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# A peptide inhibitor of exportin1 blocks shuttling of the adenoviral E1B 55 kDa protein but not export of viral late mRNAs

S.J. Flint*, Wenying Huang, Joseph Goodhouse, Saw Kyin

*Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA*

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**Abstract**

The human subgroup C adenoviral E1B 55 kDa and E4 Orf6 proteins are required for efficient nuclear export of viral late mRNAs, but the cellular pathway that mediates such export has not been identified. As a first step to develop a general approach to address this issue, we have assessed the utility of cell-permeable peptide inhibitors of cellular export receptors. As both E1B and E4 proteins have been reported to containing a leucine-rich nuclear export signal (NES), we synthesized a cell-permeable peptide containing such an NES. This peptide induced substantial inhibition of export of the E1B protein, whereas a control, non-functional peptide did not. However, under the same conditions, the NES peptide had no effect on export of viral late mRNAs. These observations establish that viral late mRNAs are not exported by exportin1, as well as the value of peptide inhibitors in investigation of mRNA export regulation in adenovirus-infected cells.


*Keywords:* Adenoviral E1B 55 kDa protein; mRNA export; Exportin1; Peptide Inhibitor

## Introduction

The selective transport of viral late mRNAs from the nucleus and cytoplasm and concomitant inhibition of cellular mRNA export that are characteristic of the late phase of human, subgroup C adenovirus infection require the viral E1B 55 kDa and E4 Orf6 proteins (reviewed in Dobner and Kzhyshkowska, 2001; Flint and Gonzalez, 2003). Genetic experiments have implicated the complex containing these viral early proteins that is formed in infected cells (Sarnow et al., 1984) in regulation of mRNA export (Bridge and Ketner, 1990; Cutt et al., 1987). Furthermore, selective export of viral late mRNAs has been correlated with the E4 Orf6 protein-dependent localization of the E1B protein to the peripheral zones of viral replication centers (Gonzalez and Flint, 2002; Ornelles and Shenk, 1991), which are the sites of synthesis, and at least initial processing, of viral late pre-mRNAs (Bridge et al., 1996; Pombo et al., 1994; Puvion-Dutilleul et al., 1994; reviewed in Bridge and Pettersson, 1996). In cells infected by an adenovirus type 5 (Ad5) mutant carrying a small insertion in the E1B 55 kDa protein coding sequence that inhibits interaction with the E4 protein, export of viral late mRNAs is impaired, and the association of the E1B with the E4 protein at peripheral zones of replication centers is disrupted (Gonzalez and Flint, 2002). By contrast, the association with, and organization at, replication centers of the E4 Orf6 protein are not altered (Gonzalez and Flint, 2002). These observations suggest that the E4 Orf6 protein is primarily responsible for recognition of some unknown features of these specialized nuclear sites, while the E1B 55 kDa protein directs viral late mRNAs for nuclear export. The cellular export pathway by which viral mRNAs leave the nucleus during the late phase of infection has not been identified. However, properties of the E1B 55 kDa protein suggest several possible candidates.

Among the numerous cellular proteins with which the E1B 55 kDa protein has been shown to associate in cells infected by Ad5 are the E1B-Ap5 and pp32 proteins (Gabler et al., 1998; Harada et al., 2002). The former, which was identified by virtue of its direct binding to the E1B protein

* Corresponding author. Fax: +1 609 258 4575.
*E-mail address:* sjflint@molbio.princeton.edu (S.J. Flint).

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in an in vitro assay, is an RNA-binding protein belonging to the heterogeneous nuclear ribonucleoprotein family (Gabler et al., 1998). The E1B-Ap5 protein binds to human Tap (Bachi et al., 2000), one subunit of the Tap-p15 (NFX1-NFX2) heterodimer, which is a major export receptor for mRNAs in eukaryotes (reviewed in Cullen, 2003; Vinciguerra and Stutz, 2004; Weis, 2002). The pp32 protein is a ligand of the human HuR protein, which has been shown to serve as an export adaptor for short-lived mRNAs encoded by early response genes, such as c-*fos* (Brennan et al., 2000; Gallouzi and Steitz, 2001). The pp32 protein is not itself an export receptor. Rather it contains a leucine-rich nuclear export signal (NES) analogous to that present in the human immunodeficiency virus type 1 Rev protein, the first export signal to be identified (Fischer et al., 1995; Fornerod et al., 1997; Fukuda et al., 1997; Malim et al., 1989, 1991; Pasquinelli et al., 1997). Like the Rev NES, that presents in pp32 is recognized by the export receptor exportin1 (Crm1) (Brennan et al., 2000). It is not known whether the interactions of the E1B 55 kDa protein with these cellular proteins are important for regulation of mRNA export in Ad5-infected cells, although it has been reported that overproduction of the E1B-Ap5 protein induces a modest increase in the accumulation of viral late mRNAs in the cytoplasm (Gabler et al., 1998).

The E1B 55 kDa protein itself contains an autonomous leucine-rich NES, which is both necessary and sufficient for exit of the viral protein from the nucleus in the absence of other viral proteins (Kratzer et al., 2000) and in Ad5-infected cells (Dosch et al., 2001). Such NES-dependent export, and hence the shuttling activity of the E1B protein, are inhibited by the HIV-1 Rev protein and leptomycin B, a specific inhibitor of exportin1 (Dosch et al., 2001; Kratzer et al., 2000). The E4 Orf6 protein has also been reported to contain a leucine-rich NES required for shuttling of the E1B 55 kDa-E4 Orf6 protein complex in the absence of other viral protein (Dobbelstein et al., 1997). In addition to its role in export of specific cellular mRNAs described in the previous paragraph, exportin1 transports unspliced and partially spliced HIV-1 mRNAs that contain the Rev recognition element from the nucleus to the cytoplasm (Bogerd et al., 1998; Fischer et al., 1995; Fornerod et al., 1997). This receptor has also been implicated in export of at least some herpesviral mRNAs that lack introns (Soliman and Silverstein, 2000a, 2000b). In conjunction, these observations suggest that viral late mRNAs could leave the nucleus via the exportin1 pathway.

