Adenovirus E4orf6 targets pp32/LANP to control the fate of ARE-containing mRNAs by perturbing the CRM1-dependent mechanism

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E4orf6 plays an important role in the transportation of cellular and viral mRNAs and is known as an oncogene product of adenovirus. Here, we show that E4orf6 interacts with pp32/leucine-rich acidic nuclear protein (LANP). E4orf6 exports pp32/LANP from the nucleus to the cytoplasm with its binding partner, HuR, which binds to an AU-rich element (ARE) present within many protooncogene and cytokine mRNAs. We found that ARE-mRNAs, such as c-fos, c-myc, and cyclooxygenase-2, were also exported to and stabilized in the cytoplasm of E4orf6-expressing cells. The oncodomain of E4orf6 was necessary for both binding to pp32/LANP and effect for ARE-mRNA. C-fos mRNA was exported together with E4orf6, E1B-55kD, pp32/LANP, and HuR proteins. Moreover, inhibition of the CRM1-dependent export pathway failed to block the export of ARE-mRNAs mediated by E4orf6. Thus, E4orf6 interacts with pp32/LANP to modulate the fate of ARE-mRNAs by altering the CRM1-dependent export pathway.

Introduction

In cells infected with viruses, viral gene products usually export their mRNA transcripts from the nucleus to the cytoplasm by using the RNA export machinery of the host cell. Furthermore, several viruses inhibit the export of cellular mRNAs to maximize the production of their virions. Research into these viruses has identified essential nuclear RNA export factors such as CRM1 and TAP (Cullen, 2003a). CRM1 has been shown as the cellular target for the nuclear export signal (NES) of human immunodeficiency virus type 1 Rev protein to export viral mRNAs (Cullen, 2003a). In the case of cellular mRNAs, although TAP-NXT is usually the crucial nuclear export factor of the majority of mRNAs (Cullen, 2003b), only a few mRNAs are exported by the CRM1-mediated pathway. One such mRNA is an AU-rich element (ARE)–containing mRNA.

Adenovirus E4orf6 is a protein encoded in the E4 region of the adenovirus. It forms a complex with the adenovirus E1B-55kD (Shenk, 1996). This complex promotes the nuclear export of viral mRNAs and contributes to the shutoff of cellular mRNAs during the late phase of adenovirus infection (Shenk, 1996). E4orf6 shuttles between the nucleus and the cytoplasm (Goodrum et al., 1996), which is almost certainly essential for the mRNA transport function of E4orf6. E4orf6 also cooperates with E1A to transform primary baby rat kidney (BRK) cells, and the expression of E4orf6 markedly enhances the ability of BRK and human 293 cells to form tumors in nude mice (Moore et
al., 1996; Nevels et al., 1997). Although some cellular proteins were found as E4orf6-associated proteins, only a few of them were identified.

Here, we show that E4orf6 protein associates with pp32/LANP. We found that E4orf6 exported pp32/LANP and HuR to the cytoplasm and that ARE-mRNAs were exported and stabilized by E4orf6. Leptomycin B (LMB), which is an inhibitor of the CRM1-dependent export pathway, failed to inhibit the export of ARE-mRNAs when cells expressed E4orf6. These findings indicate that E4orf6 controls ARE-mRNAs by overcoming the physiological CRM1-dependent export machinery.

Results and discussion

Isolation of E4orf6-associated proteins
To identify the E4orf6-associated proteins (Higashino et al., 1998), 293 cells were transfected with the expression plasmid of FLAG-tagged E4orf6. The associated proteins were then isolated using M2 affinity column chromatography and the acquired proteins were analyzed with MALDI-TOF/MS. Two proteins were identified as pp32/LANP and HuR. Leptomycin B (LMB), which is an inhibitor of the CRM1-dependent export pathway, failed to inhibit the export of ARE-mRNAs when cells expressed E4orf6. These findings indicate that E4orf6 controls ARE-mRNAs by overcoming the physiological CRM1-dependent export machinery.

Export and stabilization of ARE-mRNAs by E4orf6
We observed the export of HuR protein with its target mRNA in the presence of E4orf6 using in vivo UV cross-linking. The results show that HuR protein was exported with its target mRNA to the cytoplasm in BRK E1 cells, whereas it existed in the nucleus of BRK E1 cells (Fig. 2 A, top). Furthermore, reverse immunoprecipitation using BRK #9 cells, which express E1 and FLAG-E4orf6, showed the same interaction (Fig. 1 B, top).

