Adenovirus E4-34kDa requires active proteasomes to promote late gene expression

Kara A. Corbin-Lickfett and Eileen Bridge*

Department of Microbiology, 32 Pearson Hall, Miami University, Oxford, OH 45056, USA

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Abstract

A complex of the Adenovirus (Ad) early region 1b 55-kDa protein (E1b-55kDa) and the early region 4 ORF6 34-kDa protein (E4-34kDa) promotes viral late RNA accumulation in the cytoplasm while inhibiting the transport of most newly synthesized cellular mRNA. The E4 ORF3 11-kDa protein (E4-11kDa) functionally compensates for at least some of the activities of this complex. We find that the same large central region of E4-34kDa that is required for proteasome-mediated degradation of p53 (J. Virol. 75, (2001) 699 –709) is also required to promote viral late gene expression in a complementation assay. E4-34kDa does not promote late gene expression in complementation assays performed in the presence of proteasome inhibitors. A proteasome inhibitor also dramatically reduced late gene expression by a virus that lacks the E4-11kDa gene and therefore relies on E4-34kDa for late gene expression. Our results suggest that E4-34kDa activity in promoting late gene expression depends on the proteasome.

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Introduction

Adenovirus (Ad) DNA replication, nuclear and cytoplasmic late mRNA accumulation, late viral protein synthesis, and virus particle assembly depend on gene products from early regions 1b (E1b) and 4 (E4) (Babiss et al., 1985; Bridge et al., 1993; Halbert et al., 1985; Pilder et al., 1986). Single deletions of either E4 ORF3 encoding an 11-kDa protein (E4-11kDa) or E4 ORF6 encoding a 34-kDa protein (E4-34kDa) have only modest effects on virus growth and each are sufficient to substitute for the whole E4 region during Ad lytic infection (Bridge and Ketner, 1989; Huang and Hearing, 1989). The E4-34kDa and E1b-55kDa proteins from a complex in infected cells (Sarnow et al., 1984) that is important for promoting viral late gene expression. This complex acts posttranscriptionally to facilitate the accumulation of viral late mRNAs while inhibiting the nucleocytoplasmic transport of newly synthesized cellular mRNA (Babiss et al., 1985; Bridge and Ketner, 1990; Halbert et al., 1985; Huang and Hearing, 1989; Pilder et al., 1986). The E4-34kDa/E1b-55kDa complex also promotes the degradation of the tumor suppressor p53 (Querido et al., 1997, 2001a, b) and the Mre11/Rad50/NBS1 components of the mammalian DNA repair system in infected cells (Stracker et al., 2002). The E4-34kDa/E1b-55kDa complex interacts with a cellular cullin-containing E3 ubiquitin ligase which subsequently polyubiquitinates p53 and targets it for proteasome-mediated degradation (Harada et al., 2002; Querido et al., 2001a).

A computer generated model for the structure of the E4-34kDa protein described by Flint and colleagues suggested the existence of a core classic triose phosphate isomerase barrel-like structure and an unusual binuclear zinc coordination structure involving histidine and cysteine residues (Brown et al., 2001). In support of this model, E4-34kDa is able to bind zinc and E4-34kDa proteins with point mutations in several conserved histidine and cysteine residues lost the ability to bind E1b-55kDa, degrade p53, and promote expression of late viral genes (Boyer and Ketner, 2000). The presence of a C-terminal amphipathic...
α-helix characterized previously (Orlando and Ornelles, 1999) was predicted in the computer model. A proline substitution which disrupts the integrity of the C-terminal 

expression of viruses with an intact E4-11kDa gene. Our 

some inhibitor MG132 did not dramatically affect late gene 

E4-34kDa protein was proteasome-dependent, the protea-

somes and this requirement was conserved among several 

functions of the E4-34kDa protein.

A role for the E4-34kDa/E1b-55kDa complex in selective 

RNA export has been suggested since mutant viruses lacking the E4-34kDa and E1b-55kDa genes show reduced 

levels of late viral mRNA accumulation in the cytoplasm and fail to prevent host mRNA transport. Both the E4-

34kDa and the E1b-55kDa proteins have leucine-rich nu-

clear export signals (NES) of the HIV-1 Rev type and shuttle between the nucleus and cytoplasm during Ad in-

fection (Dobbelstein et al., 1997; Dosch et al., 2001; Kratzer 

et al., 2000; Rabino et al., 2000). Leucine-rich NESs utilize 

the cellular CRM1 export pathway (Fornerod et al., 1997; 

Fukuda et al., 1997) and leptomycin B, a specific inhibitor 

of the CRM1 export pathway (Kudo et al., 1998), prevents 

the cytoplasmic accumulation of E1b-55kDa during Ad in-

fection (Dosch et al., 2001). However, a role for CRM1 in 

E4-34kDa export remains unresolved (Dobbelstein et al., 

1997; Dosch et al., 2001; Rabino et al., 2000; Weigel and 

Dobbelstein, 2000). E1b-55kDa also has the ability to bind 

RNA (Horridge and Leppard, 1998), suggesting a model in 

which the E4-34kDa/E1b-55kDa complex could access the 

cellular CRM1 export pathway and preferentially transport 

viral mRNA bound by E1b-55kDa. However, we have previ-

ously shown that an E4-34kDa protein with leucines sub-

stituted with alanines in the NES complements the late gene 

expression defect of an E4 mutant as well as the wild-type 

protein, and leptomycin B does not prevent Ad5 viral late 

gene expression (Rabino et al., 2000). These results sug-

gested that NES activity is not essential for the function of 

the E4-34kDa protein in promoting late gene expression.

