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Adenovirus Exploits the Cellular Aggresome Response To Accelerate Inactivation of the MRN Complex

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Results reported here indicate that adenovirus 5 exploits the cellular aggresome response to accelerate inactivation of MRE11-RAD50-NBS1 (MRN) complexes that otherwise inhibit viral DNA replication and packaging. Aggresomes are cytoplasmic inclusion bodies, observed in many degenerative diseases, that are formed from aggregated proteins by dynein-dependent retrograde transport on microtubules to the microtubule organizing center. Viral E1B-55K protein forms aggresomes that sequester p53 and MRN in transformed cells and in cells transfected with an E1B-55K expression vector. During adenovirus infection, the viral protein E4orf3 associates with MRN in promyelocytic leukemia protein nuclear bodies before MRN is bound by E1B-55K. Either E4orf3 or E4orf6 is required in addition to E1B-55K for E1B-55K aggresome formation and MRE11 export to aggresomes in adenovirus-infected cells. Aggresome formation contributes to the protection of viral DNA from MRN activity by sequestering MRN in the cytoplasm and greatly accelerating its degradation by proteosomes following its ubiquitination by the E1B-55K/E4orf6/elongin BC/Cullin5/Rbx1 ubiquitin ligase. Our results show that aggresomes significantly accelerate protein degradation by the ubiquitin-proteosome system. The observation that a normal cellular protein is inactivated when sequestered into an aggresome through association with an aggresome-inducing protein has implications for the potential cytotoxicity of aggresome-like inclusion bodies in degenerative diseases.

There has been intense interest in the functions of adenovirus 5 (Ad5) E1B-55K protein because a viral deletion mutant in this gene (2) is reported to be an effective oncolytic agent against some types of human tumors (3, 49). A better understanding of E1B-55K function might allow prediction of which tumors would be candidates for therapy with this mutant. It might also allow the design of more specific E1B-55K mutants that might be effective against a wider spectrum of tumors.

During a productive Ad5 infection, the viral E1B-55K and E4orf6 proteins associate with each other and with several cellular proteins, including elongins B and C, Cullin5, and Rbx-1, to generate a high-molecular-weight E3 ubiquitin ligase complex that polyubiquitinates p53, stimulating its degradation by proteosomes (25, 48). The MRE11 and RAD50 subunits of the MRE11-RAD50-NBS1 (MRN) complex are also degraded by proteosomes in Ad5-infected cells by a mechanism requiring both E1B-55K and E4orf6, strongly suggesting that they are also substrates of the adenovirus E1B-55K/E4orf6 ubiquitin ligase complex (56). MRN complexes, comprised of the cellular proteins MRE11, RAD50, and NBS1, are required for DNA double-strand break repair (13, 46). The MRN complex binds to DNA ends and has both exo- and endonucleolytic activities that prepare them for nonhomologous end joining (13). MRN complexes bound to DNA ends also activate the ATM kinase that phosphorylates and activates proteins that initiate cell cycle arrest and DNA repair or apoptosis (8, 34). Ad5 inactivates the MRN complex, because failure to do so, following infection with an E4 deletion mutant, results in concatenation of the linear viral DNA (56, 59), interfering with viral DNA packaging into virions, and inhibition of viral DNA replication by a process that does not require viral DNA concatenation (18, 55).

In cells oncogenically transformed by Ad5, E1B-55K is constitutively expressed, but E4orf6 is generally not expressed because the E4 region is not integrated into cellular DNA (54). The viral ubiquitin ligase complex cannot assemble in the absence of E4orf6 (25, 48). Instead of being degraded in transformed cells, p53 is bound by E1B-55K (51) and stabilized (36), and much of it is found together with E1B-55K in large juxtanuclear cytoplasmic bodies (66) enriched for HSP70 and associated with the microtubule organizing center (MTOC) (6).

We report here that the cytoplasmic inclusion bodies of E1B-55K in transformed 293 cells also contain MRN complexes and fit the criteria of aggresomes, cytoplasmic inclusion bodies formed at the MTOC by the coalescence of individual small protein aggregates into a single or a few cellular foci by a process that requires dynein-based retrograde transport on microtubules (21, 33). Aggresome formation in response to the accumulation of misfolded, aggregated proteins is proposed to be the mechanism by which cytoplasmic inclusion bodies form in neurons in Parkinson's disease (42), familial amyotrophic lateral sclerosis (30), and spinobulbar muscular atrophy (58). Misfolded, aggregated proteins are thought to accumulate when their rate of synthesis exceeds their rate of degradation. Aggresomes are proposed to increase the rate of proteosomal degradation of misfolded proteins by concentrating substrates with components of the ubiquitin-proteosome system (21, 33, 58). However, most studies of aggresomes have employed pro-

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teosomal inhibitors or the overexpression of aggregation-prone proteins to cause their accumulation into aggresomes, raising the question of whether similar mechanisms underlie the formation of cytoplasmic inclusion bodies in untreated cells.

We also observed that Ad5 E1B-55K expressed at normal levels during the course of a productive viral infection forms aggresomes in the absence of proteosome inhibitors or any other drug treatments. Cellular MRN complexes are exported from the nucleus into the cytoplasmic E1B-55K aggresomes, but unlike the situation in Ad5-transformed 293 cells, during a productive infection most p53 remains nuclear. The results indicate that Ad5 exploits the cellular aggresome response to facilitate the inactivation and degradation of the MRN complex. Sequestration of MRN complexes into aggresomes inhibits their function in advance of their ultimate degradation and significantly stimulates the rate of their degradation by proteosomes. The results strongly support the suggestion (21, 33, 58) that aggresomes do indeed facilitate the proteosomal degradation of polyubiquitinated proteins.

MATERIALS AND METHODS

Antibodies and reagents. Rabbit polyclonal antibodies were to MRE11 and NBS1 (Novus Biologicals), vimentin (Lab Vision Corp.), γ-tubulin (Sigma), and p53 (Santa Cruz). Monoclonal antibodies were to E1B-55K (2A6) (52), E4orf3 (6A11) (40), E4orf6 (M45) (41), RAD50 (GeneTex), and Flag epitope (M2; Sigma). Fluorescein isothiocyanate-conjugated goat anti-mouse antibody and Cy3-conjugated goat anti-rabbit antibody were from Jackson Immunology Research and Rockland Inc., respectively. DAPI (4′,6′-diamidino-2-phenylindole; Molecular Probes), nocodazole (used at 10 μg/μl; Sigma), lepotomycin B (20 nM; LC Laboratories), and MG132 (10 μM; Calbiochem) were from the indicated suppliers.

DNA constructs and viruses. The E1B-55K expression vector pSRαE1B-55K and mutant derivatives were described previously (63, 64). The p50/dynamitin expression vector (pCMVH50myc) (17) was a gift from Richard B. Vallee, Columbia University, New York, N.Y. Adenovirus mutant *dl*1520 (2) and mutants expressing E1B-55K with four-amino-acid insertions (64) were described previously. Ad5 mutants *dl*355 (defective in E4orf6) (12), E4*in*ORF3, and E4*in*ORF3/*dl*355* (29) were gifts from Pat Hearing, State University of New York, Stoneybrook. Ad5 and *dl*1520 were plaque assayed on 293 cells (53), and *dl*355, E4*in*ORF3, and E4*in*ORF3/*dl*355* were plaque assayed on W162 cells (60). Ad5 was plaque assayed simultaneously on 293 and W162 cells, and the ratio of PFU/ml on 293 to that on W162 (30) was used to correct the W162 cell titers of *dl*355, E4*in*ORF3, and E4*in*ORF3/*dl*355* to equivalent titers on 293 cells (35). All experiments shown were performed with a multiplicity of infection of 25, based on the 293 cell titer.

