressing GFP(1-160), such treatment resulted in a clear accumulation of the fusion protein inside the nucleus (Fig. 2c). Interestingly, the subcellular localization of the GFP(231-372) fusion protein was not altered upon LMB treatment (Fig. 2c).

As an alternative approach to evaluate the involvement of the CRM1 receptor in the nuclear export of these fragments, RNA interference studies targeting the CRM1 receptor were performed. For this purpose, the subcellular localization of full-length p37 protein and p37 fragments was assessed in Vero cells transfected simultaneously with an siRNA targeted to the CRM1 receptor (29) and with each of the GFP fusion plasmids.

As expected, knockdown of the CRM1 receptor had no effect on the subcellular localization of GFP, whereas a clear nuclear accumulation of GFPp37 and GFP(1-160) fusion proteins was observed (Fig. 2d). Consistent with the results obtained using LMB, the subcellular localization of the GFP(231-372) fusion protein was not affected by depletion of the CRM1 receptor (Fig. 2d). At 48 hours posttransfection, a specific and significant reduction of the expression levels of the CRM1 receptor was confirmed by Western blot analysis, whereas a nonsilencing control siRNA had no effect on the protein expression levels (Fig. 2e).

Overall, these results demonstrate that two different pathways are responsible for the nuclear export process of full-length p37 protein: the CRM1-dependent nuclear export pathway and an unidentified CRM1-independent pathway.

The strength of the nuclear export of the N terminus of p37 protein is similar to that of the C terminus. To assess the strength of the nuclear export activities of the N- and C-terminal fragments of p37 protein, as well as their relative contribution to the nuclear export of full-length p37 protein, the Rev(1.4)-GFP nuclear export assay (19) was used. This assay is based on the expression of fusion proteins, consisting of Rev(1.4), an export-defective HIV-1 Rev protein mutant, the sequence to be tested, and GFP (19). The strength of the NESs is evaluated by analyzing their capacity to promote nuclear export of the Rev(1.4)-GFP fusion protein.

The Rev(1.4)-GFP fusion protein, which was used as a negative control for this assay, was localized exclusively in the nucleus of COS-7 cells, presenting a clear nucleolar accumulation (Fig. 3a and b). Following actinomycin D treatment, which blocks the nuclear import mediated by the Rev NLS, this fusion remained nuclear (Fig. 3a and b), further demonstrating that no NES is present in this construct. Rev(1.4)-NES–GFP, a fusion protein that contains the Rev NES, which was used as a positive control for this assay, was localized in the nucleus and the cytoplasm of transfected cells (Fig. 3a and b). The number of cells presenting exclusively cytoplasmic localization was enhanced by ActD treatment (Fig. 3a and b). The cytoplasmic localization of the Rev(1.4)-NES–GFP fusion protein was completely blocked by the addition of leptomycin B (Fig. 3a and b), as expected for a protein that is exported through the CRM1-dependent nuclear export pathway.

Consistent with previous observations obtained in our laboratory demonstrating that p37 protein is actively exported from the nucleus (12), full-length p37 protein readily promoted a relocation of the Rev(1.4)-GFP fusion protein from the nucleus to the cytoplasm of a significant number of cells (Fig. 3a and b). Upon treatment with ActD, the number of cells presenting exclusively cytoplasmic fluorescence increased significantly (Fig. 3a and b). In contrast to what was observed for Rev(1.4)-NES–GFP, upon LMB treatment, the Rev(1.4)-p37–GFP fusion protein was still detectable in the cytoplasm of the majority of cells (93.2%), which is consistent with the involvement of a CRM1-independent pathway in the nuclear export of p37 protein.

When fused with Rev(1.4)-GFP, both the N- and C-terminal fragments of p37 protein were able to induce a cytoplasmic relocation of the fusion protein in a significant number of cells, which was further increased by ActD treatment (Fig. 3a and b). Interestingly, distinct results were obtained upon LMB treatment of cells expressing each of these two constructs: the fusion protein containing the p37(1-160) fragment accumulated inside the nucleus of 68.8% of cells, whereas for the fusion protein containing the p37(231-372) fragment, no effect was observed (nuclear in 15.7% of cells) (Fig. 3a and b).

The fusion protein containing the p37(158-234) fragment remained, under all experimental conditions, exclusively nuclear in the majority of cells (Fig. 3a and b), similar to what was observed for the negative control, further demonstrating that no NES is present in this fragment.

To facilitate the comparison of the strengths of the nuclear export processes of the two fragments of p37 protein, a relative nuclear export activity was assigned to each construct according to the NES scoring system developed by Henderson and Eleftheriou (19). An export rate of 7+ denotes a strong NES, which corresponds to a sequence that is able to induce exclusively cytoplasmic localization of the Rev(1.4)-GFP fusion protein in 20 to 50% of cells in the absence of ActD and in more than 80% of cells upon ActD treatment (19).

