Nuclear export of the E1B 55-kDa and E4 34-kDa adenoviral oncoproteins mediated by a rev-like signal sequence

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The E1B 55-kDa and E4 34-kDa oncoproteins of adenovirus type 5 (abbreviated here as E1B-55kD and E4-34kD) promote the export of viral mRNA and inhibit the export of most cellular mRNA species. We show that the intracellular complex containing E1B-55kD and E4-34kD continuously shuttles between the nucleus and the cytoplasm, and may thus serve as a nucleocytoplasmic transporter for viral mRNA. We present evidence that within this complex, it is the E4-34kD protein that directs both nuclear import and nuclear export. E4-34kD contains a functional nuclear export signal similar to corresponding sequences found in the retroviral proteins rev and rex. This sequence element is required for nuclear export of the complex, and it can function autonomously when fused to a carrier protein and microinjected in HeLa cell nuclei. When E4-34kD is expressed alone, a portion of the protein that contains a predicted arginine-rich amphipathic α-helical structure mediates nuclear retention of the protein. This retention, however, can be abolished by the association with E1B-55kD or by a specific point mutation within the arginine-rich motif. The export of E4-34kD can be blocked by an HTLV-1 rex-derived competitive inhibitor and overexpressed E4-34kD inhibits rev-mediated transport, suggesting that the export pathways accessed by the adenoviral and retroviral proteins share components. The interplay between two polypeptides as well as the involvement of a dominant nuclear retention domain are novel features that might contribute to the efficiency and regulation of the adenovirus export system.

Keywords: adenovirus/HIV/mRNA export/nucleocytoplasmic transport/viral oncogenes

Introduction

The export of RNA from the nucleus to the cytoplasm appears to involve distinct pathways for different classes of RNA (Jarmolowski et al., 1994). Some of these pathways are utilized by viruses to allow efficient nucleocytoplasmic transport of viral RNA. The human retroviruses HIV and HTLV encode the gene products rev and rex that mediate the export of their genomic RNA. These proteins interact with specific viral RNA elements, and the complex of RNA and protein is carried from the nucleus to the cytoplasm. This transport is mediated by a short sequence termed the nuclear export signal (NES) (Simos and Hurt, 1995; Gorlich and Mattaj, 1996). The NES from the HIV rev protein has been shown to mediate transport on a pathway overlapping at least in part with that supporting the export of 5S ribosomal RNA but not mRNA (Fischer et al., 1994; Fischer et al., 1995). The mechanism of this export is currently under intense investigation, and several candidate protein-transport mediators have been identified (Bogerd et al., 1995; Fritz et al., 1995; Stutz et al., 1995; Bevec et al., 1996).

During the late phase of infection with adenovirus type 5, the nuclear export of most cellular mRNA species is inhibited while viral mRNA accumulates efficiently in the cytoplasm (Beltz and Flint, 1979; Babiss et al., 1985; Pilder et al., 1986). The effect depends on two viral gene products: The E1B 55-kDa protein (E1B-55kD) and the E4 34-kDa protein (E4-34kD, or E4 orf 6) (Babiss et al., 1985; Halbert et al., 1985; Pilder et al., 1986; Shenk, 1996). The two adenoviral proteins seem to associate not only within infected cells (Sarnow et al., 1984), but also when transiently expressed (Goodrum et al., 1996). E1B-55kD localizes mainly in the cytoplasm when expressed alone, whereas E4-34kD localizes in the nucleus. However, when coexpressed, both proteins are found predominantly in the nucleus, indicating that E4-34kD redirects the location of E1B-55kD, presumably by complex formation. Within the infected cell nucleus, the E4-34kD protein directs the E1B-55kD protein to the periphery of the viral replication/transcription centers during the late phase of infection (Ornelles and Shenk, 1991). It is not known how these proteins affect the export of both viral and cellular mRNA, but it has been suggested that the complex containing E1B-55kD and E4-34kD proteins binds a cellular factor required for mRNA export and relocates it to the vicinity of viral replication/transcription centers, limiting the availability of this factor largely to viral mRNA (Ornelles and Shenk, 1991).