Cell culture and transfection. 293, A549, IMR90, and H1299 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin, 100 μg/ml streptomycin at 37°C in 5% CO_2. Murine embryonic stem cells were grown in Knockout-Dulbecco's modified Eagle's medium (GIBCO) supplemented with leukocyte inhibitory factor. Cells growing on eight-chamber coverslips were transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Immunofluorescence microscopy. Cells grown on coverslips were washed three times in phosphate-buffered saline (PBS) and fixed with methanol for 15 min at −20°C. The coverslips were then washed six times with PBS-0.1% Tween 20 and then blocked in PBS, 10% bovine serum, and 0.1% Tween 20 for 1 h. Cells were incubated with primary antibody diluted in PBS, 0.1% Tween 20 for 1 h at 37°C or overnight at 4°C. Coverslips were washed six times for 1 min each time with PBS and 0.1% Tween 20. Secondary antibodies were diluted in PBS, 5 or 10% bovine serum, and 0.1% Tween 20 and incubated on coverslips for 1 h at 37°C. The coverslips were washed as described above and mounted on slides in medium containing DAPI or Topro 3 as indicated in the figures.

Protein mass spectrometry. E1B-55K was immunoprecipitated directly from nuclear extract (25) of 293 cells with monoclonal antibody 2A6 and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and silver-stained bands were excised and digested in gel with trypsin as described previously (25). Proteins were identified by matrix-assisted laser desorption ionization peptide mapping.

RESULTS

E1B-55K forms aggresomes in transformed cells. The E1B-55K substrate binding subunit of the Ad5-induced ubiquitin ligase binds the MRN complex in addition to p53 (8, 51). Consistent with this, the MRE11 and RAD50 subunits of the MRN complex were found at levels comparable to that of p53 in an anti-E1B-55K immunoprecipitate of an extract of Ad5-transformed 293 cells that express E1B-55K but not E4orf6 (Fig. 1A). The NBS1 subunit of the MRN complex was less prominent in the silver-stained gel of the immunoprecipitate but could be distinguished by immunoblotting (data not shown). Since p53 colocalizes with E1B-55K in large juxtanuclear cytoplasmic bodies in Ad5-transformed cells (66), we asked if the MRN complex is also found in these structures. Immunofluorescence microscopy showed that MRE11 and NBS1 do indeed colocalize with E1B-55K in large juxtanuclear cytoplasmic bodies similar to those observed with p53 (Fig. 1B to D). Costaining with antibody to RAD50 and p53 showed that the two proteins localize to the same cytoplasmic bodies, as revealed by the merged image (Fig. 1E), although some of the cytoplasmic bodies were enriched for either p53 or RAD50, as shown by their red or green color, respectively, in the merged image.

We also observed the intermediate filament protein vimentin and HSP70 in anti-E1B-55K immunoprecipitates from 293 cell extract at levels comparable to those of p53 and the MRN complex subunits (Fig. 1A). This, and the colocalization of these cytoplasmic bodies with the MTOC noted earlier (6), raised the question of whether these cytoplasmic bodies are examples of aggresomes, which are localized to the region of the MTOC, associated with a high concentration of HSP70, and often surrounded by a cage of vimentin (21, 33).

Similar large juxtanuclear cytoplasmic bodies containing E1B-55K were observed in cells transfected with an E1B-55K expression vector, showing that no other viral proteins are required for the formation of these subcellular structures. The cytoplasmic localization of Ad5 E1B-55K expressed in the absence of other viral proteins has been reported previously (4, 8, 15, 22, 35, 43, 61). These large juxtanuclear cytoplasmic bodies were observed in mouse embryonic stem cells, human diploid fibroblasts, and A549 p53-plus and H1299 p53-minus cells derived from human lung tumors (Fig. 2A). In 293 cells and human diploid fibroblasts (Fig. 2B), this cytoplasmic body colocalized with γ-tubulin, indicating its association with the MTOC, a characteristic of aggresomes (32). Costaining of E1B-55K vector-transfected A549 and H1299 cells with antibodies to E1B-55K and vimentin revealed that the cytoplasmic body is surrounded by a vimentin cage (Fig. 2C), another characteristic of aggresomes (32).

Aggresome formation is inhibited by nocodazole, which causes the depolymerization of microtubules (32, 62). Treatment of 293 cells with nocodazole for 12 h led to the formation of multiple smaller aggregates of E1B-55K throughout the cytoplasm in >90% of cells (Fig. 2D). Nocodazole treatment of E1B-55K vector-transfected A549 cells also greatly increased the fraction of E1B-55K-expressing cells that con-