Since we did not identify a critical role for the E4-34kDa NES in promoting viral late gene expression, we undertook a genetic analysis of the protein to identify other regions of the protein that were important for its function. Querido et al. (2001a,b) mapped regions of the E4-34kDa protein important for interacting with E1b-55kDa and targeting p53 for ubiquitination and proteasome-mediated degradation. Here we show that these same regions are also critical for the ability of E4-34kDa to complement late gene expression. The ability of E4-34kDa to promote viral replication and late gene expression depended on functional protea-

somes and this requirement was conserved among several 

E4-34kDa proteins from other human Ad serotypes. Inter-

estingly, although promotion of late gene expression by the 

E4-34kDa protein was proteasome-dependent, the protea-

some inhibitor MG132 did not dramatically affect late gene 

expression of viruses with an intact E4-11kDa gene. Our 

results suggest that the ability of the E4-34kDa/E1b-55kDa complex to promote degradation of target proteins via the proteasome is important for the efficient transition of infected cells to the late phase, in the absence of the redundant functions of E4-11kDa.

**Results**

**Complementation of H5dl1011 late gene expression by Ad5 E4-34kDa mutants and Ad E4-34kDa variants**

We used a complementation assay to determine what regions of the E4-34kDa protein are important for promoting viral late gene expression. Fig. 1 shows the location of the E4-34kDa mutations that we studied. HeLa cells were transfected with increasing amounts of wild-type pcDNA E4-34kDa plasmid to generate a standard curve of wild-type protein expression with which to compare cells transfected with mutant E4-34kDa constructs. At 24 h posttransfection, cells were infected with 20 fluorescence forming units (FFU)/cell of the Ad5 E4-deletion virus H5dl1011. This multiplicity of infection ensured that greater than 90% of the cells were infected as determined by detection of the early 72-kDa DNA binding protein at 24 h postinfection (hpi) (data not shown). Cell extracts from transfected/infected cultures were collected at 24 hpi and subjected to Western blotting using antibodies against E4-34kDa and the late proteins penton and fiber.

A representative Western blot with a standard curve of wild-type E4-34kDa expression and two C-terminal deletions (dl277–294 and dl271–294) is shown in Fig. 2A. H5dl1011 was defective for viral DNA replication (data not shown) and expression of late proteins penton and fiber when no E4-34kDa plasmid was transfected (Fig. 2A, mock). Increasing amounts of wild-type E4-34kDa plasmid resulted in more E4-34kDa protein expressed (Fig. 2A, top).
Fig. 2. Complementation of late gene expression by Ad5 E4-34kDa deletion mutants and E4-34kDa from other human Ad serotypes. (A) HeLa cells were either mock transfected or transfected with increasing amounts (0.25–2.0 μg) of the wild-type Ad5 E4-34kDa expression construct or 2.0 μg of the E4-34kDa mutants (dl277–294 and dl271–294) for 24 h. Cells were then infected with 20 FFU/cell of H5d/l1011 and cell extracts were collected at 24 hpi. We used 60 and 30 μg of total proteins for SDS–PAGE for detection of E4-34kDa and penton/fiber, respectively. Mouse monoclonal antibody RSA3 was used for detection of wild-type and C-terminal deletions of E4-34kDa (top) and a rabbit polyclonal antiserum raised against penton and fiber was used to detect viral late proteins (bottom). (B) Quantification of complementation by N- and C-terminal deletions of Ad5 E4-34kDa. HeLa cells were either mock transfected or transfected for 24 h with increasing amounts (0.25–2.0 μg) of the wild-type Ad5 E4-34kDa expression construct or 2.0 μg of the E4-34kDa mutants (dl1–20, dl1–55, dl271–294, dl277–294) and then infected with 20 FFU/cell of H5d/l1011 for 24 h. Cells extracts from transfected/infected cultures were subjected to Western blotting and the amount E4-34kDa and penton and fiber proteins expressed was determined by phosphorimager analysis. A standard curve of wild-type E4-34kDa complementation was used to quantify the percentage of wild-type complementation achieved by each E4-34kDa mutant shown in Fig. 2B. We used phosphorimager analysis to quantify the amount of E4-34kDa and penton and fiber proteins expressed. Levels of late proteins were normalized for the amount of E4-34kDa expressed to determine the percentage of wild-type complementation achieved by each mutant; wild-type E4-34kDa complementation was set at 100% (see Materials and methods). A standard curve of wild-type E4-34kDa and late protein expression was used in every replicate experiment to account for E4-34kDa expression differences between the wild-type and E4-34kDa mutants. An E4-34kDa protein lacking 20 amino acids from the N-terminus (dl1–20) was able to promote late gene expression as well as wild-type but a larger deletion of 55 amino acids (dl1–55) was defective for complementation (Fig. 2B). Since deletion of the first 55 amino acids of E4-34kDa produces a protein that is unable to form a complex with E1b-55kDa (Querido et al., 2001b; Rubenwolf et al., 1997), our results confirm the importance of this complex for promoting viral late gene expression. An E4-34kDa protein with 17 amino acids deleted from the C-terminus (dl277–294) was only two-fold reduced (50% of wild-type) for complementation, and surprisingly, removal of only six more amino acids (dl271–294) rendered the E4-34kDa protein unable to complement late gene expression (Figs. 2A and B). These data show that the region between amino acids 20 and 276 is important for the ability of E4-34kDa to promote viral late gene expression.

