Role of E1B55K in E4orf6/E1B55K E3 Ligase Complexes Formed by Different Human Adenovirus Serotypes

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The E4orf6 protein of serotypes representing all human adenovirus species forms Cullin-based E3 ubiquitin ligase complexes that facilitate virus infection by inducing degradation of cellular proteins that impede efficient viral replication. This complex also includes the viral E1B55K product believed to bind and introduce substrates for ubiquitination. Heterogeneity in the composition of these ligases exists, as some serotypes form Cul5-based complexes whereas others utilize Cul2. Significant variations in substrate specificities also exist among serotypes, as some degrade certain substrates very efficiently whereas others induce more modest or little degradation. As E1B55K is believed to function as the substrate acquisition component of the ligase, we undertook studies to compare the ability of representative E1B55K proteins to bind substrates with the efficacy of degradation by their respective E4orf6-based ligases. Interestingly, although efficient degradation in some cases corresponded to the ability of E1B55K to bind to or relocate substrates, there were several examples of substrates that bound efficiently to E1B55K but were not degraded and others in which substrates were degraded even though binding to E1B55K was low or undetectable. These results suggest that transient interactions with E1B55K may be sufficient for efficient substrate degradation and that binding alone is not sufficient, implying that the orientation of the substrate in the ligase complex is probably crucial. Nevertheless, we found that the substrate specificity of certain E4orf6-based ligases could be altered through the formation of hybrid complexes containing E1B55K from another serotype, thus confirming identification of E1B55K as the substrate acquisition component of the complex.

It is well known in the case of the best-studied human adenovirus serotype, Ad5, that the viral E4orf6 and E1B55K proteins, along with cellular proteins elongin B and C, Cul5, and Rbx1, form an E3 ligase complex that degrades a wide range of substrates, including p53 (1, 2), Mre11 (3, 4), DNA ligase IV (5), integrin α 3 (6), Bloom helicase (BLM) (7), ATRX (S. Schreiner and T. Dobner, personal communication), and Tip60 (8), to optimize the infectious process. Two adenovirus-associated viral proteins, Rep52 and capsid protein, are also targeted by the Ad5 E3 ligase (9). In addition, other cellular proteins were shown to be degraded by ligase subcomplexes formed with only E4orf6 or E1B55K. With Ad5, degradation of Daxx by E1B55K in the absence of E4orf6 was demonstrated (10), whereas with Ad12 (but not Ad5), E4orf6 alone promoted degradation of TOPBP1 (11, 12).

Recent studies have shown that considerable heterogeneity exists in the composition and substrate specificities of the E3 ligase complexes among human adenoviruses. From our previous work (13), E4orf6 and E1B55K from seven model human serotypes chosen to represent their respective species, Ad12 (species A), Ad16 (B1), Ad34 (B2), Ad5 (C), Ad9 (D), Ad4 (E), and Ad40 (F), were shown to form functional E3 ligase complexes similar to that formed by Ad5. However, Ad12 and Ad40 primarily form a Cul2-based E3 ligase whereas Ad4, Ad5, Ad9, and Ad34 primarily form a Cul5-based complex (12, 13). Ad16 can bind both Cullins. In addition, different serotypes also degrade various subsets of substrates. p53 is significantly degraded only by Ad5, Ad12, and Ad40. Mre11 is completely degraded by Ad5, Ad12, and Ad40 and partially degraded by Ad9, Ad16, and Ad34. Integrin α 3 is targeted by Ad4, Ad5, Ad12, and Ad40. DNA ligase IV is the only substrate that is efficiently degraded by all serotypes (13). These findings

suggest a degree of complexity among adenoviruses in the evolution of both the complex itself and the specificity of substrate selection.

Our current model for the assembly of this E4orf6/E1B55K E3 ligase suggests that E4orf6 usually forms the core complex with elongins B and C, Cul5 or Cul2, and Rbx1. E1B55K then binds to this complex and recruits substrates for ubiquitination (2, 3, 14–19). Recruitment of p53, Mre11, and DNA ligase IV, and probably other substrates, was shown to be independent and mapped to different locations on the Ad5 E1B55K protein (15).

In the present report, to gain a better understanding of the substrate specificity and recruitment of substrates to these complexes, a series of studies was conducted to examine interactions of various target proteins with and degradation by the ligase complexes of representatives of all adenovirus species except for species G, which had not been identified at the start of these analyses. We found that although in some cases the ability to degrade or not to degrade various substrates correlated well with their ability to bind to E1B55K, such was not always the case, suggesting that efficient degradation can occur even with weak or transient associations with E1B55K. In addition, we found several examples of proteins that bound efficiently to E1B55K but were not signifi-

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cantly degraded, suggesting that the proper orientation of the potential substrate to the E2 ubiquitin-conjugating enzyme within the ligase complex may be critical. Furthermore, we found that Ad5 E1B55K was able to form functional ligase complexes with E4orf6 proteins from Ad4 and Ad9 and that these hybrid Cullin-based complexes exhibited the substrate specificities characteristic of Ad5 and not these serotypes. These results clearly confirm that E1B55K is the major substrate recognition component of the E4orf6/E1B55K E3 ubiquitin ligase.

MATERIALS AND METHODS

Cells and cell lines. Human small cell carcinoma H1299 cells (ATCC CRL-5803), carrying a deletion of the p53 gene (20), were cultured in α minimal essential medium (Gibco) supplemented with 10% fetal calf serum (FCS; Gibco) containing 0.292-mg/ml L-glutamine (Gibco).

Plasmids. The pcDNA3 plasmids expressing FLAG-tagged E4orf6 and hemagglutinin (HA)-tagged E1B55K from each serotype (13), and human p53 (19), human HA-tagged p53 (3), and Myc-tagged DNA ligase IV (5), were described previously.

To create the pcDNA3 plasmid expressing the V5 tag, the V5 forward primer (5'-CGCAAGCTTATGGGTAAGCCTATTCCGAACCCGCTTC TCGGCTGGACTCCACCGAATTCACGCG-3') and the V5 reverse primer (5'-CGCGTGAATTCGGTGGAGTCCAGGCCGAGAAGCGGG TTCGGAATAGGCTTACCCATAAGCTTGCG-3') were annealed and digested with restriction enzymes HindIII and EcoRI. The oligonucleotides were then ligated to pcDNA3 expression vector. cDNAs encoding the Bloom helicase (BLM) gene were cloned by PCR using the pYES2 plasmid expressing BLM as the template (a gift from Ian Hickson) (21) with the following primers: forward (5'-AACTCGAGGCTGCTGTTCCCTCAAAA TAATC-3') and reverse (5'-AAGGGCCCTTATGAGAATGCATATGAA GGC-3'). The PCR product was then digested with restriction enzymes XhoI and ApaI and cloned into the pcDNA3 plasmid expressing the V5 tag described previously, at the position downstream of the V5 tag.