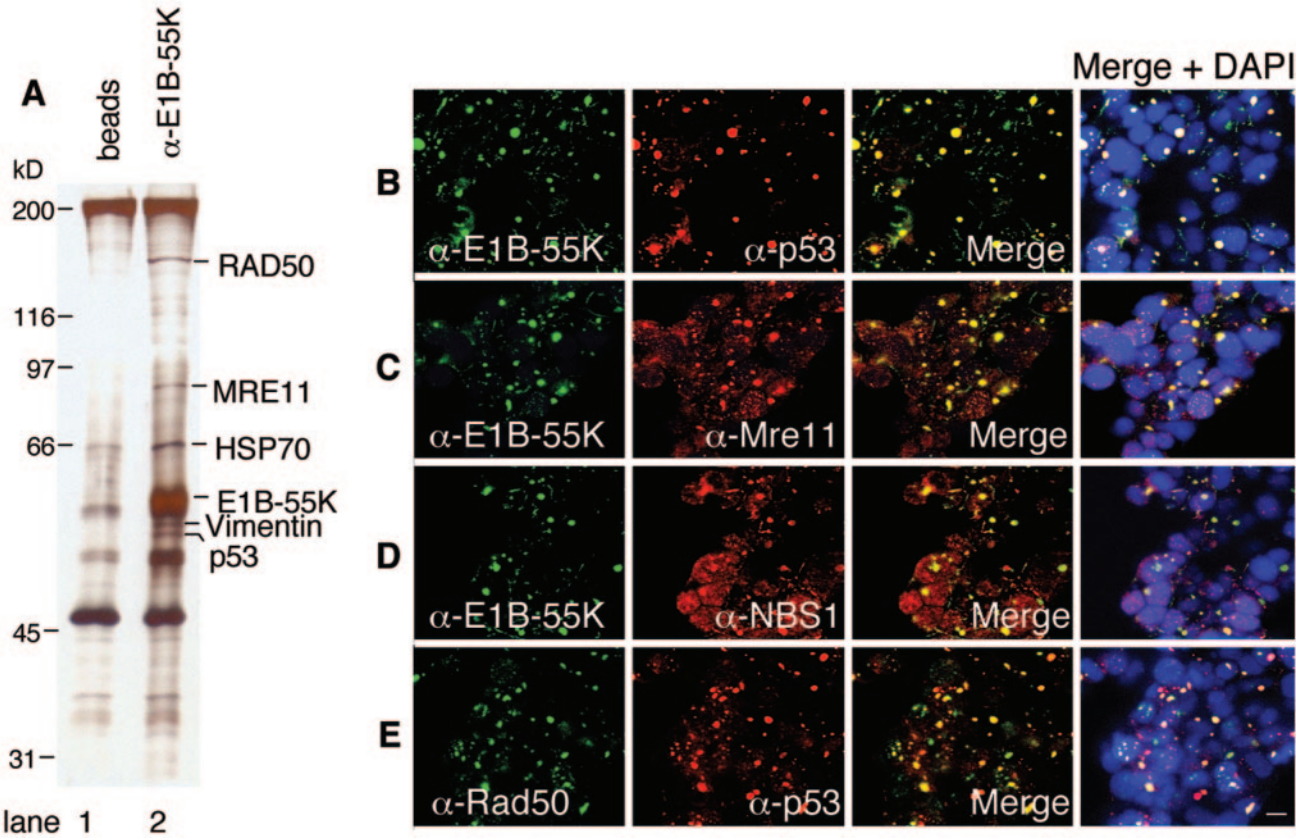


FIG. 1. The MRN complex is localized to cytoplasmic juxtanuclear bodies with p53 in Ad5-transformed 293 cells. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of protein immunoprecipitated from 293 cell extract by anti-E1B-55K monoclonal antibody (lane 2) and bound to the protein A-Sepharose beads alone (lane 1). The identifications shown on the right were from matrix-assisted laser desorption ionization–mass spectrometry. (B to E) Immunofluorescence of fixed and permeabilized 293 cells using the indicated antibodies. The first two micrographs in each row are merged in the third panel and merged again with DAPI signal in the fourth panel. Bar, 5 μm.

tained similar smaller dispersed aggregates of E1B-55K (Fig. 2E).

Transfection of a strong cytomegalovirus immediate-early promoter/enhancer-based expression vector for the p50/dynamitin component of the dynein/dynactin complex (27) inhibits dynein transport (17) because excess p50/dynamitin disrupts the connection between cargo and the dynein motor protein. As a consequence, overexpression of p50/dynamitin disrupts the formation of aggresomes (20, 31). It also resulted in a large increase in the fraction of E1B-55K vector-transfected A549 cells containing dispersed small E1B-55K aggregates (Fig. 2F and G). In contrast, expression of E4orf6 or Cullin5 from a similar cytomegalovirus immediate-early promoter/enhancer expression vector did not disrupt aggresome formation (Fig. 3A and B). It is unlikely that the observation of these dispersed E1B-55K aggregates in the cytoplasm (Fig. 2F) was the result of a fixation artifact, since several nonfunctional E1B-55K insertion mutants were evenly distributed through the cytoplasm following the same fixation protocol (Fig. 3C). Based on these results, we conclude that the juxtanuclear cytoplasmic bodies in Ad5-transformed cells containing E1B-55K and p53 observed for 20 years (66) are examples of aggresomes that also contain a large fraction of the cellular MRN complexes.

E1B-55K actively participates in aggresome formation. During a productive infection of permissive cells with Ad5, most E1B-55K associates with E4orf6 (25, 50). The fact that E4orf6 is not expressed in 293 cells or most Ad5-transformed rodent cells (54) raised the question of whether E1B-55K is found in aggresomes in these transformed cells, because it aggregates in the absence of its normal E4orf6 partner. Aggregation could occur either because the protein does not fold correctly in the absence of E4orf6 or because a hydrophobic surface that normally interacts with E4orf6 becomes exposed. As a result of such aggregation due to the absence of E4orf6, E1B-55K might be sequestered into aggresomes entirely through the action of cellular processes without any active participation in the aggresome formation process by the viral protein. However, several observations indicate that E1B-55K in 293 cell aggresomes is not extensively misfolded or aggregated and that it actively participates in the formation of these aggresomes.

First, since E1B-55K binds p53 and the MRN complex in 293 cell aggresomes (Fig. 1B to E), the E1B-55K protein domains involved in these interactions must be properly folded. Second, when an E4orf6 expression vector was transfected into 293 cells, the ~800-kDa viral ubiquitin ligase with E1B-55K, E4orf6, elongins B and C, Cullin5, and Rbx1 assembled in

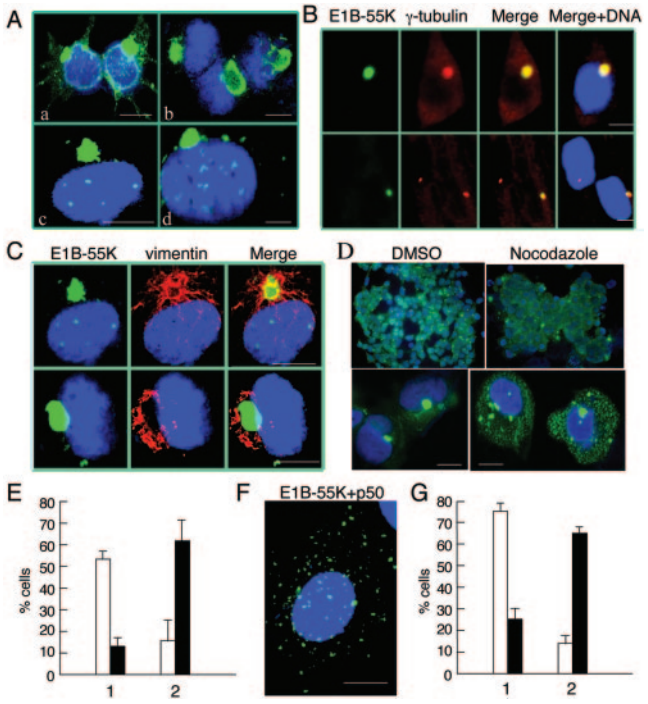


FIG. 2. E1B-55K is sequestered in aggresomes. (A) An E1B-55K expression vector was transfected into (a) mouse embryonic stem cells, (b) H1299 cells (p53-minus), (c) A549 cells (p53-plus), and (d) IMR90 diploid human fibroblasts, and the cells were examined by immunofluorescence with anti-E1B-55K monoclonal antibody. (B) 293 cells (top) and IMR90 cells transfected with an E1B-55K expression vector (bottom) were analyzed by immunofluorescence for E1B-55K (green) and γ-tubulin (red). (C) A549 cells (top) and H1299 cells (bottom) were transfected with an E1B-55K vector and stained for E1B-55K (green) and vimentin (red). (D) 293 cells at low and high magnifications treated for 12 h with nocodazole (right) or DMSO vehicle control (left). (E) Fractions of E1B-55K-expressing A549 cells transfected with an E1B-55K vector and treated with nocodazole (black bars) or DMSO vehicle control (white bars) exhibiting (bars 1) a large juxtanuclear body or (bars 2) dispersed aggregates of E1B-55K as in panel F. (F) A549 cell cotransfected with E1B-55K and p50/dynamitin expression vectors and stained with anti-E1B-55K (green) and DAPI. (G) Fractions of E1B-55K-expressing A549 cells cotransfected with an E1B-55K and p50/dynamitin expression vector (black bars) or an E1B-55K vector and the empty vector used for p50 expression (white bars) exhibiting E1B-55K staining as described in the legend to panel E. The error bars indicate standard deviations; $n = 4$ for panels E and G. The total percent cells is <100% because the remaining fraction of cells expressed E1B-55K at such a high level that the E1B-55K signal filled >30% of the cytoplasm, making it difficult to distinguish type 1 and 2 cells. Bars, 5 μm.