Efforts to evaluate the functional importance of these adenoviral export signals have yielded seemingly contradictory findings and conclusions. Whether the E4 Orf6 protein NES directs efficient exportin1-dependent export is not clear: under conditions in which the E1B 55 kDa protein was sufficient to mediate export of a GST–NES–GFP fusion protein, the E4 Orf6 NES failed to do so (Kratzer et al., 2000). It has also been reported that, compared to the E1B protein, the E4 protein shuttles between the nucleus and cytoplasm at a slow rate, and that such activity is only partially inhibited by leptomycin B (Dosch et al., 2001). Two groups of investigators have assessed the role of the E4 Orf6 NES using an assay in which deletion of the E4 coding sequence from the viral genome was complemented by transient synthesis of wild type or altered E4 Orf 6 proteins. In one case, substitutions that blocked the function of the NES were reported to be required for efficient accumulation of viral late mRNAs in the cytoplasm (Weigel and Dobbelstein, 2000), whereas in the other no effect on synthesis of viral late proteins was observed (Rabino et al., 2000). Bridge and colleagues also observed that inhibition of exportin1 by leptomycin B blocked cytoplasmic accumulation of the E4 and E1B 55 kDa proteins, but did not reduce viral late protein synthesis (an indirect measure of viral late mRNA export) (Carter et al., 2003; Rabino et al., 2000). It has been argued that differences in such experimental parameters as multiplicity of infection with E4 mutant viruses, the concentrations of exogenous, complementing E4 proteins achieved by different transient expression protocols, and the concentration and duration of leptomycin B treatment could account for these apparent discrepancies (Bridge, 2000; Dobbelstein, 2000). It should also be noted that the effects of NES mutations or drugs on viral late mRNA export have never been examined directly. The use of experimental surrogates, such as accumulation of cytoplasmic mRNA or the efficiency of late protein synthesis, could well be misleading. Any defect or delay in entry into the late phase of infection would result in reduced and delayed accumulation of both viral late mRNAs and late proteins in the cytoplasm, even though viral late mRNA export was not specifically inhibited. Furthermore, when export of viral late mRNAs is impaired, for example, in cells infected by E1B 55 kDa protein null mutants of Ad5, the consequent decreases in viral late protein synthesis result in secondary defects. In the first place, the efficiency of ML transcription declines from about 16 h p.i. (Leppard, 1993; Pilder et al., 1986), presumably because of production of reduced quantities of the viral $IVa_2$ protein (Pardo-Mateos and Young, 2004a, 2004b; Tribouley et al., 1994). Secondly, late protein synthesis is reduced to a substantially greater degree than expected from the decrease in the cytoplasmic concentrations of the corresponding mRNAs (Harada and Berk, 1999; Williams et al., 1986). This phenotype is most likely a result of impaired synthesis of the viral L4 100 kDa protein, which is necessary for efficient translation of all late mRNAs (Hayes et al., 1990; Williams et al., 1986; Xi et al., 2004).

Reagents that specifically inhibit each of the several pathways by which macromolecules are exported from mammalian cell nuclei would greatly facilitate elucidation of the mechanism of selective export of adenoviral late mRNAs. Knock-down of expression of mammalian genes using small interfering RNAs can be both sufficiently specific and effective (see Dykxhoorn et al., 2003; Hannon and Rossi, 2004; Mittal, 2004). However, inhibition of

essential export pathways by such reagents for the period necessary to reach the late stage of adenovirus infection could well induce cytotoxicity, or impair early steps in the infectious cycle upon which entry into the late phase depends. We have therefore assessed the feasibility of an alternative approach that avoids such complications, the use of cell-permeable peptides designed to inhibit particular export pathways.

## Results

### *Design, uptake, and localization of peptide inhibitors of exportin1*

The nuclear export receptor exportin1 mediates export from the nucleus of various proteins and of RNAs bound to proteins, including unspliced and partially spliced human immunodeficiency virus RNAs bound to the viral Rev protein, U snRNAs, and 40S and 60S ribosomal subunits (see Cullen, 2003; Mattaj and Englmeier, 1998; Pollard and Malim, 1998; Weis, 2002 for reviews). In the presence of Ran-GTP, exportin1 binds cargo proteins via recognition of a short, leucine-rich nuclear export signal (NES). Peptides containing such a sequence specifically block exportin1-dependent export when microinjected into Xenopus oocytes (Fischer et al., 1995; Pasquinelli et al., 1997) and when translocated into human cells in culture via attachment to a cell-permeable peptide (Gallouzi and Steitz, 2001). The latter corresponded to residues 43–58 (the third helix) of the *Drosophila* antennapedia protein, which had previously been shown to enter living cells at high efficiency, even when covalently linked to hydrophilic moieties (reviewed in Derossi et al., 1998). We therefore designed an analogous peptide inhibitor of exportin1 that comprised the 16 amino acid antennapedia sequence described above, which has been termed penetratin-1 (Derossi et al., 1994), linked to the NES of the HIV-I Rev protein. A second, control peptide contained an altered NES carrying substitutions of leucine residues known to be essential for function of the export signal (Malim et al., 1989, 1991). As illustrated in Fig. 1, the penetratin-1 and NES sequences were linked via four amino acids with small side chains, and both peptides were biotinylated at their N-termini to allow detection within cells. Modification at this position was chosen, because it has been established that a free N-terminus of the penetratin-1 peptide is not required for efficient translocation across the plasma membrane (Derossi et al., 1998). The modified peptides were synthesized, purified, and characterized by mass spectroscopy as described in Materials and methods.