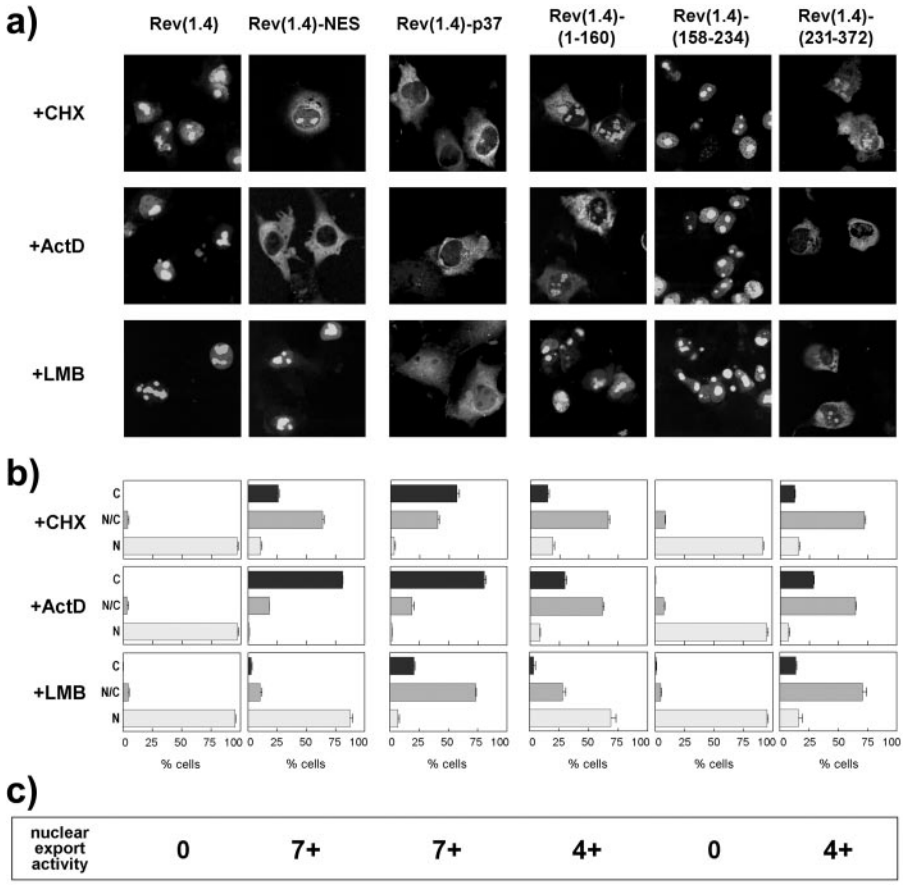

FIG. 3. The strengths of the nuclear export processes of the N and C termini of p37 protein are similar. (a) Full-length p37 protein and three p37 fragments (1 to 160, 158 to 234, 231 to 372) were fused to an export-deficient mutant of Rev [Rev(1.4)-GFP], and their subcellular distributions were analyzed in transfected COS-7 cells. Rev(1.4)-NES–GFP, which contains the HIV-1 Rev NES, was used as a positive control. Forty-eight hours after transfection, all cell samples were incubated with 10 μg/ml cycloheximide and either left untreated (+CHX), treated with 5 μg/ml actinomycin D (+ActD), or treated with 20 ng/ml leptomycin B (+LMB) for 3 hours. The subcellular distribution of the different Rev(1.4)-GFP fusion proteins was examined by confocal fluorescence microscopy (magnification, ×600), and representative images are shown. (b) The subcellular localization of each construct is indicated as a percentage of the total number of cells counted. C, exclusively cytoplasmic; N/C, both nuclear and cytoplasmic; and N, exclusively nuclear. Each experiment was performed at least three times, and approximately 200 cells were counted per experimental condition. Error bars indicate standard deviations. (c) A scoring scale based on cell counts was used to assign relative export activities to each of the tested sequences as described by Henderson and Eleftheriou (19). A relative nuclear export strength of 7+ denotes a strong NES.

As estimated from the results of the cell counts obtained under the different experimental conditions (Fig. 3b), the strengths of the nuclear export of p37 protein and that of the HIV-1 Rev NES are comparable (7+) (Fig. 3c), clearly demonstrating that the nuclear export of p37 protein is a strong process.

Interestingly, the strength of the nuclear export of the N-terminal fragment of p37 protein (4+) is similar to that of the C-terminal fragment (4+) (Fig. 3c), suggesting that the CRM1-dependent and CRM1-independent nuclear export pathways may contribute equally to the overall nuclear export of full-length p37 protein.

The CRM1-dependent nuclear export of the N terminus of p37 protein is mediated by two independent NESs. To investigate the location of the NES responsible for the nuclear export of the N-terminal fragment of p37 protein, the subcellular localization of several GFP-tagged p37 fragments and deletion mutants was analyzed in Vero cells by confocal fluorescence microscopy.