Antisera. HA epitopes were detected using mouse monoclonal anti-HA HA.11 (BabCO) for Western blotting or rat monoclonal 3F10 (Roche) for immunofluorescence (IF) or rabbit polyclonal HA.11 anti-HA (BabCO) for coimmunoprecipitation with p53 or anti-HA mouse monoclonal HA.11 (BabCO) for coimmunoprecipitation with FLAG-E4orf6; FLAG epitopes were detected with anti-FLAG M2 mouse monoclonal peroxidase-conjugated antibody (Sigma) for Western blotting and for coimmunoprecipitation with HA-E1B55K or with rabbit polyclonal anti-FLAG antibody M2 (Sigma) for immunoprecipitation with HA-p53; V5 epitopes were detected with anti-V5 mouse monoclonal antibody SV5-pK1 (abcam); Myc epitopes were detected with anti-Myc mouse monoclonal 9E10 (Covance); p53 was detected with anti-p53 pAb1801 hybridoma supernatants prepared as described previously (19) or for immunofluorescence (IF) with mouse monoclonal DO-1 (Calbiochem) or for immunoprecipitation with anti-p53 mouse monoclonal peroxidase-conjugated antibody DO-1 (Santa Cruz); Mre11 was detected with rabbit polyclonal antiserum NB 100-142D3 (NOVUS Biologicals); eEF2 was detected with rabbit polyclonal antibody 2332 (Cell Signaling); and actin was detected with mouse monoclonal anti-actin C4 (Millipore).

DNA transfections, cell lysis, and preparation of samples for degradation assays and immunoprecipitations. Cells growing in 6-well plates for degradation assays or 100-mm-diameter dishes for binding assays were transfected with plasmid DNAs using Lipofectamine 2000 (Invitrogen) as described by the manufacturer. The amount of Lipofectamine 2000 used was calculated based on a ratio of 3 μ l of transfection reagent to 1 μ g of DNA. For the p53 and Mre11 degradation assays, 0.25 μ g of pcDNA3-p53, 2 μ g of the appropriate pcDNA3-FLAG E4orf6, and 1.75 μ g of the appropriate pcDNA3-HA E1B55K were transfected for 24 h. When required, the total amount of DNA was equalized by addition of pcDNA3 empty vector DNA. For V5-tagged BLM degradation assays, the same conditions were used except that 1 μ g of pcDNA3 V5-BLM was transfected instead of p53. Cells were lysed for 15 min on ice with an

NP-40-based lysis buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 5 mM EDTA, 0.15% NP-40, 2 mM dithiothreitol [DTT], 4 mM NaF, 2 mM NaPP, 500 μ M Na₃VO₄, 200 μ g/ml phenylmethylsulfonyl fluoride [PMSF], 2 μ g/ml aprotinin, 5 μ g/ml leupeptin) for p53 and Mre11 degradation and with a more stringent buffer (10 mM sodium phosphate [pH 7.2], 150 mM NaCl, 2 mM EDTA, 1% NP-40, 24 mM sodium deoxycholate, 0.1% SDS, 2 mM DTT, 4 mM NaF, 2 mM NaPP, 500 μ M Na₃VO₄, 200 μ g/ml PMSF, 2 μ g/ml aprotinin, 5 μ g/ml leupeptin) for V5 BLM degradation, followed by three cycles of freeze/thaw and then two rounds of sonication at 100% power output for 30 s with a VCX130 sonicator (Sonics & Materials). Aliquots of cell extracts containing 15 to 50 μ g of protein were examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

For binding assays, cells in 100-mm-diameter dishes were transfected with different quantities of plasmid DNAs expressing products of each of the seven serotypes in order to attempt to obtain comparable levels of protein expression. Nevertheless, there remained some unavoidable variability in expression with individual cDNAs. To ensure that our results were unequivocally correct, every experiment was repeated (4 to 6 times and even more in some cases). For these reasons, it was not possible to make specific comparisons on the relative levels of binding for all the serotypes. Rather, it was more appropriate to make semiquantitative conclusions, which should be sufficient for the purposes of this study. pcDNA3 empty vector DNA was added to each preparation to yield 9 μ g for E1B55K and 9 μ g of FLAG-E4orf6. In addition, 2 μ g of plasmid DNAs expressing p53, 6 μ g of plasmid DNAs expressing HA-tagged p53, or 3 μ g of plasmid DNA expressing Myc-tagged DNA ligase IV was also cotransfected in the respective binding assays. For all binding assays except for the one with V5-BLM, cells were then lysed for 20 min on ice with the lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 5% glycerol, 2 mM DTT, 4 mM NaF, 2 mM NaPP, 500 μ M Na₃VO₄, 200 μ g/ml PMSF, 2 μ g/ml aprotinin, 5 μ g/ml leupeptin). For binding assays with V5-BLM, cells were lysed for 20 min on ice with a different lysis buffer (10 mM Tris-HCl [pH 7.6], 60 mM NaCl, 0.4% NP-40, 2.4 mM sodium deoxycholate, 4% SDS, 2 mM DTT, 4 mM NaF, 2 mM NaPP, 500 μ M Na₃VO₄, 200 μ g/ml PMSF, 2 μ g/ml aprotinin, 5 μ g/ml leupeptin) followed by a preclearing step. Aliquots of cell extracts containing 400 to 1,000 μ g of protein (constant with each experiment) were used for immunoprecipitation using 1 μ l of rabbit polyclonal antibody anti-HA (HA. 11) (BabCO) for binding assays involving HA-E1B55K and p53, 1.5 μ l of rabbit polyclonal anti-FLAG antibody M2 (Sigma) for E4orf6 and HA-p53, 1 μ l of mouse monoclonal anti-HA antibody (HA. 11) (BabCO) for HA-E1B55K and Mre11, 1 μ l of 9E10 anti-Myc antibody for Myc-DNA ligase IV and E1B55K, and 2 μ l of mouse monoclonal anti-HA antibody (HA. 11) (BabCO) for HA-E1B55K and V5-BLM, followed by incubation with a mixture containing 50% protein G and 50% protein A agarose (Upstate). The beads were extensively washed in the lysis buffer and eluted, and bound proteins were examined by SDS-PAGE. Proteins were examined by Western blotting, essentially as described previously (3, 22). Briefly, proteins separated by SDS-PAGE were transferred to PVDF membranes and blocked using 5% nonfat dry milk-Tris-buffered saline and Tween 20 (TBST). Membranes were incubated with the indicated primary antibodies in TBS-1% Tween 20 followed by the appropriate peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories). Polypeptides on membranes were then visualized by enhanced chemiluminescence with ECL plus Western blotting reagents (Amersham-Pharmacia Biotech).