We also tested E4-34kDa proteins from other human Ad serotypes for complementation. Many regions of the E4-34kDa protein are conserved between serotypes such as the histidine and cysteine residues predicted to coordinate zinc (Boyer and Ketner, 2000) and the amphipathic α-helix (Orlando and Ornelles, 1999). The E4-34kDa proteins from Ad9, Ad12, and Ad40 have 58, 51, and 39% amino acid identity, respectively, when aligned in a BLAST analysis with the Ad5 protein. All Ad E4-34kDa proteins were epitope tagged on the C-terminus with the influenza hemagglutinin amino acid sequence (YPYDVPDYA) to facilitate their detection. The Ad9, Ad12, and Ad40 E4-34kDa proteins were able to rescue the late gene expression defect of H5d/l1011 as determined by the presence of penton and fiber proteins in Western blots of protein extracts prepared from transfected/infected cultures (Fig. 2C). This suggests that E4-34kDa proteins from several human serotypes have conserved the ability to promote late gene expression.
The central region of the E4-34kDa protein is important for complementing Ad late gene expression independent of its role in degrading p53

Mapping studies of the Ad5 E4-34kDa protein by Querido et al. (2001b) described a large central region of the E4-34kDa protein (amino acids 44–274) that was important for promoting the degradation of p53 via a complex with E1b-55kDa and an E3 ubiquitin ligase. The proteasome inhibitors MG132 and lactacystin prevent E4-34kDa/E1b-55kDa-mediated degradation of p53 (Querido et al., 2001a,b). Interestingly, N- and C-terminal deletions that did not complement late gene expression in our assay overlapped with regions described by Querido et al. (2001b) required for p53 turnover (amino acids 44–274). To determine if there is a correlation between regions of the protein needed to promote p53 degradation and viral late gene expression, we tested several small internal E4-34kDa deletions used by Querido et al. (2001b) to characterize the importance of the central portion of the protein for p53 degradation. Two deletion mutants in the N-terminus, dl35–43 and dl49–64, and two C-terminal deletion mutants, dl249–274 and dl275–294 (Fig. 1), were tested for complementation in HeLa cells as described for Fig. 2B. The dl35–43 and dl275–294 mutants, that degrade p53, were reduced two-fold or less for complementation (Fig. 3A). In contrast, mutants that do not degrade p53, dl49–64, and dl249–274 were unable to rescue viral late gene expression of H5dl1011 in HeLa cells (Fig. 3A). Other internal E4-34kDa deletions tested by Querido et al. (2001b) that could not degrade p53 (dl123–126 and dl154–180) also failed to complement late gene expression in our assay (data not shown). These results show that regions of the E4-34kDa protein important for promoting p53 degradation are also needed to promote late gene expression.

HeLa cells express very low levels of p53 due to the presence of the human papilloma virus 18 E6 protein, which targets p53 for proteasome-mediated degradation (Scheffner et al., 1990). Several groups have shown that the replication efficiency of E1b-55kDa mutant viruses does not correlate with p53 status, suggesting that neutralizing p53 activity is not critical for Ad lytic infection (Goodrum and Ornelles, 1998; Harada and Berk, 1999). Thus, it was somewhat surprising that regions of the E4-34kDa protein important for p53 degradation were also important for promoting late gene expression. We tested the role of p53 status for E4-34kDa function by performing the complementation assay in a p53 null non-small lung carcinoma cell line, H1299. In H1299 cells, H5dl1011 was still defective for viral late gene expression when no plasmid was transfected (Fig. 3B, mock). The same E4-34kDa mutants (dl35–43 and dl275–294) that could degrade p53 and complement late gene expression in HeLa cells could also complement late gene expression in H1299 cells (Figs. 3A and B). Likewise, E4-34kDa mutants that were unable to degrade p53 or complement late gene expression in HeLa cells (dl49–64 and dl249–274) were also defective for late gene expression in H1299 cells (Figs. 3A and B). This indicates that the central region of the E4-34kDa protein is important for complementing viral late gene expression independent of its role in degrading p53.