To detect the region of E4orf6 required for binding, in vitro interaction was confirmed using a series of deletion mutants of E4orf6. To further confirm the interaction, we observed the subcellular localization of E4orf6 and pp32/LANP. Although pp32/LANP was located mainly in the nucleus in BRK E1 cells (Fig. 1 D, top, arrows), it was localized in both the nucleus and the cytoplasm with E4orf6 in BRK E1/H11001 E4 cells (Fig. 1 D, top). Additionally, pp32/LANP was in the nucleus when cells expressed E4orf6 dl210-294 (Fig. 1 D, bottom). These data suggest that E4orf6 has the potential to export pp32/LANP to the cytoplasm by the use of the oncodomain.

pp32 has also been identified as an associated protein of E1B-55kD protein (Harada et al., 2002). In that study, as E1B-55kD failed to interact with pp32 without E4orf6 expression, it was concluded that E4orf6 is required to induce the assembly of E1B-55kD with pp32. We agree with this hypothesis, as we confirmed E4orf6 and pp32/LANP binding without the presence of E1B-55kD (Fig. 1 C).

Export and stabilization of ARE-mRNAs by E4orf6
We observed the export of HuR protein with its target mRNA in the presence of E4orf6 using in vivo UV cross-linking. The results show that HuR protein was exported with its target mRNA to the cytoplasm in BRK E1+E4 cells, whereas it existed in the nucleus of BRK E1 cells (Fig. 2 A, left). The
amount of HuR in total extract of each cell was not changed by E4orf6 (Fig. 2 A, middle) and cell fractionation was confirmed by immunoblotting (Fig. 2 A, right).

We examined the accumulation of three ARE-mRNAs, c-fos, c-myc, and cyclooxygenase-2 (COX-2) mRNAs, in the cytoplasm of BRK cells using quantitative real-time RT-PCR. In the cytoplasm of BRK E1+E4 cells, these mRNAs were severalfold more abundant than those of BRK E1 cells. On the other hand, the quantity of GAPDH mRNA was almost the same in both BRK cells (Fig. 2 B, left). The same results were obtained using 293 cells transfected with E4orf6 expression construct and HeLa cells infected with wild-type adenovirus type 5 dl309 (Ad5 dl309) or E4orf6-deficient virus dl355 (Ad5 dl355). As shown in Fig. 2 B (middle and right), all ARE-mRNAs were accumulated in the cytoplasm of E4orf6-expressing cells (Fig. 2 B, E4orf6 and dl309), whereas there was no such accumulation in control cells (Fig. 2 B, pCMV and dl355). It is noteworthy that cellular mRNA was accumulated in the cytoplasm of wild-type adenovirus-infected cells because usually cellular mRNAs are shut off in the nucleus by adenovirus infection. Because another ARE-mRNA, hsp70, has also been shown to escape the viral export block (Shenk, 1996), ARE-mRNAs presumably have the potential to be exported even if almost all cellular mRNAs are shut off. We confirmed that the transcription of c-fos mRNA is not activated by E4orf6 using a luciferase assay (unpublished data).

The cytoplasmic accumulation was confirmed by Northern blot analysis using a luciferase assay system. We constructed a pGL3-based luciferase reporter plasmid with the 3’-UTR of c-fos cDNA including the AREs (Fig. 2 C, left). The amount of the cytoplasmic luciferase mRNA in 293 cells expressing E4orf6 was much higher than that of control cells (Fig. 2 C, right). We obtained the same result using BRK cells (unpublished data).

Because HuR is involved in protecting ARE-mRNA from degradation in the cytoplasm, E4orf6 may not affect ARE-mRNA export, but rather facilitate HuR-mediated stabilization. To address this question, we examined the half-life of ARE-mRNA of the cytoplasmic fraction. After actinomycin D treatment, the quantity of ARE-mRNAs was measured by quantitative real-time RT-PCR. The half-lives of three ARE-mRNAs in BRK E1+E4 cells were longer than those of BRK E1 cells, and furthermore, these cytoplasmic ARE-mRNAs increased 15 or 30 min after treatment even if the product of these mRNAs was inhibited (Fig. 2 D). These results suggest that mRNAs were both exported to and stabilized in the cytoplasm.

Export was confirmed by in situ hybridization. In BRK E1+E4 cells, the majority of c-fos, c-myc, and COX-2 mRNAs existed in both the cytoplasm and nucleus, whereas these mRNAs were in the nucleus or perinuclear region of BRK E1 cells (Fig. 2 E, compare a–c with d–f). In BRK dl210-294 cells,
which express E4orf6 dl210-294 lacking the oncodomain (Fig. 1 D), these mRNAs were not exported (Fig. 2 E, g–i). On the other hand, mutation of the NES (Fig. 1 D), which has been shown as leucine-rich Rev-like NES (Dobbelstein et al., 1997), did not affect the export (Fig. 2 E, j–l). Together, these results suggest that ARE-mRNAs are exported to the cytoplasm of cells expressing E4orf6 and the oncodomain, but not NES, is critical for the export.