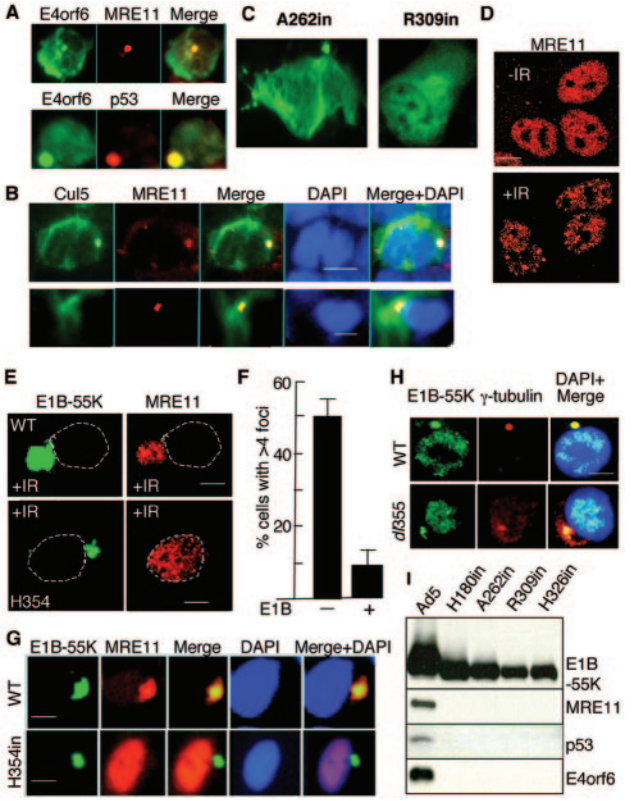
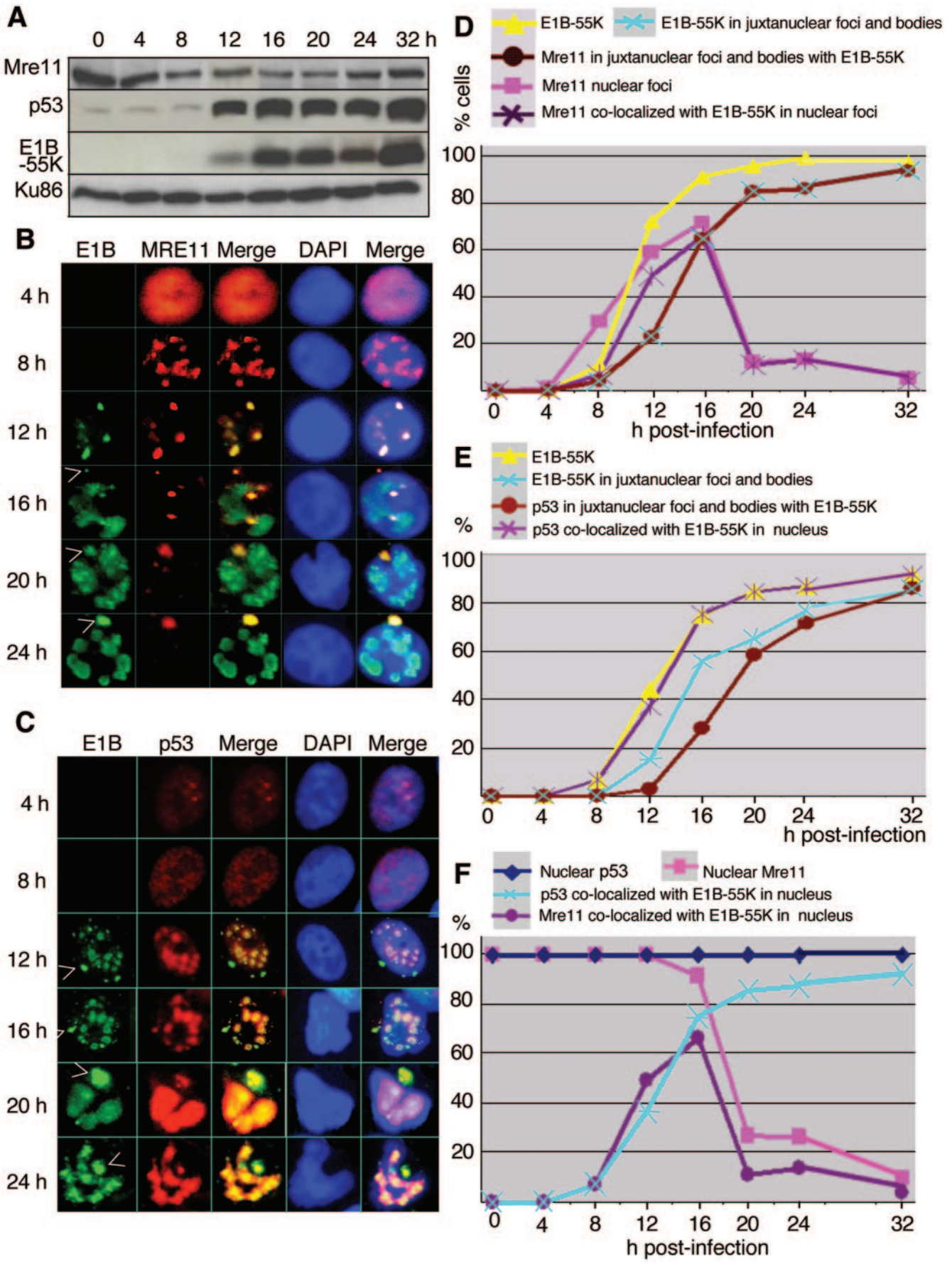


FIG. 3. E1B-55K in aggresomes is functional and inactivates MRN complexes. (A) 293 cells transfected with an E4orf6 vector and immunostained with anti-E4orf6 (green) and anti-MRE11 (red). (B) 293 cells transfected with expression vectors for E4orf6 and Flag-tagged Cullin5 and immunostained with anti-Flag (green) and anti-MRE11 (red). (C) E1B-55K mutants A262in and R309in expressed in A549 cells and detected with anti-E1B-55K. (D) Confocal microscopy of A549 cells stained with anti-MRE11. +IR, 8 h after 12-Gy gamma-irradiation. −IR, no irradiation. (E) Confocal micrograph of A549 cells transfected with expression vectors for wt E1B-55K (top) or insertion mutant H354 (bottom) irradiated as in panel D and stained for E1B-55K (green) and MRE11 (red). Nuclei are shown by dotted lines. (F) Fraction of A549 cells transfected with an E1B-55K expression vector and irradiated as in panel E that exhibited >4 MRE11 nuclear foci. + and −, cells that did or did not express E1B-55K. (G) A549 cells transfected with a vector for wt (top) or insertion mutant H354in (bottom) and immunostained for E1B-55K (green) and MRE11 (red). (H) A549 cells 20 h after infection with *dl*355 or wt Ad5 stained for E1B-55K (green) or γ-tubulin (red) and with DAPI. (I) A549 cells were infected with the indicated E1B-55K four-amino-acid insertion mutants. Extracts were prepared 16 h p.i., immunoprecipitated with anti-E1B-55K monoclonal antibody 2A6, and Western blotted with the antibodies indicated on the right. Bars, 5 μm.