The GFP(46-234) fusion protein was exported to the cytoplasm by a CRM1-dependent mechanism (Fig. 4). A detailed analysis of the amino acid sequence of this p37 fragment led to the identification of a leucine-rich sequence between amino acids 122 and 129 (LEGLDLYL), which constituted a good candidate to mediate the nuclear export of the N-terminal fragment of p37 protein. This sequence resembles the well-characterized, leucine-rich NESs present in cellular and viral proteins, such as the NESs present in PKI or in the HIV-1 Rev protein (Fig. 5), which consist of four closely spaced hydrophobic amino acids, most often leucine or isoleucine residues. Surprisingly, a deletion mutant lacking this putative NES [GFP(46-234)Δ122-129] was still efficiently exported from the nucleus to the cytoplasm of transfected cells through the CRM1 pathway (Fig. 4), clearly indicating the existence of an alternative sequence responsible for the nuclear export of the p37(46-234) fragment.

Following the systematic analysis of the subcellular localization of several other p37 fragments (data not shown), it was

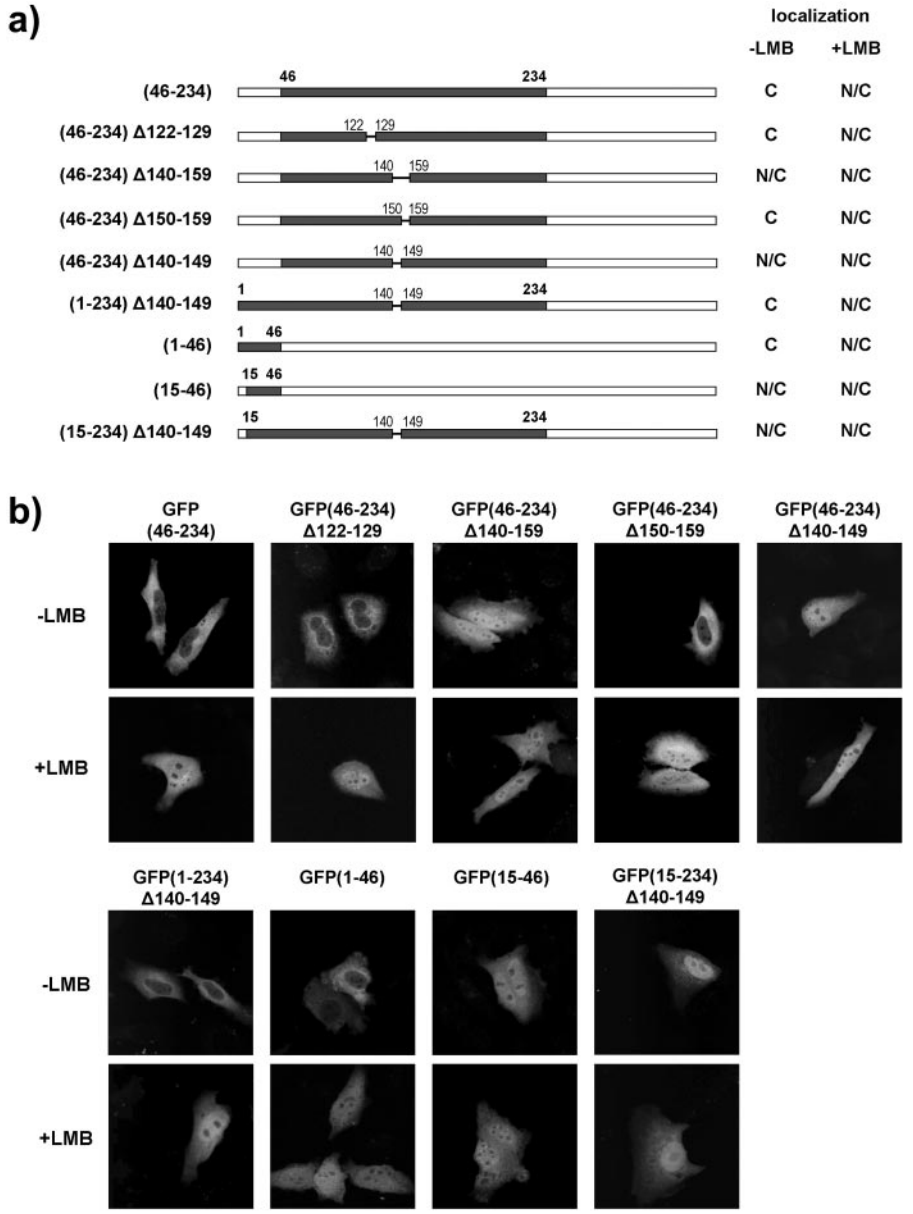

FIG. 4. The nuclear export of the N terminus of p37 protein is mediated by two LMB-sensitive NESs. (a) Schematic representation of the different N-terminal fragments and deletion mutants of p37 protein and respective subcellular localization in untreated (−LMB) or LMB-treated (+LMB) Vero cells. C and N/C indicate that in the majority of transfected cells, the fusion protein being tested presents an exclusively cytoplasmic or both cytoplasmic and nuclear localization, respectively. (b) Forty-eight hours after Vero cells were transfected with the indicated constructs, the subcellular distributions of the different GFP fusion proteins were examined in Vero cells by confocal fluorescence microscopy (magnification, ×600). The cells were left untreated or treated with LMB (20 ng/ml) at 37°C for 3 hours. Experiments were performed at least three times, and representative images are shown.