Proteasome inhibitors prevent E4-34kDa from complementing late gene expression

Recently Stracker et al. (2002) showed that Ad oncoproteins are able to disrupt the ability of cellular double-strand break repair (DSBR) enzymes to form concatemers of viral DNA genomes. The E4-34kDa/E1b-55kDa complex and the E4-11kDa proteins are independently able to prevent Ad genome concatemerization. The E4-11kDa protein redistributes DSBR proteins away from viral replication centers. The E4-34kDa/E1b-55kDa proteins promote the
degradation of DSBR proteins; degradation is prevented by the proteasome inhibitor MG132 (Stracker et al., 2002). Therefore, a target for E4-34kDa/E1b-55kDa-mediated degradation includes DSBR enzymes in addition to p53. We used the proteasome inhibitors MG132, lactacystin, and clasto-lactacystin β-lactone to investigate the role of the proteasome for E4-34kDa function in complementation.

MG132 is a synthetic peptide that reversibly inhibits the catalytic subunit of the proteasome as well as other proteases such as cathepsins and calpains (Lee and Goldberg, 1996; Rock et al., 1994). Lactacystin is a naturally occurring compound that rearranges to form clasto-lactacystin β-lactone in aqueous solutions. Clasto-lactacystin β-lactone irreversibly and specifically inhibits the proteasome (Fenteany et al., 1995). MG132 and lactacystin were previously shown to prevent Ad5 E4-34kDa/E1b-55kDa-mediated p53 degradation (Querido et al., 2001a,b). We first demonstrated the effectiveness of these inhibitors by confirming that they could prevent degradation of p53. HEK 293 cells, which express E1a and E1b proteins (Graham et al., 1977), were either cotransfected with pcDNA p53 and the wild-type Ad5 E4-34kDa expression plasmid or mock transfected. Transfected cells were either not treated or treated from 24 to 40 h posttransfection with 10 μM of the proteasome inhibitors MG132 (lanes 2, 4, and 6), lactacystin (lanes 7), or clasto-lactacystin β-lactone (lane 10). Cell extracts were collected and 100 μg of total protein was subjected to SDS-PAGE and Western blotting. Mouse monoclonal Ab1801 was used to detect p53.

The HEK 293 cells had low levels of endogenous p53 (Fig. 4, lanes 1 and 2). p53 degradation was observed in cells cotransfected with pcDNA p53 and Ad5 E4-34kDa expression constructs (Fig. 4, compare lanes 3 with 5, and 8 with 9), as expected (Querido et al., 2001a,b). p53 degradation was prevented in pcDNA p53/E4-34kDa cotransfected cells cultured with 10 μM of the proteasome inhibitors MG132, lactacystin, and clasto-lactacystin β-lactone (Fig. 4, lanes 6, 7, and 10). MG132 did not significantly affect endogenous or overexpressed p53 levels in the absence of the E4-34kDa protein (Fig. 4, compare lanes 1 with 2, and 3 with 4). The p53 protein is transcriptionally inactive in HEK 293 cells due to the expression of Ad E1a and E1b proteins in these cells (Grand et al., 1995). Transcriptionally inactive p53 should not induce its negative regulator MDM2 and will therefore not be targeted for proteasome-mediated degradation by the normal cellular pathway in HEK 293 cells. This is consistent with our observation that proteasome inhibitors do not affect p53 levels in HEK 293 cells in the absence of the E4-34kDa protein.

We next investigated the effect of proteasome inhibitors on E4-34kDa-mediated complementation of H5d1011 late gene expression. HeLa cells were transfected with an Ad5 E4-34kDa expression construct for 24 h. Transfected cells were treated with 10 μM MG132, lactacystin, or clasto-lactacystin β-lactone 5 h prior to infection with 20 FFU/cell of H5d1011 to ensure that proteasome activity was inhibited at the time of infection. Infected cells were then treated with proteasome inhibitors from 2–24 hpi as indicated. Total cell extracts were analyzed by Western blotting for expression of the late proteins penton and fiber. The Ad5 E4-34kDa protein was unable to complement H5d1011 late gene expression in cells cultured with MG132, lactacystin, or clasto-lactacystin β-lactone (Fig. 5A). Levels of the early DNA replication protein E2-72kDa detected in infected cultures treated with inhibitors were not reduced compared to the levels seen in untreated H5d1011-infected cells (data not shown). This indicates that pretreatment of cells with proteasome inhibitors did not interfere with H5d1011 infection or early gene expression. Complementation by the Ad9, Ad12, and Ad40 E4-34kDa proteins was prevented by treatment with MG132 from 2–24 hpi (Fig. 5B). These results suggest that E4-34kDa requires functional proteasomes for its activity in promoting late gene expression and this requirement is conserved among E4-34kDa proteins from other human adenoviruses. Complementation of H5d1011 by the E4-34kDa protein was only inhibited when transfected/infected cells were pretreated with lactacystin and clasto-lactacystin β-lactone. MG132 could still inhibit complementation when added to transfected/infected cells directly after virus adsorption (2 hpi) (Fig. 5B), but it failed to inhibit complementation when added at 8 or 10 h postinfection (Fig. 5C). This indicates that E4-34kDa function requires active proteasomes during the early stages of infection.