To observe the export of ARE-mRNAs with HuR protein directly, we performed an RNP immunoprecipitation (RIP) assay. As shown in Fig. 2 F, these ARE-mRNAs all existed in the cytoplasm of BRK E1 cells with HuR protein, whereas they were only found in the nucleus of BRK E1 cells. These results indicate that these ARE-mRNAs are exported together with HuR protein.

**E4orf6, E1B-55kD, pp32/LANP, and HuR are associated with c-fos mRNA**

We performed a RIP assay to observe the interaction between the protein complex and c-fos mRNA. In BRK E1 cells, c-fos mRNA was coprecipitated with HuR and pp32/LANP proteins only in the nuclear fraction. On the other hand, it was coprecipitated with HuR, pp32/LANP, E4orf6, and E1B-55kD in both the nuclear and cytoplasmic fractions of BRK E1+E4 cells with HuR protein, whereas they were only found in the nucleus of BRK E1 cells. These results indicate that these ARE-mRNAs are exported together with HuR protein.

**E4orf6 exports ARE-mRNA in a CRM1-independent manner**

(A) Coprecipitated CRM1 in the precipitates of pp32/LANP-specific antibody was confirmed using 293 cells as described in Fig. 3 B. The expressions of CRM1, pp32/LANP, and E4orf6 are shown in the bottom three panels. (B) The quantity of cytoplasmic ARE-mRNA was measured by quantitative real-time RT-PCR using heat-shocked (45°C, 1 h) BRK E1 (E1 HS) and BRK E1+E4 (E1+E4) cells treated with (-) or without (+) LMB. Data are mean ± SEM of three independent experiments. (C) The effect of LMB observed in B was confirmed by RIP analysis using the same cells. Mouse IgG (mIgG) was used as a control. (D) The interaction between E4orf6 and pp32/LANP was analyzed in the presence of RNase A using 293 cells transfected with indicated plasmids. The expression of pp32/LANP and FLAG-E4orf6 are shown.

**E4orf6 formation of a protein complex and associated with ARE-mRNA.**

We examined whether E4orf6 affected pp32/LANP–HuR binding. Considering coprecipitated HuR with pp32/LANP, the binding intensity of the pp32/LANP–HuR complex was not altered by the expression of E4orf6 (Fig. 3 B). Furthermore, E4orf6 was able to interact with HuR in vivo (Fig. 3 C, left), and in vitro–translated E4orf6 protein bound to GST-HuR (Fig. 3 C, right). These results indicate that E4orf6 interacts with both pp32/LANP and HuR without disturbing pp32/LANP–HuR binding.

Additionally, as E4orf6 was able to bind to pp32/LANP in the presence of RNase A, this interaction is not mediated by RNA (Fig. 3 D). Together, we concluded that E4orf6 formed a protein complex and associated with ARE-mRNA.
E4orf6 exports ARE-mRNAs by a CRM1-independent mechanism

It is known that CRM1 binds to pp32/LANP and that ARE-mRNAs are exported to the cytoplasm in a CRM1-dependent manner when cells are stimulated by heat shock or serum (Brennan et al., 2000; Gallouzi et al., 2001). To observe the influence of E4orf6 on pp32/LANP–CRM1 interaction, we examined the binding intensity of both proteins. Because the quantity of coprecipitated CRM1 with pp32/LANP was almost the same between cells with and without E4orf6 expression (Fig. 4 A), E4orf6 does not disturb the binding of these proteins.

To examine the CRM1 dependence of the export, we estimated the cytoplasmic accumulation of ARE-mRNAs in the presence of LMB, a specific inhibitor of CRM1 (Kudo et al., 1998), using heat-shocked BRK E1 cells and BRK E1 + E4 cells. In the presence of LMB, the heat shock-mediated accumulation of c-fos, c-myc, and COX-2 mRNAs was inhibited very efficiently (Fig. 4 B). On the other hand, they still accumulated in the cytoplasm, even if BRK E1 + E4 cells were treated with LMB (Fig. 4 B). The amount of control GAPDH mRNA was not changed by LMB.

To further confirm these results, the behavior of c-fos mRNA with HuR, pp32/LANP, and E4orf6 proteins in both BRK cells was analyzed by RIP assay. C-fos mRNA of heat shock–treated BRK E1 cells was exported to the cytoplasm with its associated proteins, whereas LMB treatment blocked this export completely. On the other hand, in BRK E1 + E4 cells, c-fos mRNA existed in the cytoplasm with the proteins after inhibition of the CRM1-dependent pathway (Fig. 4 C).

In addition, the CRM1-independent export was confirmed by in situ hybridization. C-fos mRNA was exported to the cytoplasm of BRK E1 + E4 cells in the presence of LMB, whereas it was shut off in the nucleus of BRK E1 cells by LMB (Fig. 4 D). We observed that HuR and pp32/LANP were exported by E4orf6 in the presence of LMB, although the heat shock–mediated export of these proteins was blocked by LMB (Fig. 4 E). The export of E4orf6 in the presence of LMB was also confirmed by immunofluorescence (Fig. 4 F). Thus, in cells expressing E4orf6, ARE-mRNAs were exported to the cytoplasm, even if the cells were treated with LMB. We conclude that E4orf6 changes the CRM1-dependent ARE-mRNA export pathway.