observed that the deletion mutant lacking amino acids 140 to 159 [GFP(46-234)Δ140-159] was not able to undergo efficient nuclear export (Fig. 4). By analyzing the nuclear export ability of two additional deletion mutants [GFP(46-234)Δ150-159 and GFP(46-234)Δ140-149], the nuclear export sequence was further restricted to amino acids 140 to 149 (Fig. 4).

In contrast to GFP(46-234)Δ140-149, the GFP(1-234)Δ140-149 fusion protein was exported from the nucleus to the cytoplasm of transfected cells by a LMB-sensitive nuclear export pathway (Fig. 4), strongly suggesting the existence of an additional NES localized between amino acids 1 and 46, which is also responsible for the nuclear export of the N-terminal fragment of p37 protein. This hypothesis was confirmed by the observation that the fusion protein GFP(1-46) was exported from the nucleus to the cytoplasm by the CRM1 pathway (Fig. 4). In this context, the absence of nuclear export of GFP(15-46) in cells expressing this fusion protein (Fig. 4) demonstrated that the additional nuclear export signal of the N-terminal fragment of p37 protein is localized between residues 1 and 14.

As shown in Fig. 4, the GFP(15-234)Δ140-149 deletion mu-

PKIα	37	ELALKLAGLDIN 46
MKP-3	164	VLGLGGLRIS 171
HIV-1 Rev	77	PLPPLERLTLD 83
HSV-1 ICP27	7	MLIDLGLDLS 16
Consensus		$\Phi X_{1-3}\Phi X_{2-3}\Phi X\Phi$
p37 NES1	2	ALTVEELGLS 11
p37 NES2	140	IDSIQTVQQM 149
p37 NES3	239	NIINAFARI 247

FIG. 5. Comparison of nuclear export signals identified in p37 protein with well-characterized NESs present in cellular and viral proteins. NESs identified in PKIα (48), MAP kinase phosphatase 3 (MKP-3) (24), Rev protein from HIV-1 (HIV-1 Rev) (13), and ICP27 protein from herpes simplex virus type 1 (HSV-1 ICP27) (39), as well as the three NESs identified in the ASFV p37 protein, are shown. Residues critical to NES activity are highlighted in bold type. In the consensus NES sequence, ϕ indicates a large hydrophobic residue, such as leucine, isoleucine, valine, or metionine, and X indicates any amino acid.

tant, lacking both amino acid sequences 1 to 14 and 140 to 149, was distributed throughout the nucleus and cytoplasm of transfected cells. This finding demonstrates that these two independently active nuclear export sequences, identified at the N terminus of p37 protein, are collectively responsible for the CRM1-dependent nuclear export of this viral protein.

Identification of the NES responsible for the CRM1-independent nuclear export of the C terminus of p37 protein. To investigate the localization of the C-terminal NES of p37 protein, the subcellular localization of several constructs, obtained by expanding the p37(158-234) fragment toward the C terminus, was analyzed.

Results from this analysis revealed that the fusion protein GFP(158-249) is localized exclusively in the cytoplasm (Fig. 6) and that the fusion protein GFP(158-239) is localized both in the cell nucleus and in the cytoplasm (Fig. 6), suggesting that the region between amino acids 239 and 249 is a nuclear export signal.

As shown in Fig. 6, the fusion protein GFP(251-372) was localized both in the nucleus and in the cytoplasm of transfected cells, excluding the existence of an additional NES in the C terminus of p37 protein.

In agreement with these results, the fusion protein GFP(158-372) was localized exclusively in the cytoplasm of transfected cells (Fig. 6), whereas its deletion mutant lacking amino acids 239 to 249 [GFP(158-372)Δ239-249] was distributed throughout the cytoplasm and nucleus of transfected cells (Fig. 6) in a pattern similar to that of GFP alone. Overall, these results demonstrate that a functional NES is localized between amino acids 239 and 249.

As can be observed in Fig. 6, the nuclear export of the GFP(158-249) and GFP(158-372) fusion proteins was not affected by LMB treatment. These results clearly indicate that unlike the two NESs identified in the N-terminal fragment of p37 protein, the NES identified at the C terminus of p37 protein (amino acids 239 to 249) mediates the nuclear export of this fragment through a CRM1-independent pathway.