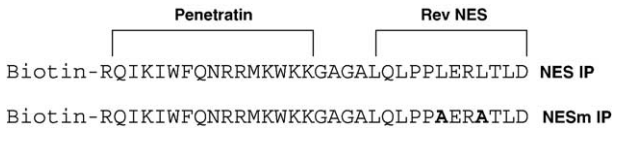


Fig. 1. Sequences of the NES and NESm peptides. The penetratin-1 (see text) and NES segments of the NES and NESm peptides are indicated on the primary sequences. The NES corresponds to that of the HIV-1 Rev protein, amino acids 73–84 (Elfgang et al., 1999; Fischer et al., 1995; Fukuda et al., 1997; Pasquinelli et al., 1997). The two NESm alanine substitutions of leucine residues that are essential for the function of the Rev NES (Malim et al., 1989, 1991) are shown in bold face.

We first assessed the entry into HeLa cells of the two peptides, and their intracellular locations. Uninfected cells were incubated in medium containing either the NES or the NESm peptide (Fig. 1) for 2–4 h at 37 °C. They were then prepared for immunofluorescence and stained with FITC-avidin as described in Materials and methods. Untreated cells exhibited faint cytoplasmic staining (Fig. 2, panels a and e) reflecting their endogenous biotin content. The uptake of both exogenous peptides was readily evident: essentially all cells exposed to 25 or 50 μM of either peptides were strongly stained with FITC-avidin (Fig. 2, panels b–d and f–h). However, the peptides differed in their intracellular locations. Although detectable in the cytoplasm, the NES peptide was concentrated at the nuclear periphery (Fig. 2, panels b and c), the major site of accumulation of exportin1 in mammalian cells, as well as in budding and fission yeasts (Adachi and Yanagida, 1989; Fornerod et al., 1997; Kudo et al., 1997). This peptide was also observed concentrated at discrete, dot-like structures within nuclei (arrows in Fig. 2, panel d), similar in appearance to intranuclear structures reported to contain exportin1 (Fornerod et al., 1997). By contrast, the NESm peptide was not concentrated at either the nuclear rim or within the nucleoplasm, but exhibited diffuse cytoplasmic staining (Fig. 2, panels f–h). These observations suggest that the NES peptide binds exportin1, while the substituted, non-functional derivative does not.

The results shown in Fig. 2 also illustrate the reproducible observation that exposure of cells to either the NES or the NESm peptide, for 4 h in the example shown, induced no cytotoxicity or visible change in cell morphology, in agreement with a previous report (Gallouzi and Steitz, 2001). Nor did entry of either peptide into Ad-5-infected cells alter cell morphology (e.g., Fig. 3A).

### *Inhibition of exportin1-dependent export of the E1B 55 kDa protein by the NES peptide*

We wished to determine whether our NES peptide indeed blocked exportin1-dependent export from the nucleus in Ad5-infected cells. We therefore examined the effects of the peptide on transport of the viral E1B 55 kDa protein from the nucleus, a process previously established to be mediated by exportin1 (Dosch et al., 2001; Kratzer et al., 2000), initially using a modification of the classic heterokaryon shuttling assay (Piñol-Roma and Dreyfuss, 1992). In this approach, HeLa cells were infected with Ad5 and plated with an equal number of uninfected HeLa cells. At 12–14 h after infection, infected and uninfected cells were fused as described in Materials and methods. They were then

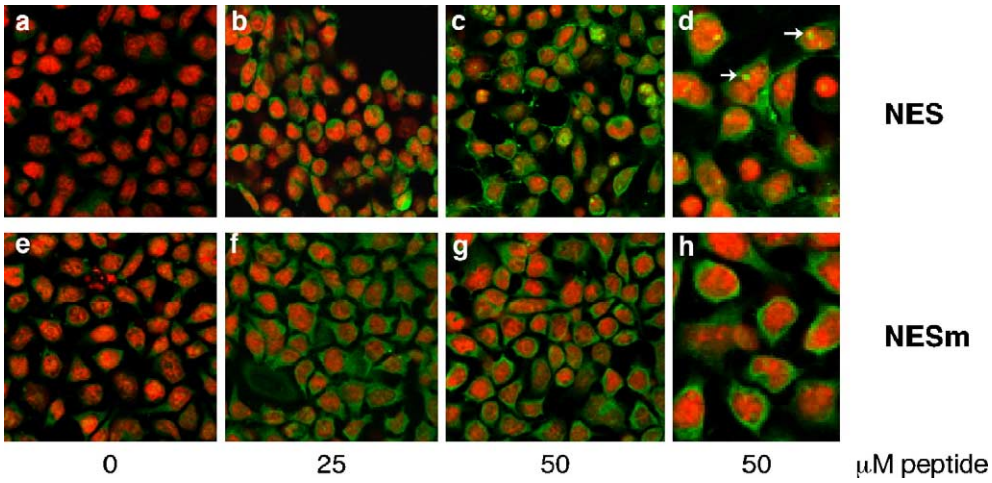


Fig. 2. Localization of the NES and NESm peptides in HeLa cells. HeLa cells were incubated with the concentrations of the NES or NESm peptides indicated for 4 h at 37 °C. The peptides were labeled with FITC-avidin (green) and their locations then examined by immunofluorescence, as described in Materials and methods. Nuclei were stained with DAP1 (red). Panels d and h show higher magnification views of parts of the fields shown in panels c and g, respectively. The white arrows in panel d indicate nucleoplasmic dots containing the NES peptide.

incubated in medium containing cycloheximide to block further protein synthesis, in the absence or presence of the NES or control NESm peptides for 3–4 h at 37 °C. Infected cells were then distinguished from their uninfected siblings by virtue of the formation within them of characteristic replication centers that contain the viral 72-kDa, single-stranded DNA-binding protein (DBP) (Puvion-Dutilleul et al., 1994; Reich et al., 1983; Sugawara et al., 1977; Volderking and Klessig, 1986). The formation of such structures, which appear as discrete dots or rings (Fig. 3A, panels b, f, and j), depends on viral DNA synthesis (Volderking and Klessig, 1986) and is restricted to adenovirus-infected cells. Shuttling of the E1B 55 kDa protein from infected to uninfected cell nuclei was therefore examined by simultaneous labeling of this protein and the DBP in syncytia formed as described above.