We present here that the adenovirus E4orf6 interacts with pp32/LANP to export and stabilize ARE-mRNAs in a CRM1-independent manner neglecting the physiological conditions of cellular mRNA export. pp32/LANP has been shown to interact with the oncodomain of E4orf6, indicating that the control of ARE-mRNA plays an important role for the oncogenic activity of E4orf6. We are currently examining the effect of the export and stabilization of ARE-mRNAs on oncogenic activity of E4orf6.

Materials and methods

Cells, plasmids, and viruses

HeLa, 293, and BRK cells were cultured in DME containing 10% FBS with antibiotics. BRK E1 + E4 cells expressing E1A, E1B, and E4orf6 and BRK E1 cells expressing E1A and E1B and pcDNA3-Flag-E4orf6 and pcDNA3-E4orf6 NES [+] have been described previously (Higashino et al., 1998; Aoyagi et al., 2003). Some cells were treated with heat shock (45°C, 1 h) or LMB (5 ng/ml). HeLa cells were infected with Ad5 dl309 as wild type or Ad5 dl355, which fails to express E4orf6, at a multiplicity of 50 PFU per cell. pCMV-E4orf6 dl1-203 containing HA-tag and Ad5 dl355 were gifts from T. Dobner (Universitat Regensburg, Regensburg, Germany).

Isolation of E4orf6-associated proteins

293 cells were transfected with pcDNA3-Flag-E4orf6 by calcium phosphate–mediated transfection. After 24 h, the cells were lysed as described previously (Higashino et al., 1998) and the extract was applied to an M2 (anti-FLAG antibody) affinity column (Sigma-Aldrich); then, FLAG-E4orf6 was eluted by FLAG-peptide (Sigma-Aldrich). Mass spectrometry was performed using a Voyager DE-STR MALDI time-of-flight mass spectrometer (ABJ). For interpretation of the mass spectrometry spectra of protein digests, we used the MS-Fit program available on the website of the University of California, San Francisco (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm).

Protein binding assay

All immunoprecipitation and immunoblotting were performed as described previously (Aoyagi et al., 2003). The antibodies used were specific to E4orf6 (RSA#3; a gift from T. Shenk, Princeton University, Princeton, NJ; Higashino et al., 1998), pp32/LANP (Matsuoka et al., 1994), HuR (Santa Cruz Biotechnology, Inc.), β-tubulin (Santa Cruz Biotechnology, Inc.), PARP (Cell Signaling), CRM1 (BD Biosciences), HA tag (12CA5; Sigma-Aldrich), β-actin (Sigma-Aldrich), and M2 affinity column. To examine whether the binding between E4orf6 and pp32/LANP is mediated by RNA, immunoprecipitation was performed in the presence of 0.5 mg/ml RNaseA. To observe the in vitro interaction, in vitro–translated E4orf6 and its mutants were subjected to GST pull down assay using GST-pp32/LANP or GST-HuR as described previously (Aoyagi et al., 2003).

Immunofluorescence

Immunofluorescence was performed as described previously (Aoyagi et al., 2003) using antibodies specific to E4orf6 and pp32/LANP followed by FITC- and rhodamine-conjugated secondary antibodies (Molecular Probes). Cells were observed using a confocal microscope (model LSM 510; Carl Zeiss Microlmaging, Inc.) equipped with a Plan-Apochromat 63×/1.4 oil objective at RT. Images were imported using the LSM-510 software (Carl Zeiss Microlmaging, Inc.).

UV cross-linking assay

In vivo cross-linking assay was performed as described previously (Pinol-Roma et al., 1989). In brief, BRK cells were exposed to UV light (6.6 × 103 ergs/mm2) for 3 min. Poly (A) RNA was treated with TRI REAGENT (Sigma-Aldrich) and the RNA was subjected to reverse transcription using Rever Tra Ace (TOYOBO). For quantitative real-time RTPCR analysis, PCR amplification was performed in DNA Engine Opticon 2 (MJ Research) with SYBR green PCR master mix (DyNAmo SYBR green qPCR kit; MJ Research).

RIP assay

RIP assay was performed as described previously (Niranjanakumari et al., 2002) using anti-HuR, pp32/LANP, E4orf6, or −E1B-55Kd antibody.

Online supplemental material

Details regarding cells, plasmids, cell fractionation, in situ hybridization, quantitative real-time RT-PCR, RIP assay, and Northern blot analysis used for this study are available at http://www.jcb.org/cgi/content/full/jcb.200405112/DC1.

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