transfected cells (25). When such cells were analyzed by immunofluorescence 24 h after transfection, p53 and MRE11 were depleted from most cells expressing E4orf6. In those cells where p53 and MRE11 were detected, they were associated with aggresomes containing E4orf6 (Fig. 3A). Similarly, when an expression vector for Flag-tagged Cullin5 was included in the transfection, Cullin5 also concentrated in aggresomes with MRE11 (Fig. 3B), whereas a control Flag-tagged protein, Flag-MED16, did not. These results suggest that E4orf6 bound to E1B-55K in the 293 cell aggresomes and assembled a functional viral ubiquitin ligase that polyubiquitinated p53 and MRE11, causing their proteosomal degradation. Consequently, it seems unlikely that the E1B-55K in 293 cell aggresomes is extensively aggregated, since it appears to interact with p53, MRE11, E4orf6, and Cullin5 and to assemble into a functional ubiquitin ligase, leading to the degradation of p53 and MRE11.

Further evidence that E1B-55K actively participates in the formation of aggresomes came from the observation that insertion mutations that inactive E1B-55K inhibit the ability of the viral protein to induce aggresome formation. We analyzed a set of 13 E1B-55K mutants with four-amino-acid insertions along the length of the 495-residue protein (64) for the ability


A
0 4 8 12 16 20 24 32 h
Mre11
p53
E1B -55K
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B
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4 h
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16 h
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24 h
C
E1B p53 Merge DAPI Merge
4 h
8 h
12 h
16 h
20 h
24 h
D
E1B-55K
E1B-55K in juxtanuclear foci and bodies
Mre11 in juxtanuclear foci and bodies with E1B-55K
Mre11 nuclear foci
Mre11 co-localized with E1B-55K in nuclear foci
% cells
100
80
60
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0 4 8 12 16 20 24 32
h post-infection
E
E1B-55K
E1B-55K in juxtanuclear foci and bodies
p53 in juxtanuclear foci and bodies with E1B-55K
p53 co-localized with E1B-55K in nucleus
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Nuclear p53
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Mre11 co-localized with E1B-55K in nucleus
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to interact with E4orf6, p53, and the MRN complex, as well as the ability to form aggresomes. Protein interactions were assayed by coimmunoprecipitation from extracts of mutant-virus-infected A549 cells. Four mutants, H180in, A262in, R309in, and H326in (the numbers indicate the amino acid immediately before the insertion), were significantly inhibited from interacting with each of these proteins (Fig. 3I). Viral replication of these mutants is decreased to the same extent as for a deletion of the E1B-55K coding region (64). The failure of these mutants to interact with any of the known E1B-55K-interacting proteins suggests that these insertion mutations cause misfolding and inactivation of the protein. These same insertion mutations also eliminated the ability of E1B-55K to induce aggresome formation. Instead, they became dispersed throughout the nucleus and cytoplasm of transfected or infected cells (results for A262 and R309 are shown in Fig. 3C). Taken together, these results indicate that the E1B-55K protein in aggresomes is correctly or largely correctly folded and functional. The observation that several insertion mutations that inactivate all known E1B-55K protein binding activities also inactivate aggresome formation strongly suggests that wild-type (wt) E1B-55K actively participates in the aggresome formation process.

MRN complex is inactivated by sequestration into an aggresome. Using retrovirus vectors, we were unable to establish stably transformed A549 or IMR90 cells that expressed E1B-55K at levels comparable to those observed in productively infected cells. Consequently, we analyzed MRN function in the presence of E1B-55K using a cell-based immunofluorescence assay of cells transiently transfected with an E1B-55K expression vector. The association of MRN complexes with DNA double-strand breaks causes the formation of nuclear foci of MRE11 in gamma-irradiated cells (Fig. 3D) (13, 46). To analyze the consequences of aggresome association for MRN function, A549 cells were transfected with an E1B-55K vector, subjected to gamma-irradiation, and assayed for E1B-55K expression and the generation of MRE11 nuclear foci. Fifty percent of cells that did not express E1B-55K contained >4 MRE11 nuclear foci. However, only 10% of the cells in the same transfected culture that did express E1B-55K contained similar nuclear foci, because most of the MRE11 was sequestered in the E1B-55K aggresomes (Fig. 3E and F). Insertion mutant H354in (64) does not bind the MRN complex (8) but formed aggresome-like structures that failed to sequester MRE11 (Fig. 3G). Cells transfected with mutant H354in formed MRE11 nuclear foci to the same extent as non-H354in-expressing cells (Fig. 3E). These results indicate that sequestration of MRN complexes into E1B-55K aggresomes inhibits MRN function.

E1B-55K forms aggresomes during a productive viral infection. To determine if E1B-55K aggresomes form during viral infection, we examined A549 cells after infection with wt Ad5 and the E4orf6 deletion mutant *dl*355 (23). Since the absence of E4orf6 prevents degradation of p53 (25, 48) and MRN (56), infection with *dl*355 makes it possible to analyze changes in the subcellular localization of these proteins in the absence of their concomitant degradation. Increasing E1B-55K expression was detected by immunoblotting beginning 8 to 12 h postinfection (p.i.) (Fig. 4A). p53 concentration increased following E1B-55K expression because of stabilization by E1B-55K in the absence of E4orf6 (36), while MRE11 levels decreased modestly. The subcellular localization of E1B-55K and MRN (Fig. 4B and D) and of E1B-55K and p53 (Fig. 4C and E) were analyzed by immunofluorescence. At 4 h p.i., MRE11 and p53 were spread diffusely through the nucleoplasm, as in uninfected cells. In a small fraction of cells, p53 also was observed in a small number of nuclear foci. By 8 h p.i., MRE11, but not p53, coalesced into distinct large nuclear foci in 30% of cells. E1B-55K was first detected in a large fraction of cells at 12 h p.i., principally in nuclear foci, most of which were colocalized with foci of MRN and stabilized p53. At 12 h p.i., E1B-55K was also detected in small juxtanuclear foci in ~20% of cells (Fig. 4C). After 16 h p.i., the percentage of cells with E1B-55K juxtanuclear foci increased dramatically to >90% by 32 h p.i., and the foci increased in size. By 20 h p.i., they resembled the E1B-55K aggresomes observed in 293 cells (Fig. 1) and cells transfected with an E1B-55K expression vector (Fig. 2A).