Role of the proteasome in Ad replication and late gene expression

Our results indicate that functional proteasomes are needed during the early phase for E4-34kDa complementation and suggest that the E4-34kDa/E1b-55kDa complex may promote efficient viral late gene expression by targeting proteins for proteasome-mediated degradation. Genetic studies have shown that the Ad E4-11kDa protein can at least partially compensate for the activity of the E4-34kDa/E1b-55kDa complex in promoting late gene expression (Bridge and Ketner, 1989; Huang and Hearing, 1989). Since
there is no evidence that E4-11kDa requires functional proteasomes for its activity, it is possible that these viral regulatory proteins promote late gene expression by proteasome- dependent and -independent mechanisms. We tested this by investigating the effect of inhibiting proteasome activity on the replication and gene expression activity of Ad5 and viral mutants lacking genes for E1b-55kDa, E4-34kDa, and E4-11kDa.

We first studied the effect of adding the proteasome inhibitor MG132 at different stages of Ad5 infection. Ad5-infected HeLa cells were treated with 10 \( \mu \)M MG132 from 2–14, 2–24, or 14–24 hpi. This concentration of MG132 was sufficient to prevent E4-34kDa/E1b-55kDa-mediated degradation of p53 (see Fig. 4). Total DNA and proteins were isolated from cells and the levels of viral DNA (Fig. 6A); early and late proteins (Fig. 6B) were determined by Southern and Western blotting, respectively. Infected cells treated with 10 \( \mu \)M MG132 from 2–14 hpi were 6.5-fold reduced for viral DNA accumulation (Fig. 6A), reduced two-fold for E2-72kDa expression, and failed to produce detectable levels of penton and fiber (Fig. 6B). However, with longer MG132 treatments from 2–24 hpi, viral DNA and late protein levels (penton and fiber) were only reduced two-fold (Fig. 6A) and three-fold (Fig. 6B), respectively, and E2-72kDa levels were unaffected (Fig. 6B). Therefore, MG132 treatment delayed the onset of the late phase but did not prevent viral replication and late gene expression. MG132 had no effect on late gene expression if it was added from 14 to 24 hpi (Fig. 6B), suggesting that the proteasome-mediated functions of the E4-34kDa/E1b-55kDa complex are most important during the early phase. This is consistent with our observation that MG132 does not inhibit complementation by E4-34kDa when added at 8 or 10 hpi (see Fig. 5C).

We next tested the effect of MG132 treatment on late gene expression by Ad mutants. H5dl110, H5dl1010, and H5dl1006 lack the genes for the E1b-55kDa, E4-34kDa, and E4-11kDa proteins, respectively (Bridge and Ketner, 1989, 1990). HeLa cells were infected with Ad5, H5dl110, H5dl1010, and H5dl1006 and were either cultured from 2–24 hpi with 10 \( \mu \)M MG132 or left untreated. Cell extracts were subjected to Western blotting to determine the levels of late proteins produced during these infections. Ad5, H5dl110, and H5dl1010-infected cells were two- to three-fold reduced for late protein levels when infected cells cultured with MG132 were compared to untreated infections (Fig. 6C). However, cells infected with the mutant H5dl1006, which fails to make the E4-11kDa protein, showed dramatically reduced levels of viral late proteins in the presence of MG132 even though early gene expression was within two-fold of untreated levels (data not shown). This indicates that a virus lacking an intact gene for the E4-11kDa protein is much more dependent on functional proteasomes to progress to the late phase than viruses that make the E4-11kDa protein.

Discussion

We have performed a genetic analysis to identify regions of the E4-34kDa protein that are important for late gene expression. Analysis of a series of nested N- and C-terminal truncations revealed that deleting amino acids 1–55 (dl1–55) of the N-terminus and 271–294 (dl271–294) of the C-terminus rendered the E4-34kDa protein unable to complement H5dl1011 (Figs. 1 and 2). Previous investigators have shown that dl1–55 fails to bind E1b-55kDa (Querido et al., 2001b; Rubenwolf et al., 1997). Thus, our data indicate that regions important for E1b-55kDa binding are also important for promoting late gene expression in a complementation assay (Fig. 2B). This is consistent with the idea that E1b-55kDa and E4-34kDa mediate their activities in late gene expression as a complex (Babiss et al., 1985; Bridge and Ketner, 1990). Although dl271–294 retains the ability to bind E1b-55kDa (Querido et al., 2001b; Rubenwolf et al., 1997), it still fails to complement H5dl1011 (Figs. 2A and B). This suggests that E1b-55kDa binding may be necessary