As expected, a mutant in which these three NESs were deleted (GFPp37ΔNES) accumulated inside the nucleus of

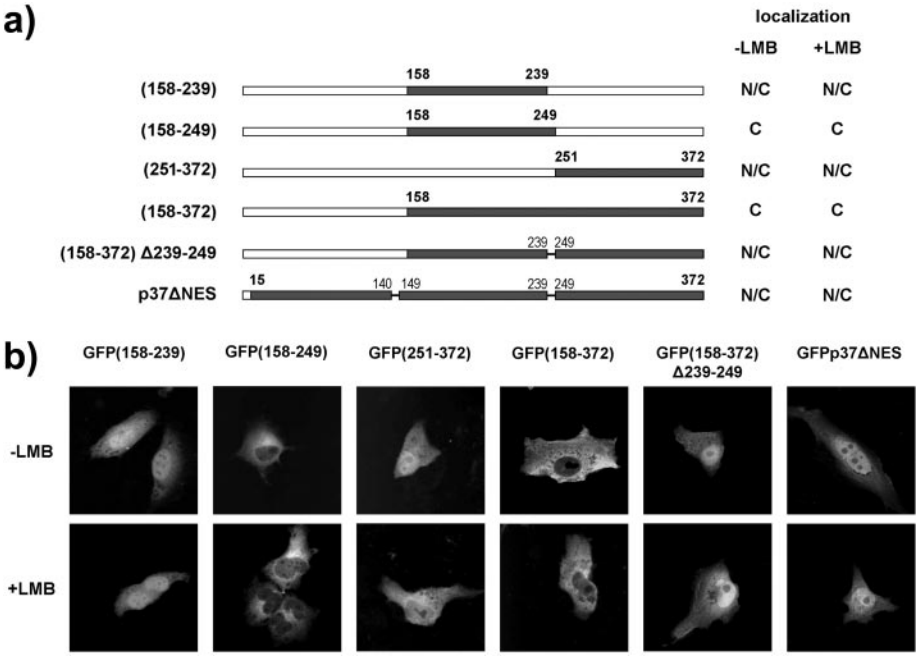

FIG. 6. The nuclear export of the C-terminal region of p37 protein is mediated by an LMB-insensitive NES. (a) Schematic illustration of the different C-terminal fragments and deletion mutants of p37 protein and their subcellular localization in untreated (−LMB) or LMB-treated (+LMB) cells. C indicates that the fusion protein presents an exclusively cytoplasmic localization, and N/C indicates that the protein is localized both in the nucleus and in the cytoplasm of the majority of transfected cells. (b) Forty-eight hours after transfection with the different constructs, Vero cells were either left untreated or treated for 3 hours with 20 ng/ml LMB, and the subcellular distribution of each fusion protein was analyzed by confocal fluorescence microscopy (magnification, ×600). Experiments were performed at least three times, and representative images are shown.