Juxtanuclear foci and bodies containing E1B-55K also appeared with a similar time course in wt Ad5-infected cells (Fig. 5). These were associated with γ-tubulin (Fig. 3H) and consequently had the properties of aggresomes. However, unlike the situation in cells transfected with an E1B-55K expression vector, where E1B-55K was the only viral protein expressed, and in 293 cells, where only the E1A and E1B viral proteins are expressed, in cells infected with *dl*355 and wt Ad5, most E1B-55K was present in large nuclear foci or domains from the time it was first detected (12 h p.i.) throughout the remainder of the infection. This internuclear E1B-55K was earlier found to be associated with viral replication centers in wt Ad5-infected cells (44).

The subcellular distribution of MRN and p53 differed markedly after 16 h after infection with *dl*355 (Fig. 4B and C). The fraction of cells with detectable nuclear MRE11 fell precipitously between 16 and 20 h p.i. (Fig. 4F). By 20 h after infection with *dl*355, most MRE11 became associated with juxtanuclear E1B-55K (Fig. 4B and D). In contrast, while p53 also was observed in juxtanuclear foci and bodies at ≥16 h p.i., most p53 remained in the nucleus associated with the nuclear E1B-55K (Fig. 4C, E, and F).

The kinetics of E1B-55K and MRE11 localization in *dl*355-infected cells (Fig. 4D) suggested that E1B-55K first associates with MRE11 in the nucleus and then is transported into the E1B-55K aggresomes in nearly 100% of cells by 32 h. p.i. We tested this hypothesis by treating *dl*355-infected cells with leptomycin B, an inhibitor of the CRM1 exportin that interacts

FIG. 4. E1B-55K aggresomes form and sequester MRN complexes in cells infected with an adenovirus mutant in the viral ubiquitin ligase. (A) Western blot of indicated proteins. In panels A to C, h is hours after infection of A549 cells with the E4orf6 deletion mutant *dl*355. Ku86 is a loading control. (B and C) Immunostaining for E1B-55K (green) and MRE11 (B) or p53 (red) (C) at the indicated time p.i. The arrowheads indicate aggresomes. (D to F) Cells (>120) were examined by immunofluorescence as for panels B and C at each time point and scored for the expression and localization of proteins as indicated.

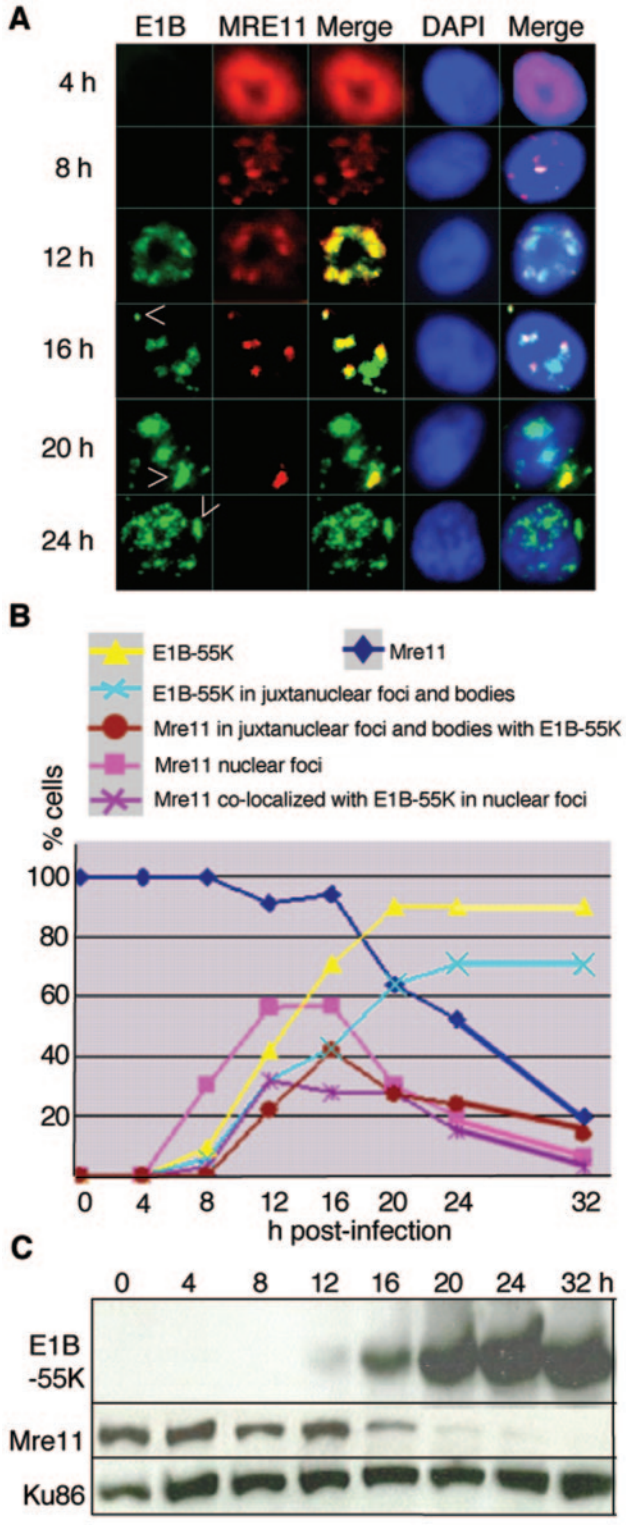

FIG. 5. E1B-55K aggresomes form in wt Ad5-infected cells. (A and B) As in Fig. 4, except that h is hours after infection with wt Ad5. Arrowheads, MRE11 observed in E1B-55K juxtanuclear foci in cells infected with wt Ad5. (C) Western blots of indicated proteins.