Fig. 5. Complementation by E4-34kDa in the presence of proteasome inhibitors. (A) HeLa cells were either mock transfected or transfected for 24 h with the Ad5 E4-34kDa expression construct. Five hours prior to infection with 20 FFU/cell of H5dl1011, cells were either treated with 10 \( \mu \)M MG132, lactacystin, or clasto-lactacystin \( \beta \)-lactone or left untreated. DMSO without inhibitor was added to control cells. Equal amounts of total cell extracts were subjected to SDS–PAGE and Western blotting for penton and fiber. (B) HeLa cells were either mock transfected or transfected with Ad5, Ad9, Ad12, and Ad40 E4-34kDa expression constructs for 24 h. Cells were infected with 20 FFU/cell H5dl1011 for 24 h and either treated with 10 \( \mu \)M MG132 from 2–24 hpi or left untreated. Western blot analysis was performed as described in part A. (C) Ad5 E4-34kDa transfected and H5dl1011-infected cells were treated with 10 \( \mu \)M MG132 from 2, 8, and 10 hpi or left untreated. Cell extracts were collected at 24 hpi and analyzed by Western blotting for the amount of penton and fiber.
but not sufficient for complementation. Querido et al. (2001b) used a series of deletions to map regions of the E4-34kDa protein important for p53 degradation. We used several of these internal deletions to further define regions of E4-34kDa that are important for complementation. E4-34kDa mutants that complement late gene expression to within 50% of wild-type levels also efficiently degrade p53. In contrast, mutants that fail to complement late gene expression do not degrade p53 (Fig. 3) (Querido et al., 2001b). Thus, E4-34kDa amino acids 44–274 are important both for both p53 degradation (Querido et al., 2001b) and for promoting late gene expression in a complementation assay (Figs. 1–3).

Several conserved structural motifs and protein-binding sites are located in the central region of the E4-34kDa protein, including the NES, a central histidine and cysteine-rich region (CCR), and an amphipathic α-helix (Fig. 1). We have previously found that point mutations in the E4-34kDa NES are not critical for complementing late gene expression (Rabino et al., 2000) and an E1b-55kDa NES mutant promotes late gene expression of a virus mutant deleted for the E1b-55kDa gene as well as wild-type (Carter et al., submitted for publication). Querido et al. (2001b) studied the same E4-34kDa NES mutant and found that it did not affect the ability of E4-34kDa to degrade p53. The E4-34kDa protein is able to bind zinc (Boyer and Ketner, 2000) and conserved cysteine and histidine residues predicted to coordinate two zinc ions are likely to be critical for the tertiary structure of the E4-34kDa protein (Brown et al., 2001). Single point mutations of specific cysteine and histidine residues (C51, H123, C124, H125, C126, C134, and H185) produced proteins that retained the ability to bind p53, but were unable to bind E1b-55kDa or degrade p53. These mutants also failed to complement late gene expression of an E4 mutant (Boyer and Ketner, 2000). A proline insertion in the amphipathic α-helix produced an E4-34kDa protein that was defective for promoting both late gene expression (Orlando and Ornelles, 1999) and p53 degradation (Querido et al., 2001b). A less disruptive mutation in the amphipathic α-helix, R248E, had little effect on either activity (Orlando and Ornelles, 1999; Querido et al., 2001b; Rabino et al., 2000). Thus, the results of several studies suggest a close correlation between the ability of E4-34kDa to degrade p53 and its ability to complement late gene expression.

Amino acids 44–274 are important for the ability of E4-34kDa to promote p53 turnover via the proteasome. Several proteasome inhibitors dramatically decreased complementation by the Ad5 E4-34kDa protein, and the E4-34kDa proteins from Ad9, Ad12, and Ad40 (Fig. 5). The observation that complementation was prevented by inactivating proteasomes with three different inhibitors (Fig. 5) strongly suggests that E4-34kDa mediates its functions in late gene expression via the proteasome. However, complementation studies done in the p53 null cell line, H1299, suggest that p53 is unlikely to be the critical target for E4-34kDa/E1b-55kDa activity (Fig. 3B). Other proteins targeted by the E4-34kDa/E1b-55kDa complex for proteasome-mediated degradation include the Mre11/Rad50/NBS1 components of the DSBR system. E4-34kDa/E1b-55kDa interfere with DSBR by degrading Mre11/Rad50/NBS1, while the E4-11kDa protein relocalizes Mre11/Rad50/NBS1 proteins away from viral replication centers.
In the absence of these viral regulatory proteins, DSBR activities ligate viral DNA genomes to form concatamers in cells infected with Ad mutants (Boyer et al., 1999; Weiden and Ginsberg, 1994). These observations raise the attractive possibility that E4-34kDa may complement late gene expression by promoting degradation of DSBR proteins and preventing the formation of DNA concatamers during viral replication. Alternatively, the E4-34kDa/E1b-55kDa complex may promote the transition to the late phase by targeting additional proteins for ubiquitination and proteasome-mediated degradation. We are currently performing experiments to address these possibilities.