In infected cells identified by the presence of replication centers, a significant fraction of the E1B protein was present at these sites, colocalized with the DBP (Fig 3A, panels d, h, and l), as reported previously (Gonzalez and Flint, 2002; Ornelles and Shenk, 1991). In the absence of any peptide, the E1B protein was readily detected in uninfected (i.e., DBP-lacking) nuclei present in syncytia with infected cell nuclei (Fig. 3A, panels a–d), indicating that this viral protein was exported from infected cell nuclei to the syncytial cytoplasm prior to entry into uninfected cell nuclei. Incubation of fused cells with the NES peptide significantly inhibited shuttling of the E1B 55 kDa protein (Fig. 3A, panels e–h), whereas the substituted NESm peptide had little or no effect (Fig. 3A, panels i–l). The results of quantification of several independent experiments like that illustrated in Fig. 3A are summarized in Fig. 3B. The NES peptide reduced the shuttling activity of the E1B 55 kDa protein by 60%, whereas the substituted derivative was not inhibitory. These data established that the NES peptide specifically blocked exportin1-dependent export of the E1B 55 kDa from Ad5-infected cell nuclei. However, the inhibitory effect of this peptide was underestimated in these experiments: to minimize subjectivity associated with assessing nuclear E1B protein concentrations by immunofluorescence, only syncytial, uninfected cell nuclei in which E1B protein staining was no greater than the background observed in non-syncytial, uninfected cells were counted as negative. We therefore examined the effects of the peptides on the cytoplasmic concentrations of the E1B protein by immunoblotting.

HeLa cells infected with Ad5 were incubated from 13 to 15.5 h after infection with 50 μM NES or NESm peptide in the presence of cycloheximide. Cytoplasmic fractions isolated by extraction of cells with NP40 under conditions that do not result in leakage of nuclear material (Yang et al., 1996) contained no more than 10% of the total E1B 55 kDa protein (data not shown), in agreement with previous observations (Ornelles and Shenk, 1991; Smiley et al., 1990). Exposure of the nuclear pellet to stronger detergents, such as 1% (v/v) Tween 40 plus 0.5% (w/v) sodium deoxycholate, released about the same quantity of additional E1B protein, again as reported previously (Smiley et al., 1990). However, treatment with these additional detergents led to leakage of nuclear contents, as judged by recovery of the viral 72 kDa DBP, which is exclusively nuclear in location (e.g., Fig. 3A; Reich et al., 1983; Volderking and Klessig, 1986) in the Tween 40/sodium deoxycholate fraction (data not shown). We therefore compared the concentrations of the E1B 55 kDa protein in cytoplasmic fractions isolated using only NP40 by immunoblotting. Despite a low concentration of E1B 55 kDa protein in this fraction of untreated, Ad5-infected cells, a substantial decrease in concentration induced specifically by the NES peptide was readily evident (Fig. 4). Indeed, this peptide inhibited nuclear export of the E1B 55 kDa protein by close to 85%, as judged by quantification of immunoblot signals