Both E1B-55kD (Sarnow et al., 1982; Yew and Berk, 1992; Yew et al., 1994) and E4-34kD (Dobner et al., 1996) bind and inactivate the tumor suppressor gene product p53 and contribute to the transformation of cells (Moore et al., 1996). To date, it is unclear why the same proteins are employed by the virus to modulate mRNA export and cell proliferation, and whether there is a link between these two activities.

We now show that the complex of E1B-55kD and E4-34kD protein shuttles between the nucleus and cytoplasm. The export is mediated at least in part by a nuclear export...
signal on the E4-34kD protein. In addition, E4-34kD contains a previously undescribed domain that mediates nuclear retention of the protein. E1B-55kD abolishes this retention and allows nuclear export of the complex. The adenoviral export pathway overlaps, at least in part, with the pathway mediating the export of HIV rev and HTLV rex proteins. As predicted from this overlap, overexpressed E4-34kD is an inhibitor of rev activity.

Results

The complex containing E1B-55kD and E4-34kD shuttles between the nucleus and the cytoplasm

Since the E1B-55kD and E4-34kD proteins from adenovirus type 5 modulate the export of RNA, we asked whether the complex containing both proteins shuttles between the nucleus and the cytoplasm. When both proteins were transiently expressed in HeLa cells, virtually all E4-34kD and the vast majority of E1B-55kD were found in the nucleus (data not shown), consistent with previous results (Goodrum et al., 1996). To assay for nucleocytoplasmic shuttling, the transfected HeLa cells were fused to murine Balb-c 3T3 cells using polyethylene glycol, to produce heterokaryons. Cycloheximide was added 15 min prior to the fusion to prevent protein synthesis. The cells were fixed and stained, 2 h after the fusion, with a monoclonal antibody against E4-34kD. Simultaneously, the cells were stained with an antibody that recognizes human but not murine nuclear Ku-antigen, an autoantigen that is part of the DNA-dependent kinase DNA-PK (Gottlieb and Jackson, 1993), thus distinguishing the nuclei of the different species from each other. Nucleocytoplasmic shuttling was indicated by the detection of E4-34kD in the murine nuclei, as previously shown in this assay for some of the hnRNP proteins (Pinol-Roma and Dreyfuss, 1992). As shown in Figure 1a–c, the E4-34kD protein shuttles between nucleus and cytoplasm when coexpressed with E1B-55kD. The same observation was made when these cells were stained for E1B-55kD instead of E4-34kD (data not shown). Thus, both E1B-55kD and E4-34kD shuttle continuously between nucleus and cytoplasm when coexpressed. In contrast, E4-34kD did not migrate from the human to the murine nuclei in the absence of E1B-55kD (Figure 1d–f), indicating that it is not efficiently exported from nuclei when expressed alone. This was observed in >200 heterokaryons and three independent experiments. Since transiently expressed E1B-55kD is localized in the cytoplasm in the absence of E4-34kD (cf. Figure 3B and Goodrum et al., 1996), export characteristics of the isolated protein could not be assessed in a heterokaryon assay. Therefore, we transiently expressed E1B-55kD fused to a nuclear localization signal (NLS) from SV40 T antigen. The stability of NLS-tagged E1B-55kD was similar to E1B-55kD, as judged by immunoprecipitation from transfected and radiolabeled cells (data not shown). The NLS sequence efficiently directed E1B-55kD to the nucleus, and the protein accumulated in a nucleolar pattern (Figure 1g, and our unpublished results) resembling transiently expressed HIV rev (Meyer and Malim, 1994). However, in contrast to rev, NLS-tagged E1B-55kD was unable to shuttle to the cytoplasm in a heterokaryon assay (Figure 1g–i). When coexpressed with E4-34kD, NLS-tagged E1B 55kD localized in the nucleoplasm, and efficient shuttling could be observed (Figure 1j–l). Hence, the two proteins must be present and presumably form a complex for nuclear export, and neither of the proteins can be efficiently exported when expressed alone.