In our complementation assay, wild-type and mutant E4-34kDa proteins were expressed from a plasmid construct under the control of a CMV promoter rather than from the viral genome. A limitation of this approach is that the expression of the E4-34kDa protein is not transcriptionally regulated in the natural context of the E4 promoter. Characterization of E4-34kDa mutants expressed from the E4 promoter during virus infection will be an important extension for further understanding E4-34kDa function. We have confirmed the importance of the proteasome for E4-34kDa function during virus infection when E4-34kDa is expressed from its own promoter in the viral genome, in infected cells treated with the proteasome inhibitor MG132 (Fig. 6). Genetic studies indicate that the activity of the E4-34kDa protein is at least partially redundant with the function of the E4-11kDa protein (Bridge and Ketner, 1989; Huang and Hearing, 1989). When cells were infected with Ad5 and mutants with an intact E4-11kDa protein, MG132 delayed, but did not block, viral replication and late gene expression (Fig. 6). However, H5/dl1006 late gene expression was more sensitive to the proteasome inhibitor MG132. H5/dl1006 lacks the E4-11kDa gene and relies on the activity of the E4-34kDa/E1b-55kDa complex for late gene expression (Bridge and Ketner, 1989). Our results indicate that while activity of the E4-34kDa/E1b-55kDa complex depends on functional proteasomes, E4-11kDa is much less dependent on proteasome activity to promote late gene expression. It will be interesting to determine if E4-11kDa-mediated redistribution of DSBR proteins and prevention of DNA concatamers can still occur in the presence of proteasome inhibitors.

In summary, our mutational analysis of the E4-34kDa protein revealed that regions needed to target proteins for ubiquitination and proteasome-mediated degradation were also critically important for the ability of E4-34kDa to complement H5/dl1011. We observe that proteasome inhibitors interfere with E4-34kDa complementation, and they dramatically affect late gene expression of a virus that depends on the function of the E4-34kDa/E1b-55kDa complex because it lacks the redundant functions of the E4-11kDa protein. Taken together, these results indicate that proteasome-mediated degradation of target proteins makes an important contribution to the ability of the E4-34kDa/E1b-55kDa complex to promote viral late gene expression.

Materials and methods

Cells and viruses

HEK 293 cells were kindly provided by J. Lingrell (Graham et al., 1977). HeLa and W162 (Weinberg and Kettner, 1983) monolayer cultures were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine, 10 U/mL penicillin, and 10 μg/mL streptomycin. H1299 cells (ATCC CRL 5803) were grown in RPMI 1640 supplemented with 10% FBS, 1 mM sodium pyruvate, 10 U/mL penicillin, and 10 μg/mL streptomycin. Wild-type Ad5 virus was originally obtained from J. Williams and propagated on HeLa and W162 cells. The E1b and E4 virus mutants H5/dl110, H5/dl1006, H5/dl1010, and H5/dl1011 have been described previously (Bridge and Ketner, 1989, 1990). H5/dl110 was propagated on HEK 293 cells. H5/dl1006, H5/dl1010, and H5/dl1011 were propagated on W162 cells. Virus titers were determined as previously described (Philipson, 1961) and expressed as fluorescence forming units per milliliter. Virus titers for Ad5 and H5/dl110 were determined in parallel on HEK 293 cells. Virus titers for Ad5, H5/dl1006, H5/dl1010, and H5/dl1011 were determined in parallel on W162 cells.