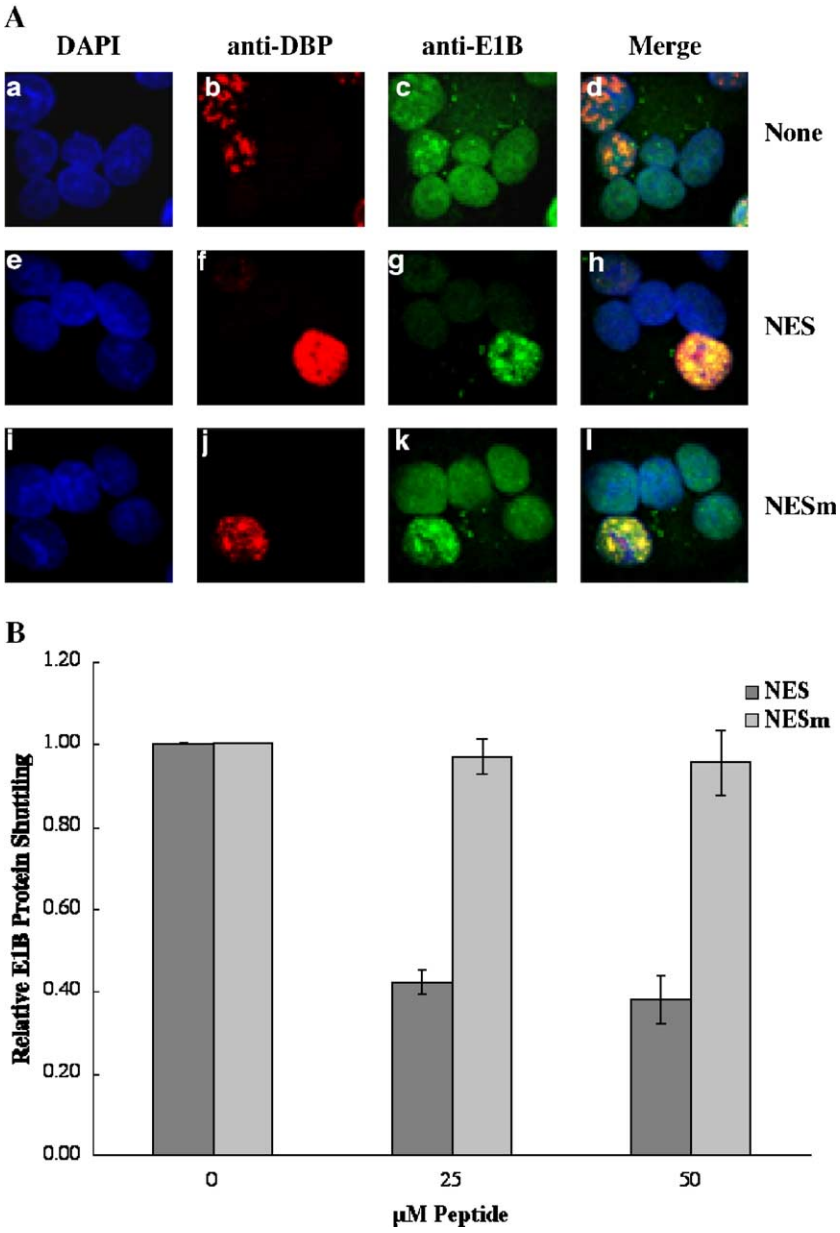


Fig. 3. Specific inhibition of E1B 55 kDa protein shuttling by the NES peptide. (A) HeLa cells were infected with 10 p.f.u./cell Ad5 and plated with equal number of uninfected HeLa cells after adsorption of the virus. Cells were fused at 14 h p.i. and incubated with 100 μg/ml cycloheximide and the peptide indicated (50 μM final concentrations) for 3 h at 37 °C. The viral 72 kDa and E1B 55 kDa proteins were examined by immunofluorescence as described in Materials and methods. (B) The number of uninfected cell nuclei present in syncytia with Ad5-infected cell nuclei and the number within this population that stained positive for the E1B 55 kDa protein (staining significantly above the background seen in non-syncytial, uninfected cells) were determined from data collected in three independent experiments like that illustrated in panel A. At least 25 syncytial, uninfected cell nuclei were examined under each condition in each experiment. The percentages of E1B protein-positive, uninfected nuclei present in the syncytia were then calculated. These values, which ranged from 40% to 58% for cells that had not been exposed to peptide, are expressed relative to that exhibited in each experiment by syncytial cells in the absence of either peptide.

using human β-actin as an internal control (Fig. 4). Cycloheximide, which does not inhibit protein synthesis completely (see Lewis and Mathews, 1980), was added to culture medium at the same time as the peptides. It is therefore likely that the small quantity of the E1B 55 kDa protein detected in the cytoplasmic fraction of cells exposed to the NES peptide represents newly-synthesized molecules that have not yet entered the nucleus.

### *Exportin1 is not required for efficient export of viral late mRNAs*

We next compared the export of viral late mRNAs from the nucleus to the cytoplasm in untreated Ad5-infected cells, in Ad5-infected cells incubated with the NES or NESm peptides, and in cells infected with the E1B 55 kDa protein null virus Hr6 (Ho et al., 1982; Williams et al.,

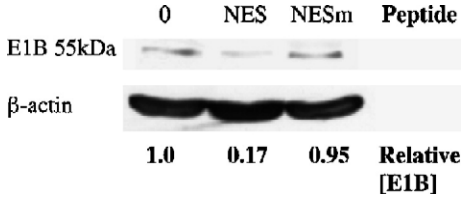


Fig. 4. Specific inhibition of export of the E1B 55 kDa protein from the nucleus by the NES peptide. Cytoplasmic proteins recovered from equal numbers of Ad5-infected cells that were untreated or incubated with 50 μM NES or NESm peptides for 2.5 h were examined by sequential immunoblotting with mouse monoclonal antibodies against the viral E1B 55 kDa protein and β-actin, as described in Materials and methods. The results of quantification of E1B protein signals using β-actin as the internal control in two experiments are summarized at the bottom of the figure. The standard deviations for the E1B protein concentrations in NES and NESm peptide-treated extracts relative to that observed in the untreated control were 0.01 and 0.02, respectively.

1986). Peptides were added soon after entry into the late phase of infection, so that any effect of export of viral late mRNAs could be readily detected. The concentrations of cytoplasmic and nuclear penton and fiber mRNAs were determined by using two-step RT-PCR. Reverse transcription was first carried out with primers specific for mature mRNAs: these primers were complementary to the junctions between the third exon of the major late (ML) tripartite leader sequence and the bodies of the viral mRNAs. Relative cDNA, and hence mRNA, concentrations were then measured by using real-time PCR to amplify a sequence within the tripartite leader common to all ML mRNAs, as described in Materials and methods. Human β-actin mRNA was reverse transcribed and amplified in the same reactions to provide an internal control. No amplification of either the ML or the β-actin sequences was observed when reverse transcriptase was omitted from primer extension reactions (data not shown), establishing that these assays detect only RNA.

Inhibition of exportin1 alters the nuclear to cytoplasmic trafficking of many cellular proteins and of cellular RNAs (see Introduction), and might therefore indirectly impair synthesis or processing of adenoviral late mRNAs. We therefore first examined the effects of the NES or NESm peptides on the nuclear concentrations of penton and fiber mRNAs. Under the conditions used in these experiments, neither peptide significantly altered the nuclear concentrations of these viral late RNAs (Fig. 5A), indicating that the reactions by which viral late mRNAs are synthesized were not perturbed by the peptides. We next determined the ratios of the steady concentrations of cytoplasmic and nuclear viral mRNAs to a measure of export efficiency: as discussed previously (Gonzalez and Flint, 2002), this parameter provides a direct assay for mRNA export. As expected (see Introduction), export of both the L2 penton and the L5 fiber mRNA was impaired in Hr6- compared to Ad5-infected cells (Fig. 5B). The degrees of inhibition induced by the Hr6 mutation observed in these experiments were similar to values we have obtained previously using different assays (Gonzalez and Flint, 2002; Yang et al., 1996). By contrast, incubation of Ad5-infected cells with the NES or the NESm peptide did not induce significant changes in the cytoplasmic to nuclear ratios of either viral late mRNA (Fig. 5B). We can therefore conclude from the results summarized in Fig. 5 that inhibition of exportin1-dependent export by the NES peptide did not reduce the efficiency of nuclear export of viral late mRNAs.