The E4-34kD protein contains a signal sequence mediating nuclear export

The E4-34kD protein contains a 10 amino acid sequence similar to characteristic arrays of hydrophobic amino acids (Figure 2A) shown previously to be crucial for export and termed nuclear export signal (Gorlich and Mattaj, 1996; Murphy and Wente, 1996). This sequence is 100% conserved between the E4-34kD proteins from types 2 and 5 of adenovirus (not shown). We therefore tested whether this sequence might be involved in the nuclear export of the E1B-55kD–E4-34kD protein complex by mutating two of the hydrophobic amino acids, L90 and I92, to alanine (data not shown). This led to nuclear retention of the protein. E1B-55kD abolishes this retention and allows nuclear export of the complex. The adenoviral export pathway overlaps, at least in part, with the pathway mediating the export of HIV rev and HTLV rex proteins. As predicted from this overlap, overexpressed E4-34kD is an inhibitor of rev activity.
complex formation by E4-34kD and E1B-55kD was apparently unaffected (see below). Like the wild-type E4-34kD protein (Figure 2B, d–f), the mutant form did not shuttle in the absence of E1B-55kD (Figure 2B, j–l). Furthermore, when fused to a carrier protein (glutathione S-transferase, GST), the E4-34kD-derived NES mediated nuclear export after intranuclear microinjection (Figure 2C, a and b), as was previously observed with the rev NES (Meyer et al., 1996). As anticipated, the majority of the protein remained in the nucleus, when the NES sequence was mutated at two of the hydrophobic regions as before (L90A and I29A; Figure 2C, c and d). Taken together, these results indicate that the E4-34kD protein contains an NES that is both necessary and sufficient to mediate efficient nuclear export.

In the absence of E1B-55kD, E4-34kD is retained in the nucleus by a C-terminal domain

If E4-34kD contains a functional NES, why is it unable to shuttle in the absence of E1B-55kD? We tested the hypothesis that E4-34kD might be actively retained in the nucleus. In a search for a domain in the protein that might mediate this retention, our attention was drawn to a sequence within the C-terminus of the protein, containing arginine residues (positions 234–251, cf. Figure 3A, top panel) that can be predicted to locate on the hydrophilic side of an amphipathic α-helical structure (J.S. Orlando and D.A. Ornelles, personal communication). To assess a possible role for this structure in nuclear retention, we mutated one of these arginine residues to glutamic acid. This mutation enabled E4-34kD to shuttle even in the absence of E1B-55kD (Figure 3A, a–c). When the NES mutation was introduced in addition, no shuttling was observed (Figure 3A, d–f), further demonstrating the presence of a functional NES within E4-34kD. Both single mutants, as well as the double mutant, retained the ability to relocalize E1B-55kD, as shown by cotransfection and double immunostaining (Figure 3B). These results show that an amino acid within an amphipathic α-helical domain near the C-terminus of E4-34kD is required for the active retention of the protein in the nucleus. This retention can be overcome by either a point mutation or by the association of E4-34kD with E1B-55kD.

The export pathway for E4-34kD includes components that also mediate the export of HIV rev and HTLV rex