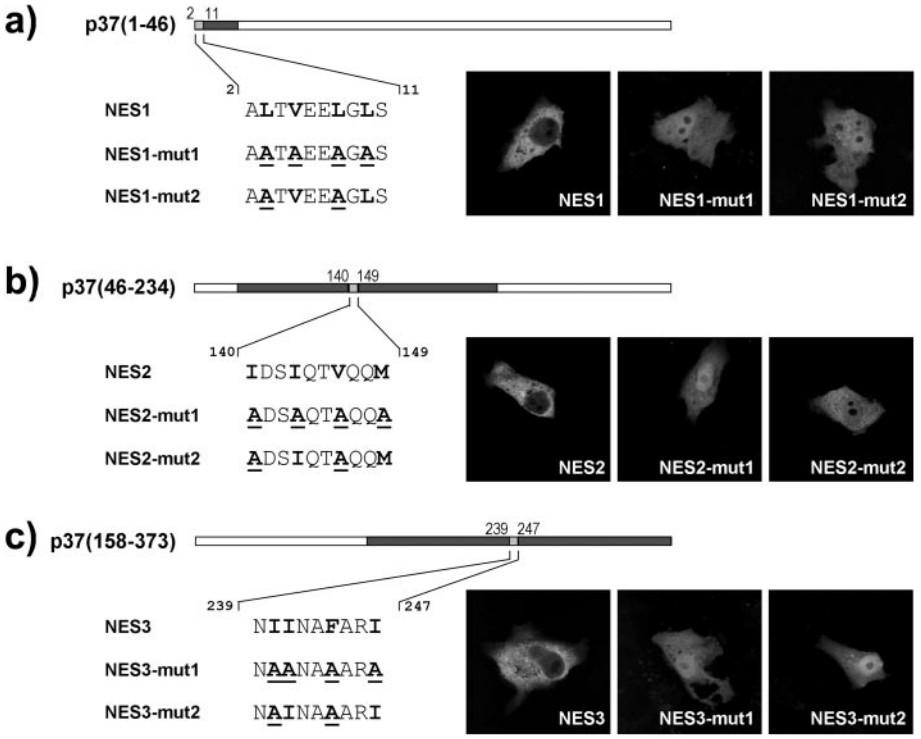

FIG. 7. Hydrophobic amino acids are critical to the nuclear export activity of the three NESs identified in p37 protein. Vero cells were transiently transfected with the control fragments and point mutants of (a) NES1 (amino acids 2 to 11), (b) NES2 (140 to 149), and (c) NES3 (239 to 247). Hydrophobic residues, which were replaced by alanines (underlined) to generate the different mutants, are shown in bold type. The subcellular localization of the GFP fusion proteins was determined by confocal fluorescence microscopy (magnification, ×600). Experiments were performed at least three times, and representative images are shown.

transfected cells (Fig. 6), confirming that the three identified signals are responsible for the nuclear export of p37 protein.

Identification of the amino acids critical to the function of the three nuclear export signals identified in p37 protein. Since it has been extensively demonstrated that hydrophobic amino acids are critical to the CRM1-dependent nuclear export activity mediated by leucine-rich NESs, the role of such amino acids in the activity of NES1 and NES2 of p37 protein was analyzed by site-directed mutagenesis.

As shown in Fig. 7a, point mutation of leucine 3, valine 5, leucine 8, and leucine 10 of NES1 to alanines (NES1-mut1) dramatically disrupted the cytoplasmic localization of the GFP(1-46) fusion protein, demonstrating the importance of these residues to the activity of NES1. A similar alteration of the subcellular localization was observed upon mutation of two (L3A and L8A) of the four hydrophobic amino acids of NES1 (NES1-mut2) (Fig. 7a).

Mutation of the residues isoleucine 140 and valine 146 of NES2 to alanines (NES2-mut2) caused a redistribution of the GFP(46-234) fusion protein throughout the nucleus and cytoplasm (Fig. 7b), whereas mutations I140A, I143A, V146A, and M149A of NES2 (NES2-mut1) resulted in an increased nuclear accumulation of this fusion protein (Fig. 7b). These results demonstrate that hydrophobic amino acids are also critical to the function of NES2, although this signal is not in agreement with the consensus sequence for leucine-rich NESs (Fig. 5).

In an approach similar to that used for the nuclear export activity of NES1 and NES2, the residues essential to the nuclear export activity of NES3 were identified by analyzing the subcellular localization of point mutants of this signal, in which the hydrophobic amino acids were replaced by alanines. As shown in Fig. 7c, neither NES3-mut1 nor NES3-mut2 was efficiently exported from the cell nucleus, as demonstrated by the fluorescence detected both in the nucleus and in the cytoplasm of cells expressing these mutants. Although the nuclear export activity mediated by NES3 was shown to be CRM1 independent, these results provide clear evidence that hydrophobic residues are also crucial to the activity of this signal.

p37 protein is localized in different subcellular compartments during ASFV infection. Aiming at determining whether p37 protein is actively transported between the nucleus and the cytoplasm in ASFV-infected Vero cells, the subcellular localization of p37 protein was determined by immunofluorescence analysis at different times postinfection. To facilitate the analysis of the subcellular localization of p37 protein, the localization of α-tubulin, which was used as a cytoplasmic marker, was also determined.

As can be observed in Fig. 8, at early times postinfection (6 hpi), the ASFV p37 protein is localized in distinct regions inside the nucleus and dispersed throughout the cytoplasm of Vero cells. On the other hand, at late times of the infection cycle (14 hpi), no signal is found in the nucleus and p37 protein is localized exclusively in the cytoplasm, clearly concentrated in denominated viral factories, specific regions where viral assembly occurs (Fig. 8). Immunofluorescence results of noninfected