## Discussion

The studies reported here establish the feasibility of using peptide inhibitors to investigate the roles of cellular export pathways in the adenoviral infectious cycle. Using an NES-containing inhibitor of exportin1, we have confirmed that exportin1 mediates nucleocytoplasmic shutting of the Ad5 E1B 55 kDa protein (Dosch et al., 2001; Kratzer et al., 2000) and shown that the NES peptide inhibits nuclear export of the E1B protein to a high degree, if not completely (Fig. 4). Nevertheless, under the same condition, the NES peptide had no effect whatsoever on export of viral late mRNAs to the cytoplasm (Fig. 5). This result confirms the finding based on use of leptomycin B and an indirect measure of late mRNA export that exportin1 is not the export receptor for viral late mRNAs (Carter et al., 2003; Rabino et al., 2000). The observation that export of cellular U snRNAs and ribosomal RNAs, which have been reported to leave the nucleus using the exportin1 receptor (reviewed in Cullen, 2003; Weis, 2002), is not perturbed during the late phase of infection (Castiglia and Flint, 1983; Smiley et al., 1990) is consistent with this conclusion. It therefore appears that the previously reported dependence of accumulation of viral late mRNAs in the cytoplasm on the NES of the E4 Orf6 protein (Weigel and Dobbelstein, 2000) represents a secondary consequence of defects in one or more earlier steps in the infectious cycle. Indeed, both a delay in viral DNA synthesis and reduced nuclear accumulation of late mRNAs were observed when this NES was altered (Weigel and Dobbelstein, 2000).

As translocation of the E1B 55 kDa protein into the cytoplasm by exportin1 makes no contribution to selective export of viral late mRNAs, such nuclear export is presumably required for some other function(s) of this adenoviral early protein. One possibility is regulation of the activity and/or concentration of the cellular p53 protein. Indeed, Dobner and colleagues have recently reported that substitutions of the leucine residues of the E1B NES with alanines improved both inhibition of p53-dependent transcription and transformation of baby rat kidney cells by the E1B protein (Endter et al., 2005). In conjunction with the viral E4 Orf6 protein, the E1B 55 kDa protein also induces accelerated turnover of the p53 protein (Nevels et al., 1999; Querido et al., 1997; Roth et