Immunofluorescence microscopy. Cells grown on coverslips in 6-well dishes were transfected as described for the figures and then fixed for 15 min with 4% paraformaldehyde and permeabilized for 15 min with 0.5% Triton X-100-phosphate-buffered saline (PBS). Following washes in PBS, immunofluorescence analysis was performed for 2 h at room temperature in a humidity chamber using rabbit mouse p53 DO1 antibodies and rat HA antibodies for the localization of p53 and with mouse HA antibodies and rabbit Mre11 antibodies for Mre11. The secondary

FIG 1 Analysis of interactions between E1B55K from all serotypes and p53. (A) Association of E1B55K with p53. H1299 cells were transfected with plasmid DNAs expressing p53 and HA-E1B55K of different serotypes as indicated. Immunoprecipitates (IP) obtained using anti-HA (E1B55K) antibodies were immunoblotted using anti-p53 antibodies and whole-cell extracts with anti-HA (E1B55K) and anti-p53 antibodies. The pattern of p53 degradation for each serotype (with species) determined in a previous study (13) is indicated at the top. (B) Relocalization of p53 by E1B55K. H1299 cells were transfected with plasmid DNAs

antibodies used for the localization of p53 were anti-mouse antibody conjugated to Alexa 488 dye and anti-rat antibody conjugated to Alexa 594 dye (Molecular Probes) and anti-mouse antibody conjugated to Alexa 594 dye and anti-rabbit antibody conjugated to Alexa 488 dye (Molecular Probes) for Mre11. Images were taken on an LSM3 confocal microscope with a 63 $\times$ objective with the LSM 4.2 Image browser software.

Luciferase assays. Cells were transfected using 25-kDa polyethylenimine (PEI) (Polysciences Europe GmbH, Eppelheim, Germany) (23) with indicated amounts of the effector plasmid DNAs (HA-E1B55K or FLAG-E4orf6 of the seven serotypes), p53-expressing plasmid DNA (p53SN3), the p53-dependent reporter constructs (pRE-Luc) for expression of firefly luciferase (24, 25), and 1.0 mg of pRL-TK (Promega, Madison, WI) as an internal control. Total cell extracts were prepared 36 h after transfection, and firefly luciferase activity was assayed in a luminometer (Lumat LB9510; Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). All samples were normalized for transfection efficiency by measuring Renilla activity.

Cell transformation assays. Subconfluent primary baby rat kidney cells (BRK) were transfected with plasmid DNAs expressing Ad5 E1A and HA-E1B55K of all seven serotypes at 2 days postplating, and numbers of multilayered cell foci were visualized by crystal violet staining after 3 to 6 weeks of incubation in a manner similar to that described in reference 26.

Phylogenetic trees. The protein sequences of E1B55K, E4orf6, hexon, and fiber of all seven serotypes were aligned using the multiple alignment program Clustal W. These alignments were then analyzed by PhyML, and the branch lengths of the trees were obtained using the program ATV. The phylogenetic trees are visualized in phenograms using Drawgram. All the programs can be found in the website (<http://www.phylogeny.fr/version2.cgi/index.cgi>).

RESULTS

Only partial correlation between binding of p53 to E1B55K and p53 degradation by adenovirus ligases. It is generally believed that adenovirus E4orf6 proteins are responsible for the formation of the Cullin-based adenovirus E3 ubiquitin ligases whereas E1B55K products bind to these complexes and function largely in substrate recruitment. As shown in previous studies (13), p53 is not degraded by the ligases of all human adenovirus serotypes. With Ad5, although E4orf6 is capable of interacting with p53, E1B55K was shown to play the major role in p53 recruitment for degradation by the ligase (3). Therefore, we believed it likely that there would be a close correlation between the ability of p53 to associate with E1B55K from the various serotypes and the ability of the respective ligases to degrade p53. To investigate this correlation, H1299 cells that had been transfected with plasmid DNAs expressing p53 and the various HA-E1B55K products were harvested and the extracts were immunoprecipitated using polyclonal rabbit anti-HA antibodies and then analyzed by Western blotting using anti-p53 antibodies. The levels of HA-E1B55K and p53 in whole-cell extracts were determined by Western blotting using monoclonal mouse anti-HA or anti-p53 antibodies. As discussed previously (13), the expression of E1B55K of different serotypes often varies significantly in individual experiments and thus, for this type of assay, as in all of our previous studies, many

repetitions were performed to ensure that results shown are wholly representative. Figure 1A shows that E1B55K from all selected serotypes coimmunoprecipitated with p53 except in the case of Ad4. For three serotypes, Ad5, Ad12, and Ad40, their ability to degrade p53, as demonstrated in previous studies (13), correlated with p53 binding to their respective E1B55K proteins. The efficient binding of Ad5 E1B55K to p53 has been demonstrated many times before (27–29), as has the somewhat weaker interaction between p53 and Ad12 E1B55K (29–31). With Ad4, the absence of p53 degradation was consistent with the absence of a detectable interaction between p53 and Ad4 E1B55K. However, no such correlation was found in Ad9, Ad16, and Ad34. Although E1B55K of all of these serotypes interacted well with p53, all were found unable to degrade p53 (13). Thus, only a partial correlation was seen between the ability of the ligase to degrade p53 and its binding to E1B55K.