Plasmids

Wild-type Ad5 E4-34kDa cDNA was obtained from T. Dobner. The gene was PCR amplified with a 5’ primer introducing HindIII and BamHI sites (5’-CGCAAGCTTGATCCATGACTACGTCCGGCGTTCCATTTGGC-3’ and a 3’ primer that mutated the natural stop codon, and added an XhoI site, a nine amino acid influenza hemagglutinin (HA) epitope tag (YPDYDIVDYA), and a new stop codon 3’ to the HA sequence to the C-terminus (5’-CGCCTCGAGCTAGCTTGCCTGAATCCCGTGACATAGGTAAGGTGTTAGTGTAAGAGTACATGCGGGGG-3’). Ad5 E4-34kDa PCR products were cloned into pCNA3.1(+) (Invitrogen) under the control of a CMV promoter. N-terminal deletions were generated by PCR using a 5’ primer engineered with a translation start codon that hybridized 3’ to the wild-type start codon at the desired nucleotide with a HindIII site (d1–20: 5’-CGCAAGCTTACCATGCGGCTCGGGC-CACTCCGTACAGTAGGAGTAGGGCCGCGAGAGAGGGTAAGGTGTTAGTGTAAGAGTACATGCGGGGG-3’; d1–55: 5’-CGCAAGCTTACCATGCGGCTCGGGC-CACTCCGTACAGTAGGAGTAGGGCCGAGGAGAGAGGGTAAGGTGTTAGTGTAAGAGTACATGCGGGGG-3’) and the same 3’ primer used for wild-type E4-34kDa. C-terminal deletions were generated by PCR using the same 5’ primer used for wild-type E4-34kDa above and a 3’ primer that hybridized internal to the translational stop codon at the desired location. The 3’ primer added the HA tag, a new stop codon to the C-terminus, and an XhoI site (d277–294: 5’-GCGCTCGAGCTAGCTTGCCTGACATGCGGCTCGGGC-CACTCCGTACAGTAGGAGTAGGGCCGAGGAGAGAGGGTAAGGTGTTAGTGTAAGAGTACATGCGGGGG-3’; d271–294: 5’-GCGCTCGAGCTAGCTTGCCTGACATGCGGCTCGGGC-CACTCCGTACAGTAGGAGTAGGGCCGAGGAGAGAGGGTAAGGTGTTAGTGTAAGAGTACATGCGGGGG-3’).
or rabbit polyclonal Y-11; Santa Cruz Biotechnology) were diluted 1:200 and 1:250, respectively. E4 ORF6 C-terminal antibody 1807 (rabbit polyclonal from P. Branton) diluted 1:5000 was also used to detect N-terminal deletions of E4-34kDa. RSA3 (mouse monoclonal antibody from T. Shenk) was diluted 1:100 and used to detect wild-type Ad5 E4-34kDa and C-terminal deletions of E4-34kDa. B610 (mouse monoclonal from A. Levine) was diluted 1:100 to detect E2-72kDa. Rabbit polyclonal antibodies to penton and fiber (from U. Pettersson) were used at a 1:1000 dilution. Antibody to p53 (mouse pAb1801; Zymed) was diluted 1:250. Protein blots were incubated with horseradish peroxidase conjugated goat anti-mouse or anti-rabbit IgG (1:1500) secondary antibodies diluted in 5% nonfat dry milk. Proteins were visualized by incubating blots with ECL reagent (Amersham Pharmacia) and the chemiluminescent signals were captured by Hyperfilm ECL (Amersham Pharmacia).

Analysis of E4-34kDa and late protein expression levels in the complementation assay

Cell extracts from complementation assays were subjected to Western blotting and wild-type and mutant E4-34kDa and late proteins were detected with the same primary antibodies described for Western blotting. An alkaline phosphatase conjugated goat anti-mouse or anti-rabbit IgG (1:2000) secondary antibody (Sigma) was used and enhanced chemiluminescence (ECF) substrate (Amersham) was used for detection of proteins on a STORM 860 phosphorimager (Molecular Dynamics). Scanned images were analyzed using ImageQuant 5.2 (Molecular Dynamics) software to quantify the amount of protein. The amount of E4-34kDa quantified from cultures transfected with increasing amounts (0.25–2.0 μg) of wild-type E4-34kDa plasmid was plotted against the amount of late proteins detected in the respective cell extracts to generate a wild-type E4-34kDa complementation standard curve for every replicate experiment. Complementation by mutant E4-34kDa proteins was determined by normalizing complementation for the amount of mutant E4-34kDa expressed relative to wild-type E4-34kDa. This was done by extrapolating the amount of E4-34kDa mutant protein expressed onto the wild-type E4-34kDa standard curve to determine the expected amount of late proteins relative to the amount of E4-34kDa expressed. This extrapolated value was then compared to the actual amount of late proteins quantified in the mutant E4-34kDa complementation assay. If the expected amount of late proteins was equal to the actual amount expressed in the mutant complementation assay, complementation by the mutant was 100% of wild-type.

Viral DNA analysis

HeLa cells seeded onto 35-mm dishes were infected with 20 FFU/cell of Ad5 virus in the presence or absence of 10
μM MG132. Total DNA was isolated as described (Bridge and Ketner, 1989). Ten micrograms of total DNA from each sample was digested with EcoRI and subjected to electrophoresis in a 1% agarose gel for 20 h at 22 V. DNA was transferred to Hybond-N nylon membrane (Amersham) according to the manufacturer’s specifications. 

14P-labeled Ad2 DNA was synthesized using the Multiprime DNA labeling system (Amersham). Hybridization was performed at 65°C for 20 h as described (Sambrook et al., 1989). The amount of viral DNA was measured by phosphorimager analysis. Scanned images were analyzed using ImageQuant 5.2 (Molecular Dynamics) software to quantify amount of DNA.

Proteasome inhibitors

In experiments where proteasome inhibitors were used, 10 μM MG132 (Calbiochem), 10 μM lactacystin (Calbiochem), or 10 μM clasto-lactacystin β-lactone (BostonBiochem) dissolved in DMSO (Sigma) was added at the indicated times posttransfection or infection. DMSO was added to control cells not treated with the inhibitors.

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