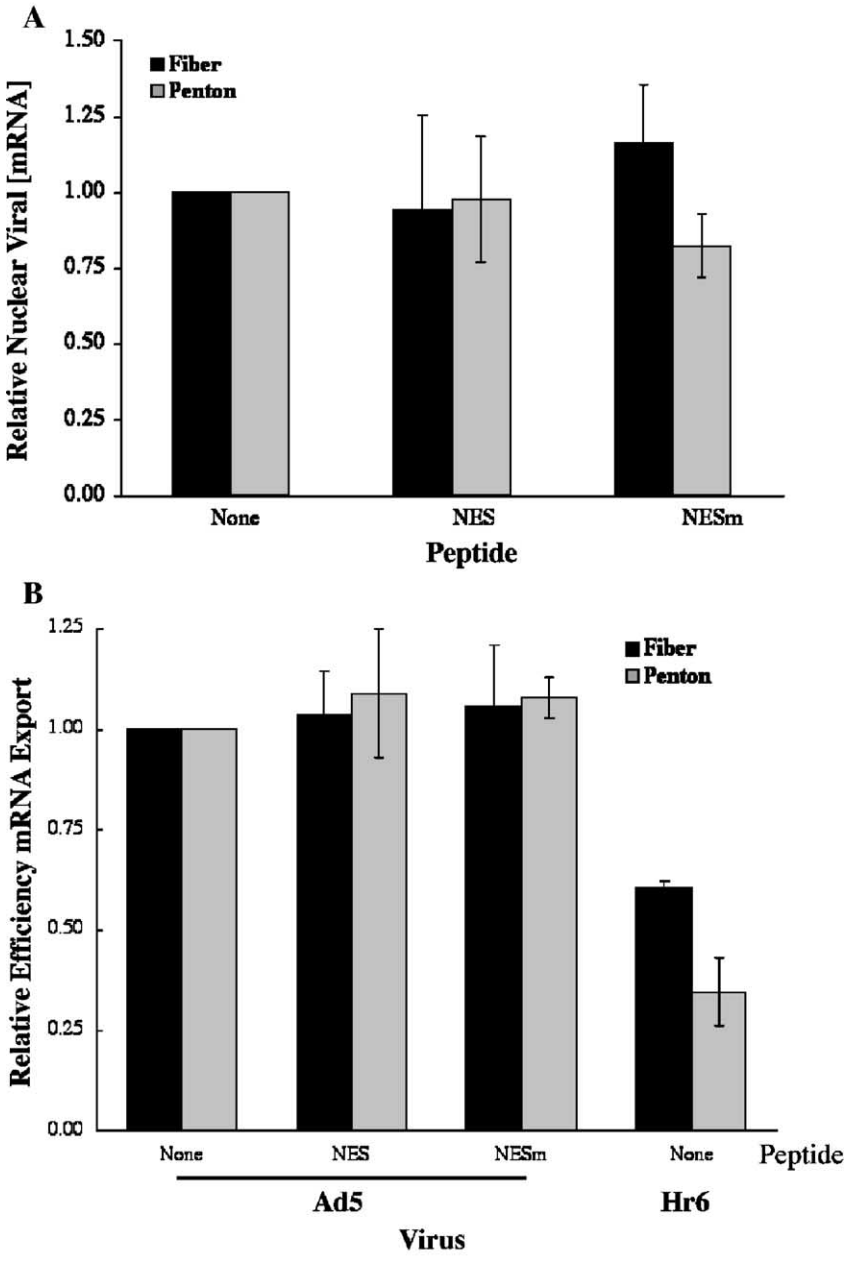


Fig. 5. The NES peptide does not alter the production or the efficiency of export of viral late mRNAs. HeLa cells were infected with 10 p.f.u./cell Ad5 or Hr6. At 14 h p.i., the NES or NESm peptides (50 μM final concentrations) were added to sets of Ad5-infected cell monolayers and incubation at 37 °C continued for 4 h. The cells were harvested and nuclear and cytoplasmic RNA fractions purified as described previously (Gonzalez and Flint, 2002). The concentrations of processed L2 penton and L5 fiber mRNAs present in cytoplasmic and nuclear fractions recovered from an equal number of cells were then determined by reverse transcription and quantitative real-time PCR, as described in Materials and methods. The values obtained were corrected for any variations in cell number or RNA recovery using the β-actin internal control. The relative concentrations of nuclear RNAs (panel A) and the ratios of cytoplasmic: nuclear late mRNAs (panel B) were then calculated. The latter are expressed relative to those determined for Ad5-infected cells that were not exposed to peptide. The values shown represent the results of 3 independent experiments, in each of which the mRNA concentrations were measured using at least two reverse transcription reactions.

al., 1998; Steegenga et al., 1998). The mechanism by which p53 degradation is induced is not fully understood, but seems likely to depend on association of the viral proteins with p53 and cellular proteins such as cullin 5, Tbx1/Roc1/Hrt, and elongins B and C in a complex that directs addition of polyubiquitin to p53 (Harada et al., 2002; Querido et al., 2001). The cellular Hdm-2 protein (Mdm-2 in mice) is a critical regulator of the concentration of p53 in uninfected cells. This protein binds to p53 and ubiquinates both the tumor suppressor and itself, targeting both proteins for degradation by the proteosome (reviewed in Lakin and Jackson, 1999; Oren, 1999; Prives, 1998; Ryan et al., 2001). Efficient degradation of p53 depends on export from the nucleus (Dobbelstein et al., 1997; Freed-

man and Levine, 1999; Tao and Levine, 1999), a process that in turn requires the E3 ubiquitin ligase activity of the Hdm-2 protein (Boyd et al., 2000; Geyer et al., 2000). The p53 tetramer is not efficiently exported from the nucleus unless C-terminal lysine residues are ubiquinated, a modification that is believed to expose export signals (Stommel et al., 1999). Signals that result in stabilization and activation of p53, such as those induced by DNA damage, result in phosphorylation of p53 at specific N-terminal Ser residues. These modifications inhibit interaction with Hdm-2 and render p53 temporarily refractory to export and degradation. It is therefore possible that in Ad5-infected cells, the E1B 55 kDa-E4 Orf6 protein-containing complex effects export and degradation of Hdm2-refractory p53 protein that accumulates in response to synthesis of viral E1A proteins (reviewed in Braithwaite and Russell, 2001). The E1B 55 kDa protein has also been implicated in induction of degradation of components of the cellular double-stranded break repair system (Stracker et al., 2002) and in relocalization of p53 and the Wt1 tumor suppressor proteins to peripheral nuclear bodies in transformed cells (Blair-Zajdel and Blair, 1988; Maheswaran et al., 1998; Zantema et al., 1985). It should be possible to use peptide inhibitors like that described here to evaluate the contribution of exportin1-dependent export to these activities of the E1B 55 kDa protein.

The failure of the NES peptide (Fig. 5) as well as of leptomycin B (Carter et al., 2003; Rabino et al., 2000) to block export of viral late mRNAs from the nucleus indicates that the E1B 55 kDa protein does not function as an NES-containing adaptor for RNA export, analogous to the HIV Rev protein (see Introduction). These observations also rule out late mRNA export by a second route, that in which the HuR RNA-binding protein is directed to exportin1 via the HuR ligands and NES-containing proteins pp32 and April: this pathway is also blocked by NES peptides (Gallouzi and Steitz, 2001). However, HuR can also direct cellular mRNAs synthesized from early response genes to a second export receptor, transportin-2 (Gallouzi and Steitz, 2001). This export mechanism depends on recognition of the amino acid sequence required for nucleocytoplasmic shuttling of the HuR protein (Fan and Steitz, 1998), to which transportin-2 appears to bind directly (Gallouzi and Steitz, 2001). Whether HuR and transportin-2 are responsible for export of adenoviral late mRNAs has not been tested. As noted in the Introduction, the E1B 55 kDa protein may interact with the Tap export receptor via the E1B-Ap5 protein (Bachi et al., 2000; Gabler et al., 1998). The Tap protein binds to FG repeat-containing nucleoporins, such as Nup214/Can (Bachi et al., 2000; Sugawara et al., 1977; Wiegand et al., 2002). This interaction is facilitated by the p15 (NFX2) component of the Tap-p15 heterodimer and required for mRNA export (Bachi et al., 2000; Fribourg et al., 2001; Kang and Cullen, 1999; Katahira et al., 1999). Although Tap binds directly to the constitutive transport element of unspliced RNAs of some simple retroviruses (Grüter et al., 1998; Kang and Cullen, 1999), recognition of cellular mRNAs depends on adaptors such as Ref/Aly, UAP56, and certain SR splicing proteins (reviewed in Cullen, 2003; Stutz and Izaurralde, 2003; Vinciguerra and Stutz, 2004). It is therefore possible that the E1B 55 kDa protein, which contains an RNP motif that mediates binding to RNA in vitro (Horridge and Leppard, 1998), and/or the E1B-Ap5 protein, function as export adaptors for Tap-dependent export of viral, late mRNAs. If Tap represents the major mRNA export receptor in mammalian cells, as it does in simpler eukaryotes (reviewed in Stutz and Izaurralde, 2003), hijacking of Tap for export of viral late mRNAs would account for the inhibition of export of cellular mRNAs during the late phase of infection. Experiments are therefore in progress to identify peptides that specifically inhibit the transportin-2 and Tap export receptors.