To confirm these results, further evidence for the interaction between E1B55K and p53 was sought by verifying the abilities of the various E1B55K proteins to relocalize p53. H1299 cells grown on coverslips were transfected with plasmid DNAs expressing HA-tagged E1B55K of different serotypes and p53, and then the localization of both HA-E1B55K and p53 was assessed 24 h later by immunofluorescence microscopy using appropriate antibodies. As shown in Fig. 1B (panels a to c), in the absence of E1B55K, p53 localized evenly throughout the nucleus, excluding the nucleoli (32). Localization of E1B55K from each serotype has been described in detail elsewhere and has been found to differ somewhat among different serotypes. Ad5 E1B55K is mostly cytoplasmic, and Ad12 E1B55K is present in both the nucleus and cytoplasm, whereas E1B55K from all other serotypes is present largely in the nucleus, concentrated in dots. E1B55K from many but not all serotypes also concentrates in perinuclear aggresomes or aggresome-like structures (33). Figure 1B (panels at the left) largely confirms these previous findings; however, in the present context the change in localization of p53 in the presence of E1B55K was of the most interest, especially any change from its usual even nuclear distribution, as demonstrated previously with Ad5 E1B55K (17, 34, 35). Figure 1B (panels p to r) clearly shows that Ad5 E1B55K relocalized a significant amount of p53 to the juxtanuclear aggresome. Ad16 E1B55K also relocalized p53 to the aggresome but did not do so as efficiently as Ad5. Ad12 E1B55K exhibited some p53 relocalization to the aggresome-like structure and throughout the cytoplasm, perhaps suggesting a weaker interaction. Ad34 and Ad40 E1B55K strongly relocalized p53 to distinct nuclear dots. Some relocalization was also evident with Ad9. Results from these serotypes tended to confirm those of the binding assay presented in Fig. 1A. However, a weak relocalization of p53 to a perinuclear structure and some concentration in nuclear dots was observed in cells expressing high levels of Ad4 E1B55K. This observation may suggest that there is a weak interaction between these two proteins that is not stable enough to be detected

encoding HA-E1B55K of different serotypes and p53. The immunofluorescence assay was carried out using rat anti-HA (E1B55K) antibody and mouse anti-p53 antibody. DNA, in blue, was detected with DAPI (4',6-diamidino-2-phenylindole). (C) Repression of p53 transactivation activity by E1B55K. H1299 cells were transfected with plasmid DNAs expressing p53 and different amounts (in μ g) of HA-E1B55K of different serotypes as indicated, p53-dependent reporter constructs for expression of firefly luciferase (pRE-Luc), and an internal construct (pRL-TK) for normalization for transfection efficiency. Means and standard deviations of the results of three independent experiments are presented. (D) Cell transformation activity of E1B55K. Primary baby rat kidney cells (BRK) were transfected with plasmid DNAs expressing Ad5 E1A and HA-E1B55K of all serotypes tested. The numbers of multilayered cell foci were scored 6 weeks posttransfection, and the transformation activity relative to Ad5 has been indicated with means and standard deviations of the results of three independent experiments.

by coimmunoprecipitation. In contrast to the clear binding between Ad9 E1B55K and p53 shown by coimmunoprecipitation, p53 relocalization by Ad9 E1B55K was very weak. This discrepancy may be due to the increased chances of interaction between proteins from different cellular compartments and structures under the conditions of coimmunoprecipitation.

As shown in Fig. 1A and B, Ad9, Ad16, and Ad34 E1B55K proteins bind to p53 even in the absence of apparent substrate degradation by the ligase. To investigate this issue further, the E1B55K/p53 interaction was examined by an additional functional approach. It has been known for some time that the E1B55K products of Ad5 and Ad12 are able to bind p53 (27–29) and to inhibit p53 transcriptional transactivation activity (29, 31, 32, 36, 37), a function that presumably requires an interaction between E1B55K and p53. To determine if such transcriptional repression occurs with other adenovirus serotypes, an assay using a luciferase reporter gene regulated by a p53-dependent promoter was performed. As shown in Fig. 1C, as expected, Ad5 E1B55K repressed p53 transactivation in a dose-dependent manner. E1B55K from all other serotypes except Ad4 also induced comparable levels of reduction of luciferase activity. Thus, these results indicated that E1B55K from all representative serotypes apart from Ad4 bound to p53 and repressed its transactivation activity.

In view of the failure of Ad4 E1B55K to either detectably bind to p53 or to repress its transactivation activity, we examined another biological function of the various adenovirus E1B55K products, that of facilitating cell transformation by E1A (27, 38). Primary baby rat kidney cells (BRK) were transfected with plasmid DNAs expressing the various E1B55K products and, in all cases, Ad5 E1A 48 h after plating. The numbers of transformed cell foci were scored 6 weeks later. Figure 1D shows that E1B55K products from all serotypes except Ad4 cooperated with Ad5 E1A to generate stable colonies of transformants. With Ad4, E1B55K colony formation was not significantly more than seen with E1A alone, thus confirming its inability to act on p53.

In summary, while it is clear that failure of E1B55K to bind p53 resulted in its failure to be degraded by the ligase (as with Ad4), binding alone with the other serotypes was not sufficient to ensure p53 degradation.

Ad5 and Ad4 E4orf6 proteins bind p53 and repress transcriptional transactivation. It is widely believed that downregulation and/or degradation of p53 is important for optimal adenovirus progeny production (39–43). Although elucidation of this issue was not a major objective of our present studies, we wondered if Ad4 (the only species E adenovirus serotype) possessed E1B55K-independent mechanisms to regulate p53. We and others had previously found that the Ad5 E4orf6 protein can bind to p53 (16, 19, 44–46). In order to verify whether this function of E4orf6 is conserved for all serotypes, a coimmunoprecipitation experiment similar to the one described previously for Fig. 1A was performed using cells transfected with plasmid DNAs expressing HA-p53 and the various FLAG-E4orf6 products. The extracts were immunoprecipitated using anti-FLAG antibody and then analyzed by Western blotting using anti-HA antibodies. The levels of FLAG-E4orf6 and HA-p53 in whole-cell extracts were determined by Western blotting using anti-FLAG or anti-HA antibodies. Figure 2A shows that significant binding was detected only between p53 and E4orf6 from Ad4 and Ad5, although perhaps low levels occurred with E4orf6 from other serotypes. Binding of Ad5 E4orf6 is also known to block p53 transcriptional transactivation activity

(16, 44–46), and thus we conducted luciferase reporter assays similar to those whose results are presented in Fig. 1C to examine this function using FLAG-E4orf6 proteins from various serotypes. Figure 2B shows that although E4orf6 from all serotypes except Ad34 and Ad9 exhibited some p53 repression activity, this function was significantly higher with Ad5 and Ad4. Thus, although Ad4 is deficient in E1B55K-mediated regulation of p53, this defect is compensated for at least partially via efficient binding of p53 by its E4orf6 product, suggesting the evolution of combinatorial strategies for the viruses to deal with p53. All the results obtained in the p53 experiments are summarized in Table 1.