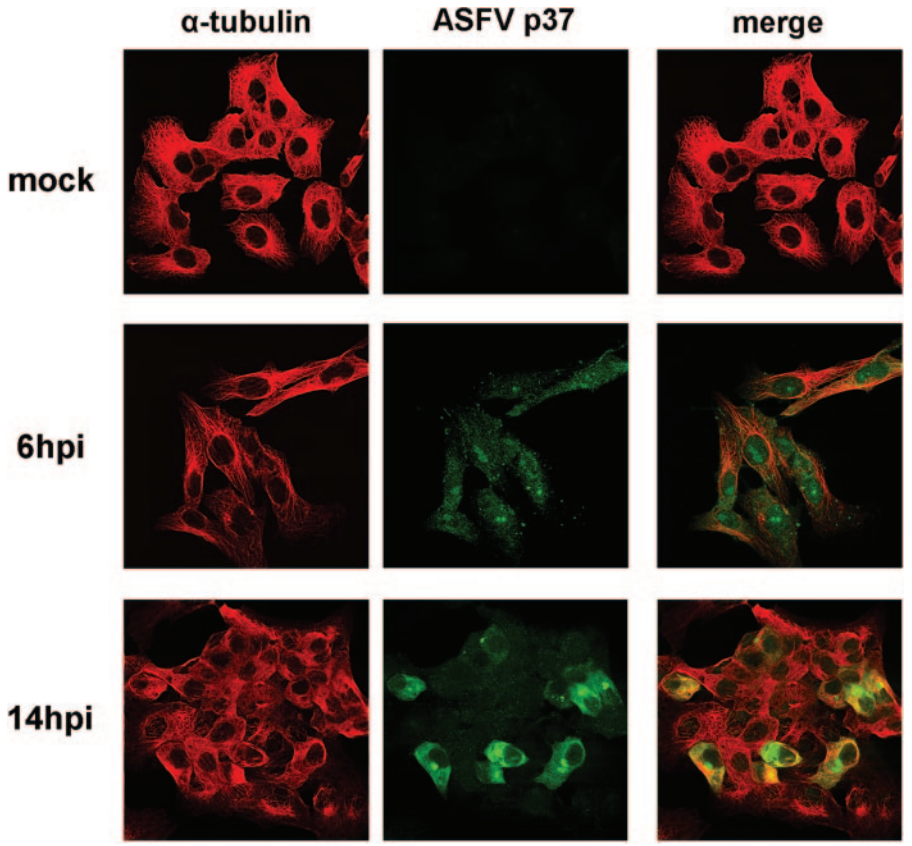

FIG. 8. Distinct subcellular localization of p37 protein in ASFV-infected cells at different times postinfection. Vero cells infected with the BA71V strain of ASFV or mock-infected cells were fixed and processed for immunofluorescence analysis at the indicated times postinfection. Samples were incubated with a rabbit antiserum raised against the p37 protein and a monoclonal α-tubulin antibody, followed by incubation with secondary antibodies conjugated to Alexa Fluor 488 (green) and Alexa Fluor 594 (red), respectively. Confocal laser scanning images acquired in green and red channels are presented separately and as a merged image. Experiments were performed at least three times, and representative images are shown.

cells (mock), which were used as a negative control, are also shown for comparison.

The observation of distinct subcellular localizations of p37 protein at early and late times of infection, particularly the presence of the viral protein inside the nucleus at the initial stages of the infection cycle and its disappearance from the nucleus at later times, is in agreement with our results for transfected cells demonstrating that p37 protein is a nucleocytoplasmic shuttling protein. Overall, these results support the existence of an active p37 transport between the nucleus and the cytoplasm during ASFV infection.

DISCUSSION

Aiming at identifying the signals responsible for the nuclear export of the ASFV p37 protein, we investigated the nuclear export ability of several protein fragments. We report that both the N- and C-terminal fragments of p37 protein are exported from the nucleus to the cytoplasm of yeast and mammalian cells, demonstrating the existence of at least two independently active nuclear export signals in this protein.

Our results showing that the N-terminal fragment of p37 protein accumulated in the nucleus upon cell treatment with LMB demonstrated that its nuclear export occurs through the CRM1 pathway. Interestingly, the nuclear export of the C-terminal segment of p37 protein was not affected by cell treatment with LMB, indicating that the nuclear export of this p37 fragment occurs via a CRM1-independent nuclear export pathway. Overall, these results undoubtedly demonstrate that the nuclear export of p37 protein occurs by the CRM1-dependent nuclear export pathway as well as by an unidentified CRM1-independent nuclear export pathway.

Using the Rev(1.4)-GFP nuclear export assay, we were able to determine that the nuclear export of p37 protein is a strong process, comparable to that of the Rev protein of HIV-1 (7+, according to the NES scoring system) (19). This finding is in agreement with previous results obtained in our laboratory demonstrating that the nuclear export of p37 protein is able to overcome the nuclear import mediated by the SV40 NLS (12).

Since the nuclear export of full-length p37 protein is LMB sensitive, it would be reasonable to assume that the nuclear export mediated by the CRM1 receptor (sensitive to LMB) would be stronger than the CRM1-independent nuclear export (insensitive to LMB). However, as determined by the Rev(1.4)-GFP assay, the strengths of the nuclear exports of the N- and C-terminal fragments of p37 are equivalent (4+), suggesting that the CRM1-dependent and CRM1-independent

nuclear export pathways contribute equally to the nuclear export of full-length p37 protein. Nonetheless, it cannot be excluded that in the context of full-length p37 protein, one of these pathways may be dominant compared to the other.

Although the nuclear export pathway mediated by the CRM1 receptor is the most extensively studied, other export receptors have been identified (34). For example, it has been shown that in addition to the CRM1 receptor, calreticulin mediates the nuclear export of protein kinase inhibitor by interacting with the leucine-rich NES present in PKI (20). More recently, a new nuclear export receptor was identified, exportin-7 (31), which was suggested to exhibit broad substrate specificity, therefore defining a new general nuclear export pathway. However, the signals that were shown to mediate nuclear export through this pathway are very different from the leucine-rich NESs commonly associated with the CRM1-dependent nuclear export pathway. Interestingly, these signals do not appear to be short linear sequences but rather signals requiring a specific three-dimensional folding. Moreover, basic residues were shown to be critical to exportin-7 recruitment.