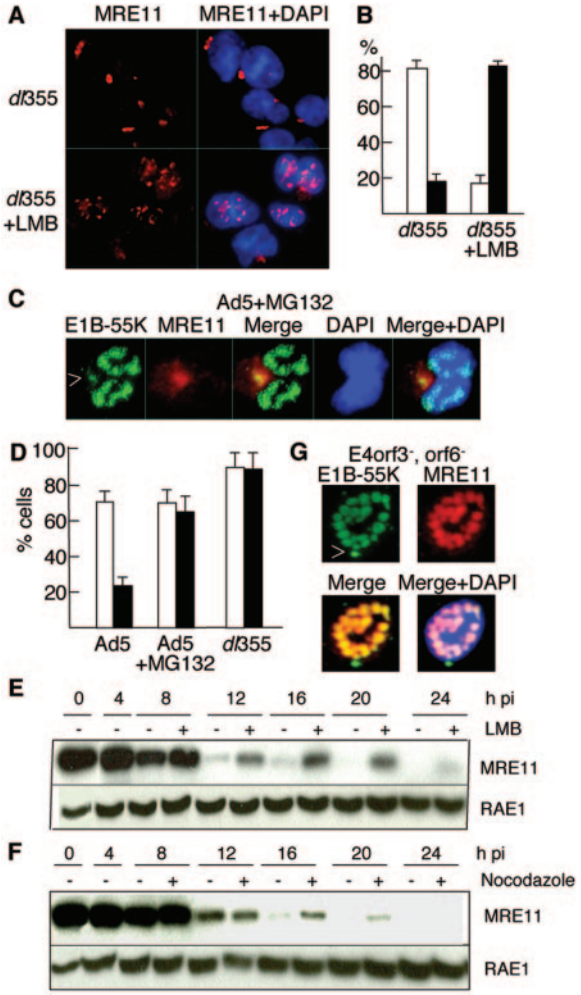

FIG. 6. Nuclear export of MRN complexes to E1B-55K aggresomes increases the rate of their proteosomal degradation. (A) Leptomycin B (LMB) was added to A549 cells infected with the E4orf6 deletion mutant *dl*355 at 8 h p.i., and treated and control cells were stained with anti-MRE11 (red) and DAPI at 24 h p.i. (B) Fractions of *dl*355-infected cells treated and untreated with leptomycin B exhibiting MRE11 in juxtanuclear bodies (white bar) and/or in nuclear foci (black bar). The error bars indicate standard deviations; $n = 4$. (C) A549 cells infected with wt Ad5 were treated with MG132 at 12 h p.i. and stained with anti-E1B-55K (green), anti-MRE11 (red), and DAPI at 24 h p.i. (D) Fractions of cells at 24 h after infection with juxtanuclear E1B-55K (white bars) and juxtanuclear E1B-55K that costained with MRE11 (black bars). The error bars indicate standard errors. (E) Western blot of MRE11 and RAE1 (as a loading control) in extracts of Ad5-infected A549 cells at the indicated times p.i. and treated with leptomycin B as indicated beginning 4 h p.i. (F) Same as panel E plus and minus nocodazole. (G) A549 cells infected with an E4orf3^{-}/E4orf6^{-} double mutant stained with anti-E1B-55K, anti-MRE11, and DAPI. The arrowheads indicate aggresomes.

with an E1B-55K nuclear export signal (9, 15). Addition of leptomycin B at 8 h p.i. greatly inhibited the appearance of juxtanuclear MRE11 at 24 h p.i. and caused MRE11 to persist in nuclear foci (Fig. 6A and B) associated with E1B-55K (not shown). Leptomycin B treatment did not decrease the expression of E1B-55K during the course of infection, as judged by Western blotting of extracts prepared at 24 h p.i. (data not shown). These results indicate that the association of MRE11

with E1B-55K aggresomes at >16 h after infection of untreated cells does not result from the association of newly synthesized E1B-55K and MRE11 in the cytoplasm but rather from the transport of E1B-55K–MRE11 complexes from the nucleus into the cytoplasm.

MRE11 also was observed in E1B-55K juxtanuclear foci in cells infected with wt Ad5 (Fig. 5A). The appearance of E1B-55K in cytoplasmic juxtanuclear foci and bodies followed a time course in wt Ad5-infected cells similar to that in *dl*355-infected cells (Fig. 5B). However, the fraction of cells with MRE11 colocalized with E1B-55K juxtanuclear foci peaked at 16 h p.i. and then declined, probably because of MRE11 degradation (Fig. 5C). In contrast to MRE11, most p53 remained in nuclear foci, as in *dl*355-infected cells (Fig. 4C and E), with only a small fraction of total p53 associated with E1B-55K aggresomes at any time point (data not shown). The intensity of the p53 signal in both nuclear foci and aggresomes decreased after 20 h p.i. as p53 was degraded.

Aggresome formation increases the rate of MRE11 degradation. The results presented above demonstrate that formation of an E1B-55K aggresome is a normal event late in wt Ad5 infection. In cells infected with wt Ad5 (Fig. 5B) and *dl*355 (Fig. 4D), E1B-55K associated with MRE11 in nuclear foci shortly before juxtanuclear foci of E1B-55K and MRE11 appeared. This time course is consistent with the conclusion from experiments with leptomycin B that aggresomes containing E1B-55K and MRE11 are generated by the nuclear export of E1B-55K/MRN complexes (Fig. 6A and B). These observations are consistent with the model that in wt Ad5-infected cells, a considerable fraction of total MRE11 degradation occurs in aggresomes.

To test this model, we treated wt Ad5-infected cells with MG132 to inhibit proteosomal proteolysis or with leptomycin B to inhibit E1B-55K nuclear export. MG132 led to accumulation of MRE11 with E1B-55K in juxtanuclear cytoplasmic regions and the concomitant depletion of MRE11 from nuclei by 24 h p.i., similar to the situation in *dl*355-infected cells (Fig. 6C and D). This indicates that MRE11 nuclear export continues throughout the course of infection in wt Ad5-infected cells as in cells infected with the E4orf6 deletion mutant *dl*355. Leptomycin B treatment led to a significant decrease in the rate of MRE11 degradation (Fig. 6E). This result indicates that aggresome formation is not required for MRE11 ubiquitination by the viral ubiquitin ligase and subsequent proteosomal degradation but that export to the aggresome accelerates MRE11 degradation significantly. Similarly, treatment of cells with nocodazole, which results in dispersed E1B-55K foci throughout the cytoplasm (as observed with p50 overexpression in Fig. 2F), also decreased the rate of MRE11 degradation (Fig. 6F). Nocodazole treatment did not reduce the expression of E1B-55K, as assayed by Western blotting of cell extracts at 24 h p.i. (not shown).

E4orf3 or E4orf6 is required for nuclear export of the MRN complex to the E1B-55K aggresome during viral infection. The E4 proteins orf3 and orf6 have redundant functions required for viral replication. Expression of either E4orf3 or E4orf6 in the absence of other E4 proteins is sufficient to allow near-normal levels of Ad5 replication, whereas failure to express both E4orf3 and E4orf6 profoundly inhibits viral replication (5, 29). E4orf3 is an 11-kDa protein that interacts with promyelocytic leukemia protein (PML) and alters the morphology of PML nuclear bodies (also known as PML oncogenic domains or nuclear domain 10) (10, 16, 56). E4orf3 is also known to associate with E1B-55K in PML bodies of altered morphology in Ad5-infected cells (35). As for E1B-55K, expression of E4orf3 in the absence of other viral proteins also leads to the appearance of MRN in cytoplasmic foci containing the viral protein (56). To determine if E4orf3 influenced MRN complex nuclear export to E1B-55K aggresomes, we infected A549 cells with the double mutant E4*in*ORF3/*dl*355*, which is mutant in both E4orf3 and E4orf6 (29). E1B-55K aggresomes formed with a much slower time course than in wt Ad5-infected cells or cells infected with *dl*355 mutant only in E4orf6. In addition, the sequestration of MRE11 into these aggresomes was greatly delayed. At 24 h p.i., while >80% of *dl*355-infected cells showed virtually all MRE11 associated with E1B-55K aggresomes (Fig. 4B and D), in the E4orf3/E4orf6 double-mutant-infected cells, only 20% of cells contained an E1B-55K aggresome, and <1% of these (0 of 131 cells) contained MRE11 (Fig. 6G). Thus, although E1B-55K expression in the absence of other viral proteins is sufficient for the formation of E1B-55K aggresomes that sequester MRE11 (Fig. 3E and G), the E4orf3 protein greatly increases the rate of E1B-55K aggresome formation and is required for MRE11 export into these aggresomes during infection with *dl*355 mutant in E4orf6 only.