To probe the relationship of the pathways mediating the nuclear export of mRNAs encoded by adenoviruses and complex retroviruses, we coexpressed the E4-34kD protein with an N-terminus-truncated form of HTLV rex fused to the nuclear localization signal of simian virus 40 (SV40) T antigen (Figure 4A). When the rex construct is overexpressed, it can inhibit the nuclear export of RNA by Fig. 2. (A) A sequence element from E4-34kD is compared with known nuclear export signals (NESs). The hydrophobic residues within the NESs believed to be critical for export function are shown in bold. Dots are inserted to equalize the pairing between conserved hydrophobic residues in the different motifs. A mutant version of E4-34kD was created with the sequence shown in the bottom line. Numbers indicate the position of the amino acids within the proteins. The references for the NES elements were: human immunodeficiency virus 1 (HIV-1) rev (Fischer et al., 1995); human T cell lymphotropic virus 1 (HTLV-1) rex (Palmeri and Malim, 1996); visna-maedi virus (VMV) rev (Tiley et al., 1991); equine infectious anemia virus (EIAV) rev (Mancuso et al., 1994); yeast mRNA transport protein Gls1 (Murphy and Wente, 1996); cAMP-dependent protein kinase inhibitor (PKI) (Wen et al., 1995); transcription factor TFIIB (Fridell et al., 1996). (B) Two amino acid changes (L90A and I92A) within the putative E4–NES element inhibit shuttling of the complex containing E1B-55kD and E4-34kD. The cells were transfected to express E4-34kD as wild type (a–f) or mutant (L90A and I92A) either together with E1B-55kD (a–c and g–l) or alone (d–f and j–l), and the heterokaryons were stained for E4-34kD (a, d, g and j) and human Ku (b, e, h and k) and photographed in phase contrast (c, f, i and l). (C) The E4 NES element can direct nuclear export of a carrier protein. The NES-like sequence from E4-34kD was thus assayed for export activity in wild type (a and b) and mutant (L90A and I92A) form (c and d).
Fig. 3. (A) Effect of a mutation within the arginine-rich amphipathic helical domain of E4-34kD (R248E) on nuclear retention. The mutant protein was expressed in HeLa cells and subjected to a heterokaryon assay (a–c). A double mutant of E4-34kD having the NES-mutation (cf. Figure 2A) in addition to R248E was assayed in parallel (d–f). (B) Effect of mutations within E4-34kD on E1B-55kD translocation from the cytoplasm to the nucleus. E1B-55kD was expressed in HeLa cells by transient transfection in the absence (a and b) or presence of E4-34kD expression plasmid (c–j). Either wild type E4-34kD (c and d) or the indicated mutants of it (e–j) were expressed. The cells were fixed 18 h after electroporation, and simultaneously stained for E4-34kD (a, c, e, g and i) and E1B-55kD (b, d, f, h and j) as described in the legend to Figure 1.

We also asked whether overexpressed E4-34kD protein can inhibit rev function. To this end, HeLa cells were cotransfected with a rev expression plasmid (pRSVrev) and a reporter construct that contained the chloramphenicol acetyl transferase (CAT) coding region within an intron that also contains the rev responsive element (RRE) of HIV RNA (pDM138RRE) (Hope et al., 1991). In this assay, export of non-spliced RNA by rev is assayed by an internal reference for normalization. The rev protein efficiently mediated nuclear export of RRE-containing RNA (Figure 5, compare lanes 1 and 8). However, when E4-34kD was coexpressed, CAT expression was reduced in a dose-dependent manner (Figure 5, lanes 2 and 3). In contrast, neither the E1B-55kD expression construct nor the empty expression vector had a detectable effect on CAT expression (Figure 5, lanes 4–6). NLS-rex inhibited rev-dependent CAT expression (Figure 5, lane 7), as predicted from previous results (see above). These results strongly suggest that E4-34kD can inhibit nuclear export mediated by the HIV rev protein.