## Materials and methods

### *Cells and virus*

HeLa cells were maintained in monolayer culture in DMEM (Gibco-Invitrogen Corp.) 5% (v/v) fetal bovine serum and 5% (v/v) calf serum (Gibco-Invitrogen Corp.). Adenovirus type 5 was propagated in HeLa cells and titered by plaque assay on the same cells as described previously (Flint et al., 1975). Uninfected or Ad5-infected cells were exposed to peptides added to the medium to final concentrations of 25 or 50 μM for 2–4 h at 37 °C.

### *Synthesis of biotinylated peptides*

Peptides were synthesized on a model 433A Peptide Synthesizer (Applied Biosystems) using FMOC Chemistry. After the last FMOC protecting group had been removed on the instrument, the peptide-resin with side-chain fully protected was reacted off-line with biotin (Anaspec) manually overnight. The biotinylated peptides were then cleaved from the resin and deprotected with TFA. They were purified using a Luna C18 preparative column (Phenomenex) with an 0.1% TFA buffer system on a reverse phase HPLC system (Agilent Technologies) The purified, biotinylated peptides were analyzed on a LC/MS system (A1100/MSD-Agilent Technologies) to confirm the presence of biotin. They were dissolved in 0.01 M Hepes-KOH, pH 7.9 containing 0.01 M NaCl and 10% (v/v) dimethyl sulfoxide and stored in small portions at −20 °C.

### *Immunofluorescence*

HeLa cells were seeded onto glass cover slips in the wells of 6-well tissue culture dishes. When 80–90% confluent, they were infected with 10 p.f.u./cell Ad5. Uninfected and infected cells were fixed with 4% (v/v) paraformaldehyde in phosphate-buffered saline (PBS,

Gibco-Invitrogen Corp.), permeabilized with 0.5% (v/v) Triton X-100 in the same buffer, and blocked as described by Ornelles and Shenk (1991). Biotin-labeled peptides and the viral E1B 55 kDa protein were detected using FITC-avidin (Santa Cruz Biotechnology) and monoclonal antibody 2A6 (Sarnow et al., 1984) IgG labeled with AlexaFlour 488 (Molecular Probes), respectively. Approximately 1 mg purified 2A6 IgG was stirred for 1 h at room temperature with AlexaFlour 488 reactive dye in 0.10 M sodium bicarbonate. The labeled antibody and unreacted dye were then separated by gel filtration chromatography in 0.01 M potassium phosphate, pH 7.2, containing 0.15 M NaCl and 0.2 M sodium azide. The degree of labeling, in this case 3.3 mol dye/mol protein, was determined according to the manufacturer's protocol. The viral 72-kDa single-stranded DNA-binding protein was examined by indirect staining using a monoclonal antibody against the DBP (B6, Reich et al., 1983) and Cy5-labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.). After incubation with these reagents, cells were stained with DAPI, washed with PBS, and mounted as described previously (Gonzalez and Flint, 2002). They were examined using a Zeiss LSM 510 confocal microscope with a C-Apochromat 40 × 1.2 NA water immersion objective.

*Analysis of E1B 55 kDa protein shuttling*

HeLa cells were infected with 10 p.f.u./cell Ad5. After adsorption of virus particles for 1 h at 37 °C, the inoculum was removed and the cells washed twice with PBS and removed from the dish by trypsinization. The infected cells were mixed with an equal number of uninfected HeLa cells and plated on cover slips as described above. At 12–14 h after infection, they were fused by exposure for 2 min to 50% polyethylene glycol (Gibco-Invitrogen Corp.) They were then incubated in medium containing 100 μg/ml cycloheximide and no peptide, or the NES or NESm peptides (Fig. 1) for 2–4 h at 37 °C. The locations of the viral E1B 55 kDa and the viral DBP were then examined by immunofluorescence as described in the previous section.

*Immunoblotting*

Cytoplasmic and nuclear fractions of Ad5-infected cells were prepared by two extractions with 0.01 M Tris–HCl, pH 7.5, containing 0.15 M NaCl, 1 mM EDTA, and 0.065% (v/v) NP40, as described previously (Gonzalez and Flint, 2002). The proteins present in cytoplasmic extracts obtained from equal numbers of infected cells that were untreated or exposed to 50 μM NES or NESm peptides were separated by electrophoresis in 10% polyacrylamide–SDS gels. They were then transferred to 0.2 μ nitrocellulose membranes (Schleicher and Schuell) using the semi-dry method. The E1B-55 kDa and human β-actin proteins were detected using the 2A6 monoclonal antibody described above and mouse monoclonal antibody AC-15 (abcam), respectively, and chemiluminescence (ECL plus, Amersham Biosciences). Signals were quantified using NIH Image software.

*Export of viral late mRNA*

Export efficiencies were determined as the ratios of cytoplasmic to nuclear concentrations of the viral L2 penton and L5 fiber mRNAs (Gonzalez and Flint, 2002). These concentrations were measured using real-time RT-PCR. Reverse transcription was performed manually using primers complementary to the splice junctions between the ML tripartite leader sequence and the bodies of L2 penton or L4 fiber mRNAs. Both primers were complementary to 11 bases upstream of the splice sites and the penton and fiber primers were complementary to 12 and 14 bases, respectively, downstream of the splice junctions. Reactions were as described previously (Gonzalez and Flint, 2002), and contained 5–10 μg RNA, 15 pmol of ML penton or fiber primers, and 4.5 pmol of the reverse primer of a human β-actin amplicon (Applied Biosystems). Multiplex, real-time PCR was carried out using the ABI Prism 7700 sequence detections system and TaqMan (Applied Biosystems) probes of an amplicon within the ML tripartite leader sequence designed using Primer Express software (Applied Biosystems) and the human β-actin amplicon mentioned above, labeled with VIC and FAM, respectively. The ML amplicon comprised a forward primer corresponding to positions 69–90 of the tripartite leader sequence, a reverse primer complementary to positions 133–150, and a probe corresponding to positions 94–114. Relative RNA concentrations were determined by the standard curve method.

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