Partial correlation between the binding of Mre11 and DNA ligase IV to E1B55K and degradation by adenovirus ligases. As is the case with p53, Mre11 is degraded efficiently by only a subset of serotypes (13). Mre11 can interact with Ad5 E1B55K in the presence or absence of E4orf6; however, its interaction with E4orf6 is E1B55K dependent (3). To determine Mre11 binding to E1B55K of the model serotypes, a study similar to that whose results are presented in Fig. 1A was conducted except that H1299 cells were transfected only with plasmid DNAs expressing the various HA-E1B55K products and binding of endogenous Mre11 was examined in coimmunoprecipitation studies using appropriate antibodies. Figure 3Ai shows some correlation between Mre11 binding and degradation in that the partial degradation of Mre11 seen with Ad16 and Ad9 correlated with weak Mre11 binding to their respective E1B55K proteins whereas both significant binding and degradation were evident with Ad5. However, Ad34 E1B55K interacted strongly with Mre11 but degraded it at a modest rate whereas Ad4 E1B55K was seen to bind Mre11, at least at low levels, but Mre11 was not detectably degraded by the Ad4 ligase. Figure 3Aii shows that with Ad12 and Ad40, even though these serotypes degraded Mre11 to the same extent as Ad5, the interactions between Mre11 and their E1B55K proteins were very weak and were detectable only at high exposures of the Western blots. Thus, with some of the serotypes, high-level stable Mre11 binding to E1B55K is not the only determinant of its degradation by the ligase complexes.

To confirm the results corresponding to the Mre11/E1B55K interaction by another approach, the ability of E1B55K of different serotypes to relocalize Mre11 was investigated using immunofluorescence microscopy. A study similar to that whose results are presented in Fig. 1B was performed except that cells were transfected only with plasmid DNAs expressing HA-E1B55K of different serotypes, and locations of E1B55K and endogenous Mre11 were determined using appropriate antibodies. Figure 3B (panels a to c) shows that, in mock-infected cells, Mre11 is evenly spread throughout the nucleus, excluding the nucleoli, as found previously (4, 47). Figure 3B shows that Mre11 was clearly relocalized to either aggresomes (Ad16, Ad5, and Ad4) or nuclear dots (Ad34) where these E1B55K proteins localize, as found previously with Ad5 (17). A very modest level of relocalization to the cytoplasm was usually evident with Ad12 (Fig. 3B, panels d to f). Little relocalization was evident with Ad9 or Ad40 E1B55K proteins. Thus, the abilities of the various E1B55K proteins to relocalize Mre11 correlated quite well with the coimmunoprecipitation results shown in Fig. 3A.

As we have previously shown, DNA ligase IV is the only substrate degraded by the ligases of all serotypes tested (13). To determine DNA ligase IV binding to E1B55K from the model serotypes, a study similar to that described for Fig. 1A was conducted

FIG 2 Analysis of interactions between E4orf6 from all serotypes and p53. (A) Association of E4orf6 with p53. H1299 cells were transfected with plasmid DNAs expressing HA-p53 and FLAG-E4orf6 of different serotypes, as indicated. Immunoprecipitates obtained using anti-FLAG (E4orf6) antibodies were immunoblotted using anti-HA (p53) antibodies (two exposures [exp] of the blots have been included) and whole-cell extracts with anti-FLAG (E4orf6) and anti-HA (p53) antibodies. (B) Repression of p53 transactivation activity by E4orf6. H1299 cells were transfected with plasmid DNAs expressing p53 and different amounts (in μ g) of FLAG-E4orf6 of different serotypes as indicated, p53-dependent reporter constructs for expression of firefly luciferase (pRE-Luc), and an internal construct (pRL-TK) for normalization for transfection efficiency. The means and standard deviations of the results of three independent experiments are presented.

except that H1299 cells were transfected with plasmid DNAs expressing the various HA-E1B55K products and Myc-tagged DNA ligase IV. **Figure 3C** shows that E1B55K from all serotypes interacted with DNA ligase IV, with the possible exception of Ad34 and Ad40, for which levels of a background species made determination of binding difficult. Thus, there might exist some correlation between binding to E1B55K and DNA ligase IV degradation.

It should be noted that our results determined using epitope-tagged E4orf6 and E1B55K with these three substrates agreed ex-

tremely well with those obtained by Forrester et al. using whole virus corresponding to most of these serotypes (12).

Bloom helicase is targeted by only a subset of serotypes. Bloom helicase (BLM) is a 3'-to-5' DNA helicase (21) involved in DNA repair (48) and genome stability (49), and it was recently shown to be a substrate of the Ad5 E4orf6/E1B55K E3 ligase (7). To determine if the E4orf6/E1B55K complexes from other serotypes also degrade BLM, H1299 cells were cotransfected with plasmid DNAs expressing human V5-tagged BLM and HA-E1B55K

TABLE 1 Summary of results determined for p53 and E4orf6 of all serotypes tested^a

Species (serotype)	p53 degradation	p53/E1B55K binding	E1B55K relocalization of p53	E1B55K repression of p53	p53/E4orf6 binding	E4orf6 repression of p53
A (Ad12)	+	+/-	+	++	-	+
B1 (Ad16)	-	+	++	++	-	++
B2 (Ad34)	-	+	+++	+++	-	-
C (Ad5)	+	+	+++	++	+	+++
D (Ad9)	-	+	+/-	++	-	-
E (Ad4)	-	-	+	-	+	+++
F (Ad40)	+	+	+++	++	-	+

^a -, none; +/-, minimal; +, moderate; ++, high; +++, very high.