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Fig. 4. (A) Schematic depiction of fusion proteins that inhibit the rev export pathway. The RNA binding domain of HTLV-1 rex was replaced by the nuclear localization signal (NLS) from SV40 T antigen. This fusion protein still retains the rex nuclear export signal. A point mutation within the NES, M90, renders this fusion protein inactive for export and competition, thus serving as a negative control. (B) Effect of an inhibitor of rev-mediated export on nucleocytoplasmic shuttling of E4-34kD. A mutated form of E4-34kD carrying an amino acid exchange (arginine 248 to glutamic acid) to allow shuttling in the absence of E1B-55kD was coexpressed with NLS-rex (plasmid pCFNrex, a–c), or with an inactive rex fragment carrying a point mutation at amino acid 90 of rex (pCFNrexM90, d–f). Shuttling assays were performed as described in the legend to Figure 1.
Fig. 5. E4-34kD as an inhibitor of rev-mediated RNA export. The ability of the rev-protein to export non-spliced mRNA was assayed by cotransfecting a rev expression plasmid (pBSrev; 0.05 μg) with a reporter plasmid containing the CAT coding sequence within an intron and along with the rev responsive element from genomic HIV RNA (pDM13RRE; 0.5 μg), and the ability of rev to export RNA was quantified by the CAT activity observed. pDM13RRE was transfected into HeLa cells in the absence (lanes 1–8) or presence (lanes 1–7) of pBSrev. Increasing amounts of the E4-34kD expression plasmid were cotransfected (lanes 2 and 3), and so was the E1B-55kD expression plasmid (lanes 4 and 5). As a positive control, a known inhibitor of rev-export was cotransfected (pCFN, lane 7) and compared with the cotransfection of its empty expression vector (pCMVneoBam; 0.5 μg of a luciferase expression construct (pGL3control, Promega) were cotransfected as an internal standard. The ratios of CAT and corresponding luciferase activities are shown (average and standard deviation of at least three independent experiments).

Discussion

Our data show that the adenoviral modulators of mRNA export, E1B-55kD and E4-34kD, shuttle between the nucleus and cytoplasm on a rev-like pathway. The E4-34kD protein (Figure 6A) appears to mediate both import (Goodrum et al., 1996) and export of the complex consisting of E1B-55kD, E4-34kD, and possibly cellular factors (Figure 6B). The export is mediated by an NES, and competition experiments (Figures 4 and 5) indicate that the E4-34kD NES interacts with the same cellular factors as the rev and rex proteins from human retroviruses. This competition leads us to infer that the adenovirus and retrovirus mRNA transport systems operate through the same or overlapping transport pathways. It is intriguing to note that in Xenopus oocytes peptides comprising the rev NES inhibit the nucleocytoplasmic transport of SS ribosomal RNA and spliceosomal U1 small nuclear RNAs (snRNAs) but not mRNA, tRNA or ribosomal subunit RNAs (Fischer et al., 1995). This result has been interpreted to suggest that rev-mediated viral mRNA transport utilizes a SS RNA/snRNA pathway rather than a cellular mRNA pathway, and the same may be true of the adenovirus mRNA transport system. Consistent with this proposal, 5S rRNA and U1 snRNA have been shown to continue to accumulate in the cytoplasm of adenovirus-infected cells where mRNA transport is blocked (Smiley et al., 1995). However, it remains possible that 5S RNA, snRNA and cellular mRNAs utilize the same pathway; the competition experiments might detect differences in the affinity of various NES motifs for an RNA transport machine that carries all classes of transported RNA to the cytoplasm. If this latter proposal is correct and the E4 NES exhibits a relatively high affinity for components of the growing family of naturally occurring NES motifs (Figure 2A) and the functional NES elements derived from an in vivo randomization-selection assay (Bogerd et al., 1996), a relatively broad range of sequences can mediate the export function when placed in a suitable context. The rev NES has been reported to interact with several different cellular factors, including the protein termed Rab (Bogerd et al., 1995) or hRIP (Fritz et al., 1995). The similarity in the E4 and rev NES motifs (Figure 2A) and the ability of the two proteins to compete for one or more limiting cellular factors (Figure 4 and 5)
suggest that rev-binding factors such as Rab/hRIP also interact with the adenovirus E4-34kD protein.