We have identified and characterized three functional NESs in p37 protein (Fig. 5). NES1 and NES2, localized in the N terminus of p37 protein, were shown to powerfully mediate the export p37 protein from the nucleus to the cytosol by the LMB-sensitive, and hence CRM1-dependent, nuclear export pathway. Although NES1 is similar to the NESs identified in PKI or the HIV-1 Rev protein, NES2 is not in agreement with the consensus sequence for leucine-rich NESs (Fig. 5). Nonetheless, mutation of the hydrophobic residues within both NESs completely abolished their nuclear export abilities. Other viral proteins have been described to contain NESs which, although rich in hydrophobic residues, do not fit the well-defined consensus for leucine-rich NESs (32, 37). Despite this lack of homology, these NESs also mediate CRM1-dependent nuclear export.

In addition to the two NESs identified in the N terminus of p37 protein, a third NES was identified at its C terminus. Although NES3 does not interact with the CRM1 receptor, the amino acids that were shown to be critical to its function are also hydrophobic. Moreover, recent reports have demonstrated the interaction of proteins bearing leucine-rich NESs with receptors other than CRM1 (17, 52). It should be stressed that the characteristics of NES3 are divergent from those that have been proposed to be required for exportin-7 recruitment, although the possibility that other motifs are recognized by this exportin or that p37 protein is most likely exported by an alternative pathway cannot be excluded. Additional studies are needed to investigate the detailed mechanism by which the CRM1-independent nuclear export of p37 protein occurs.

Similar to that of the ASFV p37 protein, which contains three NESs and is exported through two concurrent pathways, the nuclear export of other proteins has been demonstrated to be mediated by more than one NES and to occur through both the CRM1-dependent and CRM1-independent pathways. Examples include α-catenin, which was reported to contain both a weak LMB-insensitive NES and a strong LMB-sensitive NES (17), as well as receptor-interacting protein 3, which contains two leucine-rich NESs that are responsible for its nuclear export by the CRM1 pathway and an additional NES that is involved in the CRM1-independent nuclear export of this protein (52).

A possible explanation for the existence of three NESs in p37 protein is that the presence of multiple NESs in proteins, apparently redundant, constitutes a strategy that ensures successful nuclear export of certain proteins of critical function; an additional hypothesis is that the existence of multiple NESs in proteins results in an enhanced nuclear export ability, by a cooperative effect, in a mechanism similar to the one suggested for the presence of multiple NLSs in proteins (36).

We have previously shown that a fusion protein containing p37, with a molecular weight above the nuclear pore diffusion limit, accumulates inside the nucleus when nuclear export is inhibited (12). Although this finding demonstrates that p37 protein is also imported into the nucleus by an active process, analysis of p37 protein sequence did not reveal the existence of sequences with homology to classical NLSs. The best-characterized NLSs consist of a small cluster of basic amino acids, namely, lysine and arginine residues (23). Nonetheless, a variety of sequences that have no obvious resemblance to classical NLSs were also shown to mediate protein nuclear import (8, 50). Alternatively, the binding of p37 protein to some cellular or viral NLS-containing protein that is recognized by the nuclear import machinery may explain how p37 protein is imported into the nucleus.

Whatever the mechanism of nuclear import used by p37 protein, the strong cytoplasmic localization of p37 protein at the steady state strongly suggests that its nuclear import is less important than its nuclear export, which occurs through two different pathways and is mediated by the three NESs characterized in this study.

Considering the importance that nucleocytoplasmic transport of viral proteins may have at an early stage of ASFV infection, when the nuclear phase of viral DNA replication occurs, it is reasonable to assume that the nucleocytoplasmic shuttling activity of p37 protein may play an important role in the ASFV replication cycle. One possibility is that p37 protein is involved in the export of the viral genome from the nucleus to the cytoplasm of infected cells following the brief nuclear replication phase. This hypothesis is supported by the results of immunofluorescence studies of ASFV-infected cells described in the present report, which clearly demonstrate that p37 protein is localized inside the nucleus at early times postinfection (6 hpi) and localized exclusively in the cytoplasm at later times (14 hpi). Additionally, results from in situ hybridization experiments clearly showed colocalization of the viral DNA and p37 protein in specific nuclear regions at early times postinfection (unpublished data), further supporting this hypothesis.

Although studies in progress aim at further elucidating the relevance of the nuclear export of p37 protein to the replication cycle of African swine fever virus, all evidence indicates that this capacity should be crucial for its biological function, as stressed by the presence of multiple NESs and of two alternative pathways for the nuclear export of this protein.

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