and FLAG-E4orf6 products of the various serotypes. The levels of V5-BLM present in whole-cell extracts harvested at 24 h posttransfection, as well as those of E4orf6 and E1B55K, were determined by Western blotting using appropriate antibodies, as in all of our previous studies of this type (3, 13, 22). The levels of endogenous Mre11 were also examined as a control, and Fig. 4A shows that the pattern of Mre11 degradation corresponded exactly with that from a previous study (13) as summarized at the top of Fig. 3A. Figure 4A also shows that in the case of Ad5, Ad12, and Ad40, V5-BLM was degraded most efficiently when both E4orf6 and E1B55K were expressed, although in some repeat assays, degradation with Ad5 was seen in the presence of E4orf6 alone (data not shown). This finding was of some interest because with Ad16, Ad34, and Ad4, although degradation of V5-BLM in the presence of both E1B55K and E4orf6 was pronounced, similar levels of degradation were also seen consistently with E4orf6 alone, suggesting that an E1B55K-independent degradation process exists with some or all serotypes. A further level of complexity was apparent in the cases of Ad9 and Ad16. Expression of the E1B55K protein of these serotypes induced a clear and fully reproducible increase in the level of V5-BLM, although in the presence of both E1B55K and E4orf6 or with E4orf6 alone, extensive (Ad16) or modest (Ad9) degradation was apparent. Figure 4B shows that in coimmunoprecipitation experiments, only E1B55K from Ad12, Ad9, and, at low levels, Ad5 interacted detectably with V5-BLM. Because the levels of Ad34 E1B55K in this experiment (and repeated studies) were very low, we cannot be certain that there is indeed no interaction with BLM. We have also made repeated attempts to examine interactions between V5-BLM and the various E4orf6 products but have never observed any evidence of coimmunoprecipitation of these species (data not shown). Thus, these studies indicated a complicated pattern of BLM degradation with little correlation with binding to E1B55K (see Discussion for further comments about the BLM results).

Modifying the substrate specificity of different E3 ligase complexes by replacing the E1B55K protein. As discussed above, E1B55K is generally believed to be responsible for recruiting most substrates and thus acting as the main determinant of substrate specificity of E4orf6/E1B55K E3 ligase complexes (2, 15, 16, 50); however, some of the results of the present studies appeared to contradict this model. To test this idea directly, we hypothesized that formation of hybrid E3 ligase complexes with E1B55K from different serotypes may result in modification of substrate specificity. Thus, Ad5 E1B55K was coexpressed with E4orf6 from other selected serotypes to form hybrid E3 ligases. The ability of Ad5 E1B55K to form an E3 ligase with other E4orf6 proteins was tested in an assay similar to that shown in Fig. 1A except that H1299 cells

were cotransfected with plasmid DNAs expressing both FLAG-E4orf6 from various serotypes and Ad5 HA-E1B55K. Figure 5A shows that Ad5 E1B55K coimmunoprecipitated with E4orf6 proteins of all other chosen serotypes, confirming that Ad5 E1B55K can form complexes with these E4orf6 proteins.

To determine if these E4orf6/Ad5 E1B55K hybrid ligases are functional and whether the substrate specificity is altered, studies were performed in H1299 cells transfected with plasmid DNAs expressing human p53 and various E1B55K and E4orf6 proteins. The levels of exogenous p53 and endogenous Mre11 present in whole-cell extracts harvested at 24 h posttransfection, as well as those of E4orf6 and E1B55K, were determined by Western blotting using appropriate antibodies. Figure 5B shows that the hybrid ligases formed by Ad5 E1B55K with E4orf6 proteins from Ad4, Ad9, Ad12, and Ad40 were functional, whereas, with Ad16 and Ad34, activity was low at best, probably due to the fact that their associations were the weakest of all of the pairs. But, importantly, Fig. 5B also shows that whereas the original Ad4 and Ad9 ligases could not degrade p53 and Mre11, their respective hybrid ligases showed considerably enhanced degradation of both substrates. Interestingly, for Ad4, enhancement was more significant for p53 than Mre11 whereas, for Ad9, Mre11 degradation was more enhanced than for p53. The original complexes of Ad12 and Ad40 were capable of degrading both p53 and Mre11, and the substrate specificity remained the same for their hybrid complexes. However, coupling Ad5 E1B55K with Ad16 and Ad34 E4orf6 proteins had no effect on degradation of either of these substrates. These data showed that, at least in several cases, changing the E1B55K protein could modify the substrate specificity of the ligase complex, thus confirming the role of E1B55K in substrate recognition for the E4orf6/E1B55K E3 ligase complex for at least p53 and Mre11.

More examples of such hybrids were also tested for serotypes other than Ad5. As indicated in Fig. 5C, Ad12 E1B55K was combined with Ad4 E4orf6, and their interaction is shown in Fig. 5Ci. As shown in Fig. 5Cii, degradation assays performed with Ad12 E1B55K and Ad4 E4orf6 indicated an increase in degradation of p53 but not of Mre11. In contrast, as shown in Fig. 5D, Ad9 E1B55K was combined with Ad5 E4orf6, and their interaction is shown in Fig. 5Di. Figure 5Dii shows that this hybrid failed to exhibit significant degradation of either p53 or Mre11, both of which were effectively degraded by the Ad5 ligase. We attempted to examine several other combinations of E4orf6 and E1B55K proteins, but in many cases no complex formation could be detected. Nevertheless, these results strongly suggested that E1B55K functions as the major substrate-recruiting component for the ligase. Clearly, however, substrate recruitment to the complex

FIG 3 Association of E4orf6 of all serotypes with the substrates Mre11 and DNA ligase IV. (Ai and Aii) Association of E1B55K with endogenous Mre11. H1299 cells were transfected with plasmid DNAs expressing HA-E1B55K of different serotypes as indicated. Immunoprecipitates obtained using anti-HA (E1B55K) antibodies were immunoblotted using anti-Mre11 antibodies and whole-cell extracts with anti-HA (E1B55K) and anti-Mre11 antibodies. The pattern of Mre11 degradation for each serotype (with species) determined in a previous study (13) is indicated at the top of panel Ai. Panel Aii shows the interaction between Ad12 and Ad40 E1B55K with Mre11 with long exposure. (B) Relocalization of Mre11 by E1B55K. H1299 cells were transfected with plasmid DNAs encoding HA-E1B55K of different serotypes and Mre11. The immunofluorescence assay was carried out using rat anti-HA (E1B55K) antibody and rabbit anti-Mre11 antibody. DNA, in blue, was detected with DAPI (4',6-diamidino-2-phenylindole). (C) Association of E1B55K with DNA ligase IV. Immunoprecipitates obtained using anti-Myc (DNA ligase IV) antibodies were immunoblotted using anti-HA (E1B55K) antibodies and whole-cell extracts with anti-Myc (DNA ligase IV) and anti-HA (E1B55K) antibodies. ctl, control.