E4-34kD does not shuttle when it is expressed alone in the nucleus of HeLa cells (Figure 1). This is in contrast with a previous report suggesting that E4-34kD alone is a shuttling protein (Goodrum et al., 1996). The apparent discrepancy can be explained by the assumption that the nuclear retention is specific for primate cells. In our experiments, the viral proteins were expressed in human cells that were fused to rodent cells. In the previous report, E4-34kD was transiently expressed in rodent cells before cellular fusion and movement from rodent to human nuclei. The protein not only shuttled but also accumulated in the human nuclei to an extent exceeding the amount found in the rodent nuclei. This is consistent with the hypothesis that E4-34kD is retained in primate but not rodent nuclei. The mechanism of nuclear retention is currently not understood. Our results indicate that the arginine-containing amphipathic α helix within E4-34kD is important for nuclear retention (Figure 3). Perhaps the motif can interact with the nuclear matrix or a matrix-associated factor that holds the protein in the nucleus. Since E4-34kD does not shuttle in the absence of E1B-55kD, it is clear that the ‘retention domain’ is dominant over export signals by the NES. E1B-55kD might sterically block the E4-34kD retention signal when the two proteins interact to activate shuttling.

Our data suggest that E4-34kD does not require the presence of E1B-55kD to inhibit rev function (Figure 5). Cotransfection of E1B-55kD does not significantly change the inhibitory effect of E4-34kD on rev, and E4-34kD with the R248E mutation seems to work equally as well as wild-type E4-34kD to inhibit rev function (our unpublished observations). Therefore, E4-34kD apparently does not need to be liberated from retention to block the export activity of rev. Perhaps a factor necessary for rev-mediated RNA transport is bound and sequestered by E4-34kD regardless of its retention or precise intranuclear location.

E4-34kD might contain a nuclear retention signal so that it can be retained in the nucleus to perform non-transport-related functions when it is not associated with E1B-55kD. Since the retention signal in E4-34kD is rich in arginine, one might speculate that the motif functions as a nuclear localization signal as well. Indeed, a portion of E4-34kD comprising the putative amphipathic α helix (residues 230–283) can mediate nuclear import when fused to β-galactosidase, but the same is true for a fusion protein containing the R248E mutation (our unpublished observations). Therefore, nuclear localization and nuclear retention seem to be distinct activities that can be separated by a point mutation. Further, the complex of E4-34kD and E1B-55kD fused to the NLS from SV40 T antigen can exit the nucleus (Figure 1, j–l), suggesting that nuclear retention is mediated by an activity different from nuclear import. The heterogeneous ribonucleoprotein (hnRNP) C proteins also contain a nuclear retention domain that overlaps the NLS region and is rich in arginine residues (Nakielny and Dreyfuss, 1996). It is tempting to speculate that E4-34kD and the hnRNP C proteins might be retained in the nucleus by the same mechanism.

Our results are consistent with a model (Figure 6B) in which E4-34kD functions as a transporter and E1B-55kD mediates the interaction of the transporter with RNA. Although such an E1B-55kD–RNA interaction has not been demonstrated, there are several lines of evidence consistent with such a role for E1B-55kD. First, E1B-55kD does not seem to mediate nuclear export in the absence of E4-34kD, yet it is required for mRNA export during infection, consistent with the view that it provides a link between the E4-34kD transporter and the mRNA to be exported. Secondly, the expression of E1B-55kD protein fused to a nuclear localization signal blocks the export of mRNA in yeast (Liang et al., 1995), suggesting that E1B-55kD may have the capability to interact directly or indirectly with mRNA. Thirdly, NLS-tagged E1B-55kD localizes in discrete nucleolar structures in a pattern similar to that observed when transiently expressing HIV rev and in rev, the nucleolar localization maps to the same sequence elements as the interaction with RNA (Cochrane et al., 1990; Venkatesh et al., 1990; D’Agostino et al., 1995). The nucleolar localization of NLS-tagged E1B-55kD is sensitive to low concentrations of actinomycin D that block the synthesis of ribosomal RNA in the nucleolus (W.T.Kimberley, unpublished data), as has been reported for HIV rev (Richard et al., 1994; D’Agostino et al., 1995). The RNA binding domain of rev can interact either with the RRE or with the nucleolar B23 protein, but not with both (Fankhauser et al., 1991). Similarly, the domain within NLS-tagged E1B-55kD that attaches to a nucleolar structure might associate with RNA in the context of an adenoviral infection. Finally, the E1B-55kD protein contains a domain at residues 249–288 homologous to the RNP domains that mediate direct interaction with RNA (J.Horridge and K.Leppard, personal communication).

It still remains to determined why the same adenoviral proteins that mediate RNA export each also bind directly to the tumor suppressor p53, and antagonize its activity. Intriguingly, the hdm2 oncoprotein, which binds to the same region on p53 and inhibits its activity as does E1B-55kD (Lin et al., 1994), also shuttles between the nucleus and the cytoplasmin (J.Roth, M.Dobbelstein, D.A.Freedman, T.Shenk and A.J.Levine, unpublished results); and hdm2 has been shown to have RNA binding properties as well (Elenbaas et al., 1996). E1B-55kD, E4-34kD and hdm2 are the first oncogenic products shown to undergo nuclear export, raising the possibility that rev-like nuclear export might contribute to the regulation of cellular growth and oncogenic transformation.

The formation of a E1B-55kD–E4-34kD complex cannot be observed in most rodent cells. In those cells, E1B-55kD is not relocalized to the nucleus when co-expressed with E4-34kD. Fusion of these rodent cells with human cells apparently leads to complex formation, as judged by relocalization of E1B-55kD (Goodrum et al., 1996). This suggests that a primate cellular factor is required for the complex formation between the two viral proteins. Perhaps a cellular protein forms a complex with both viral proteins simultaneously. Even though both adenovirus proteins have been shown to interact with p53 (Dobner et al., 1996), the presence of p53 is not required for the relocalization of E1B-55kD by E4-34kD, since this phenomenon can also be observed in Saos-2 and H1299 cells, which both lack a p53 gene (not shown). Therefore, p53 is not a necessary partner to form a complex containing both viral proteins. Another possibility is that a factor specifically found in...
primate cells can post-translationally modify one or both of the viral proteins, thus enabling them to interact efficiently with each other. This could, for example, be achieved through a cellular kinase. Indeed, at least E1B-55KD is known to be a phosphoprotein, and the mutation of some phosphorylation sites can reduce the protein’s efficiency to inactivate p53 (Teodor et al., 1994). However, these mutations did not show an observable effect on the virus’s ability to shut down cellular protein synthesis (Teodor et al., 1994), indirectly suggesting that the modulation of mRNA export, and hence the association of E1B-55KD with E4-34KD, was not affected.

Should a cellular factor (or several of them) form a ‘bridge’ between the two viral proteins, then such a factor may function as part of a complex that mediates mRNA export in the absence of virus, and may be recruited away from cellular mRNA during adenovirus infection, explaining the observed block in the export of most, though not all (Moore et al., 1987), cellular mRNA molecules (Ornells and Shenk, 1991). An important goal for future investigations will be the identification of these putative cellular factors.

In conclusion, the adenoviral strategy to influence mRNA export comprises several unique features that are not found in retroviral RNA export: (i) the use of at least two different viral proteins that cooperate to modulate mRNA export; (ii) the growth-regulating and transforming activity of these proteins; (iii) the involvement of a dominant nuclear retention domain in addition to an NES 0.4 cm gap width using a Bio-Rad Gene Pulser with capacitance extender activity of these proteins; (iii) the involvement of a putative cellular factor. Indeed, at least E1B-55KD with E4-34KD, were not affected.