FIG 4 Analysis of Bloom helicase (BLM) with all serotypes. (A) Degradation of BLM. H1299 cells were transfected with plasmid DNAs encoding V5-tagged BLM, FLAG-E4orf6, and HA-E1B55K of different serotypes as indicated, and at 24 h posttransfection whole-cell extracts were immunoblotted for V5 (BLM), Mre11, FLAG (E4orf6), and HA (E1B55K) using appropriate antibodies. eEF2 levels were assessed by Western blotting as loading controls, Mre11 levels were assessed as a positive control, and the serotype (with species) has been indicated at the top of each set. Degradation of V5-BLM was determined by the decrease of V5-BLM in the presence of both E4orf6 and E1B55K. (B) Association of E1B55K with BLM. Immunoprecipitates obtained using anti-HA (E1B55K) antibodies were immunoblotted using anti-V5 (BLM) antibodies and whole-cell extracts with anti-HA (E1B55K) and anti-V5 (BLM) antibodies.

alone is not sufficient for the degradation (see more discussion below).

DISCUSSION

In this study, we have shown that the degradation of substrates by the E4orf6/E1B55K E3 ubiquitin ligases from different serotypes cannot be explained simply by the level of stable binding of these targets to E1B55K, the presumed substrate recognition protein. From our studies with p53, Mre11, and DNA ligase IV, we propose that several possibilities probably exist related to the degradation of substrates and their interaction with E1B55K. Figure 6 illustrates that in the first scenario, the substrate binds stably to E1B55K (i.e., with enough stability to be detected in coimmunoprecipitation experiments) and is ubiquitinated and degraded (examples: p53 and Mre11 in Ad5, Ad12, and Ad40). The second scenario is that the substrate is not degraded, as it does not detectably associate with E1B55K (example: p53 in Ad4). However, a third scenario is that the substrate can stably interact with E1B55K but is not degraded (examples: p53 in Ad9, Ad16, and Ad34 and Mre11 in Ad4). The fourth scenario is that the substrate is degraded but no stable binding to E1B55K is detected (example: BLM in Ad40). Thus, efficient degradation of a substrate does not solely quantitatively depend on its stable association with the substrate recognition protein, suggesting that, not surprisingly, proper positioning and/or orientation within the ligase complex also plays a critical role. Previous work on the structure of a cellular Cullin-based E3 ligase supports this notion. When the rigid Cul1 structure is replaced by a flexible linker in the SCF complex, the degradation of its substrate p27 is abolished, suggesting that

proper position and orientation of the lysine residue on the substrate with respect to the active site of the E2 enzyme are essential for its ubiquitination and its subsequent degradation (51). The fourth scenario could be explained by the transient binding of the substrate to E1B55K or by a more unstable interaction between the substrate and E1B55K that cannot be maintained during coimmunoprecipitation. A real consequence of these observations is the need for the design of a screen to identify new substrates of the viral ligase complex. Screens established to identify E1B55K binding proteins as potential substrates would not be as comprehensive as previously thought, as some substrates may not bind stably enough to allow detection. Additionally, from our results, we think that such a screen would lead to a considerable number of false positives, identifying proteins that bind to E1B55K but that are not actually substrates.

We have also shown that Ad5 E1B55K can form functional E3 ligases with E4orf6 of other serotypes. Formation of hybrid ligases of Ad5 E1B55K with Ad4 or Ad9 E4orf6 products can alter their original substrate specificity, confirming again that E1B55K is the substrate recognition subunit. However, we found that Ad5 E1B55K failed to function with the E4orf6 proteins of some serotypes, suggesting again that, in addition to binding, the position and orientation of the substrate in the ligase complex are critical. Interestingly, we tried several E1B55K/E4orf6 combinations and found that, for unknown reasons, Ad5 E1B55K was by far the most effective in forming functional hybrid ligase complexes.

We believe that the selection of either Cul2 or Cul5 by different serotypes has little to do with substrate specificity. Nevertheless, it is apparent that two of the serotypes that exhibit the broadest

FIG 5 Modification of substrate specificity by swapping E1B55K from different serotypes. (A) Association of Ad5 E1B55K with E4orf6 from other serotypes. H1299 cells were transfected with plasmid DNAs expressing HA-E1B55K and FLAG-E4orf6 of different serotypes as indicated. Immunoprecipitates obtained using anti-HA (E1B55K) antibodies were immunoblotted using anti-FLAG (E4orf6) antibodies and whole-cell extracts with anti-HA (E1B55K) and anti-FLAG (E4orf6) antibodies. (B) Degradation of p53 and Mre11 by E4orf6 coupled with their corresponding E1B55K and with Ad5 E1B55K. H1299 cells were transfected with plasmid DNAs encoding human p53, FLAG-E4orf6, and HA-E1B55K as indicated, and at 24 h posttransfection whole-cell extracts were immunoblotted for p53, Mre11, FLAG (E4orf6), and HA (E1B55K) using appropriate antibodies. Actin levels were assessed by Western blotting as loading controls, and the serotype (with subgroup) has been indicated at the top of each set. Within each set, the assay with Ad5 E1B55K is on the left and the one with E1B55K from their original serotype is on the right. Degradation of p53 and Mre11 was determined by the decrease of p53 and Mre11 levels in the presence of both E4orf6 and E1B55K. (C i and C ii) Increase of substrate degradation with E3 ligase formed with Ad4 E4orf6 and Ad12 E1B55K. Association of Ad12 E1B55K with Ad4 E4orf6 was assessed in a manner similar to that described for panel A. Degradation of p53 and Mre11 by E3 ligase formed with Ad12 E1B55K with Ad4 E4orf6 was assessed in a manner similar to that described for panel B. (D i and D ii) Blockage of substrate degradation with E3 ligase formed with Ad5 E4orf6 and Ad9 E1B55K. Association of Ad12 E1B55K with Ad4 E4orf6 was assessed in a manner similar to that described for panel A. Degradation of p53 and Mre11 by the E3 ligase formed with Ad12 E1B55K with Ad4 E4orf6 was assessed in a manner similar to that described for panel B.

FIG 6 Models for substrate degradation by the E4orf6/E1B55K ligase. Four scenarios representing models for substrate binding and degradation by the ligase are presented. (1) The substrate (sub) optimally binds to the complex for efficient ubiquitination and degradation. (2) The substrate cannot be recruited to the complex and thus is not degraded. (3) Although the substrate can bind to the complex, it is not in an appropriate orientation for efficient ubiquitination by E2 and thus it is not degraded. (4) Although the substrate is degraded, no association with the complex is detected by coimmunoprecipitation due to its transient, weak, or unstable binding.

range of substrate degradation, Ad12 and Ad40, both utilize Cul2. However, Ad5 exhibits an equally broad substrate range and it utilizes Cul5. We are currently conducting further studies related to Cullin selection by adenovirus E4orf6 proteins.