To construct pCFNrex and pCFNrexM90, the 3’ portion of the rex coding region was PCR-amplified using the primers TGGTCTCAAGGGGTCAAGTGGGAGGAGGGCCGCTCCTCCCGG- TTAGAAC. This resulted in a mutation of the amino acids L90 and I92 to alanine residues. Similarly, the arginine residue 248 was changed to glutamic acid using the oligonucleotides CGGAGAGCGCTTATGCT- CAGCCAGGCGAGGAAATC and GATGATGGACCCCGCTCG- AAGTAAAGCCGCTTGG. A double mutant was created by performing the two procedures serially.

Heterokaryon assays

Nucleocytoplasmic shuttling was detected by using a heterokaryon assay. The plasmids were used to express NES sequences fused to the antigens. Plasmids containing E1B-55kD coding sequences were routinely checked by restriction analysis, since the presence of these sequences in glass cover slips coated with poly-lysine (Becton Dickinson) using an automated injection system (AIS, Zeiss/Eppendorf) and an Axsovert 35M microscope (Zeiss). The cells were fixed and stained 2 h after injection with a monoclonal mouse antibody against GST (Santa Cruz) and a Texas Red-conjugated antibody against murine IgG (Jackson) as described above, and evaluated by confocal microscopy in a blinded fashion.

Microinjection

GST fusion proteins were prepared from Escherichia coli strain DH10B containing the appropriate pGex-derived construct as described previously by Dobbelstein and Shenk (1995). The proteins were added to a concentration of 3 mg/ml in PBS containing FITC-conjugated dextran 150 000 (Sigma FD 150) at 3 mg/ml. This solution was microinjected, as described by Dobbelstein et al. (1992), into the nuclei of HeLa cells that were seeded in a semiconfluent monolayer containing cycloheximide and further incubated at 37°C. The cells were then covered with a solution of 50% (w/v) polyethylene glycol 8000 (Sigma) in DMEM for 2 min at 37°C, to induce cell fusion. Subsequently, the cover slips were transferred back to DMEM/10% FBS containing cycloheximide and further incubated at 37°C. The cells were fixed 2 h later with 4% paraformaldehyde in PBS at 15 min, followed by permeabilization with 0.2% Triton X-100 in PBS. During and after fixation, all incubations were done at room temperature. The cells were incubated with a blocking solution (10% FBS in PBS with 0.01% w/v sodium azide) for 30 min, followed by primary antibodies [Rb antisem mouse monoclonal IgG2a anti-E4-34kD (Ornells and Shenk, 1991) hybridoma supernatant 1:10 or 9C10 rat IgG anti-E1B-55kD (Oncogene Science) 1: 500, in blocking solution containing 1:40 000 diluted anti-Ku-86 mouse monoclonal IgG1, Santa Cruz J51] for 1 h, three washes for 5 min in PBS, secondary antibodies [FITC-conjugated goat anti-mouse IgG2a or anti-rat IgG, depending on the primary antibody, and Texas Red-conjugated goat anti-mouse IgG1 (Caltag), both diluted 1:500 in blocking solution] for 1 h, then three washes as above followed by mounting in Aquamount solution (Lerner). The cells were examined by fluorescence microscopy using a confocal microscope in a blinded fashion.
Reporter assays
HeLa cells were transfected with the indicated plasmids by electroporation as described above. In all experiments, the total amount of cotransfected expression plasmid was kept at 3 μg by adding empty expression vector pCMVneoBam (Baker et al., 1990), and 5 μg of pGemm7ZEr (+) (Promega) were added as a carrier; 0.5 μg of a luciferase expression construct (pGL3control, Promega) were cotransfected as an internal standard. The cells were harvested in reporter buffer (Promega) 18 h after transfection; luciferase and CAT assays were performed using the appropriate reagents (Promega) according to the manufacturer’s instructions. Nuclear export antigens were determined using a luminescence (Analytical luminescence laboratory, Monolight 1010), and CAT assays were quantified after thin layer chromatography using a PhosphoImager (Molecular Dynamics).

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References

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