As discussed at least in part in recent reviews (52, 53), substrate degradation mediated by adenovirus E1B55K and E4orf6 products can occur by a variety of processes. At present, degradation of the majority of substrates by all serotypes appears to require the presence of both E4orf6 and E1B55K, where E4orf6 forms the Cullin-based ligase complex and E1B55K recruits the substrate. This situation appears to be the case with most of the substrates we have examined, as in our previous studies little or no degradation was observed in the presence of E4orf6 or E1B55K alone (13). Although E1B55K seems to be the major substrate recognition subunit, E4orf6 has also been shown to play at least a minor role in substrate recruitment in some cases. p53 can bind to Ad5 E4orf6 (3, 44) and, as shown in Fig. 2A, also to Ad4 E4orf6; however, these interactions, presumably with E4orf6 protein present in the Cullin-based ligase complex, were not seen to lead to p53 degradation (13). Nevertheless, a recently identified substrate, TOPBP1, was shown to be degraded by the Ad12 ligase formed by E4orf6 alone in the absence of E1B55K (11). It is possible that TOPBP1 is present in the complex in an orientation sufficient for its ubiquitination and degradation. It is also possible that some other TOPBP1-binding protein introduces it into the E4orf6/Cullin-based ligase complex. A similar situation may exist with Bloom helicase. We found that although degradation of BLM with some serotypes required both E1B55K and E4orf6 (Ad12 and Ad40 for certain), with others (Ad16, Ad34, and Ad4 for certain), E4orf6 alone could induce effective degradation, suggesting a process perhaps comparable to that seen with Ad12 and TOPBP1. A further complexity

FIG 7 E1B55K alignment of the seven model serotypes. Predicted E1B55K protein sequences are shown. An alignment of the E1B55K protein sequences was made using the CLUSTAL W program. The gray shading is based on the percentage of identity as analyzed by Jalview (alignment editor program). The positions of the SUMOylation and phosphorylation sites (- and *, respectively) are also indicated.

is that we consistently found with Ad16 and Ad9 that expression of E1B55K alone induced an increase in BLM levels. We currently have little insight into this effect. Perhaps it is possible that in some cases E1B55K can prevent normal cellular degradation of BLM in a manner similar to that observed with p53 in which E1B55K binding prevents its degradation by Mdm2 (54). In addition, DNA ligase IV can also bind independently to both E4orf6 (55) and E1B55K (5); however, efficient degradation requires the presence of both viral proteins. It remains possible that in addition to recruiting substrate, the binding of E1B55K to the E4orf6/Cullin ligase complex might also introduce key conformational changes to optimize ubiquitination of some substrates. Finally, there is currently one example thus far of a substrate, Daxx, that is degraded by Ad5 E1B55K in the absence of E4orf6 in a SUMOylation- and proteasome-dependent process, although the E3 ubiquitin ligase involved has not been identified (10, 26, 56). There is clearly still much to be learned about these processes.

p53 is believed by at least some investigators to represent a major roadblock for the adenovirus life cycle (39, 57, 58). Ad5 is known to counteract the effect of p53 by three different strategies. p53 is targeted for proteasomal degradation by the E4orf6/E1B55K E3 ligase (1, 2, 19, 45, 46, 59), and in some cases, both E1B55K (14, 28, 29, 32, 36, 37) and E4orf6 (16, 44, 46) can individually bind to p53 and suppress its transcriptional transactivation. Only Ad5, Ad12, and Ad40 effectively degrade p53; however, E1B55K of all serotypes except Ad4 can suppress p53 transcriptional activation. Ad4 may compensate for the failure of its E1B55K protein to bind p53 in that the Ad4 (and Ad5) E4orf6 protein binds efficiently to p53 and suppresses its transcriptional transactivation. Thus, some serotypes seem to have evolved to target p53 using different and often redundant pathways.

Ad5 E1B55K is phosphorylated at Ser 490 and 491 and Thr 495 (5, 60, 61) and SUMOylated at Lys 104 in the ψ KXE motif (where ψ is hydrophobic) (24). Recently studies suggested that phosphorylation of E1B55K at the C terminus is required for its subsequent SUMOylation (56). Figure 7 shows that the SUMOylation motif is conserved in all serotypes except for Ad12 and Ad40 (the motif for Ad9 is shifted to the right in the alignment); however, an Ad5 E1B55K mutant defective in SUMOylation still exhibits efficient p53 and Mre11 degradation (62). The first two phosphorylation sites are highly conserved, while the third is not found in Ad12 and Ad40. The Ad5 triple-phosphorylation mutant (3XPA) has been found to be defective in binding and degrading p53, Mre11, and DNA ligase IV in studies involving virus-infected cells (15), suggesting an important role of phosphorylation in substrate degradation of the ligase. However, in DNA transfection studies, only p53 degradation was found to be defective and mutation of the first two phosphorylation sites still resulted in binding and degradation of p53 (15). Although Ad12 and Ad40 do not contain the last phosphorylation site, they can still degrade p53. Thus, further studies on the posttranslational modification profiles of different E1B55K proteins could lead to a better understanding of the role of E1B55K in substrate recruitment.

Figure 8 shows the evolutionary trees of E1B55K and E4orf6 sequences and those of hexon and fiber from the serotypes we have tested. Those of hexon and fiber differ from each other, whereas those of E4orf6 and E1B55K are highly similar. These patterns strongly suggest that E1B55K and E4orf6 coevolved, probably to preserve their common functions through the formation of the E4orf6/E1B55K E3 ligase. These findings indicate the primal im-

FIG 8 Phylogenetic trees of E1B55K, E4orf6, hexon, and fiber proteins of model serotypes. Phylogenetic relationships of E1B55K, E4orf6, hexon, and fiber sequences from seven human adenovirus serotypes are shown. The protein sequences of E1B55K, E4orf6, hexon, and fiber of the seven serotypes were analyzed, and their respective phylogenetic trees (presented as phenograms with branch lengths) were created as described in Materials and Methods.

portance of the E4orf6/E1B55K E3 ligase in the survival of all human adenoviruses. E4orf6 and E1B55K sequences are also highly conserved in adenoviruses that infect other species, thus indicating that the formation and function of the ligase may be maintained and may represent a common essential function among all adenoviruses.

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