Consistent with this function of E4orf3 in stimulating nuclear export of the MRN complex into E1B-55K aggresomes, E4orf3 has been observed to associate with MRN in PML nuclear bodies in cells transfected with an E4orf3 expression vector (56), and E4orf3 and E1B-55K associate with each other and with PML during wt Ad5 infection (35). Moreover, the relocalization of MRN complexes to PML nuclear bodies with altered morphology containing E4orf3 is required for viral DNA replication in the absence of E4orf6 (19, 57). We can now add to these earlier observations the order of these events through the course of infection.

All detectable MRE11 associated with E4orf3 in nuclear foci at 8 h p.i. in *dl*355-infected (Fig. 7A) and wt Ad5-infected (not shown) cells. At this time p.i. E4orf3 colocalized with PML (shown for wt Ad5-infected cells in Fig. 7B). Since colocalization was so complete, we conclude that at 8 h. p.i. MRE11 associates with E4orf3 in PML nuclear bodies. This interaction with E4orf3 is probably responsible for the change in the localization of MRE11 from a fairly homogeneous distribution in the nucleoplasm before infection and at 4 h p.i. to the MRN nuclear foci observed in Fig. 4 and 5 at 8 h p.i.

E1B-55K was not observed at 8 h p.i. when MRE11 first associated with E4orf3, but at 12 h p.i., when E1B-55K was first detected and colocalized with MRE11 in nuclear foci (Fig. 4B and 5B), E1B-55K and E4orf3 were also associated with PML (Fig. 7B). These results indicate that E1B-55K joined the MRE11-E4orf3-PML complex by 12 h p.i., before the MRE11–E1B-55K complex was exported into aggresomes in most cells. E4orf3 then appeared with MRE11 in aggresomes in *dl*355-infected cells beginning at 12 h p.i., and then in a larger fraction of cells at later times, with MRE11 being completely exported to aggresomes containing E4orf3 in most cells by 24 h p.i. (Fig. 7A). This is the same time course as the appearance of E1B-55K and MRE11 in aggresomes (Fig. 4B and D). Thus, E4orf3 accompanies E1B-55K during the export

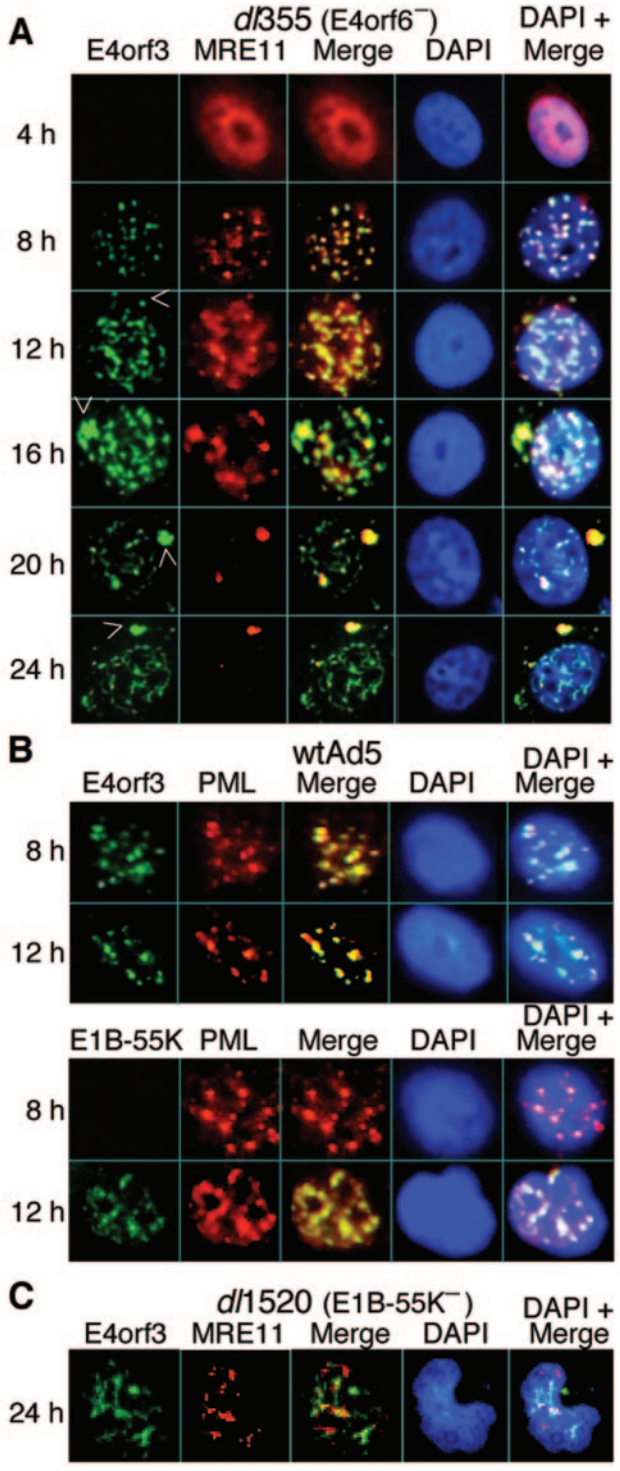

FIG. 7. Interactions between MRE11, E4orf3, PML, and E1B-55K during viral infection. A549 cells infected with the E4orf6 deletion mutant *dl*355 (A), wt Ad5 (B), or the E1B-55K deletion mutant *dl*1520 (C) were stained as indicated at 24 h p.i. The arrowheads indicate aggresomes.

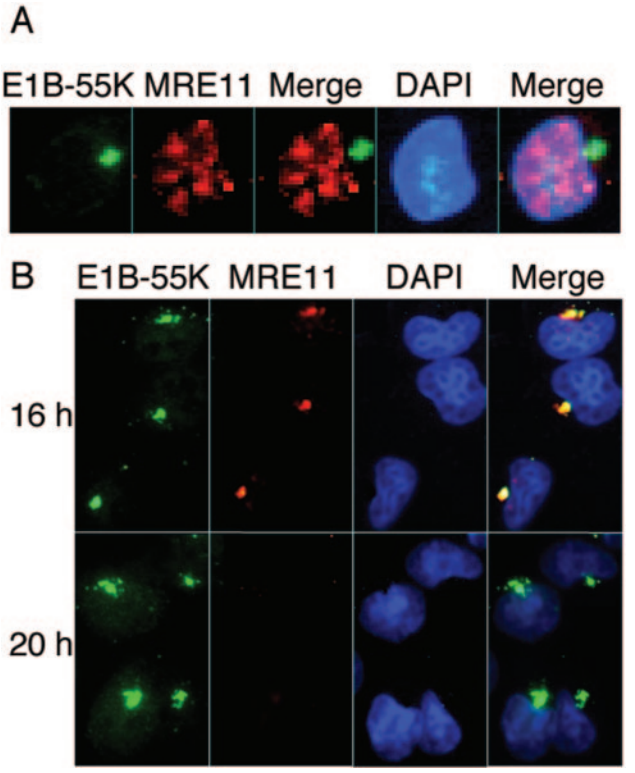

FIG. 8. MRE11 localization and degradation in E1B-55K insertion mutant-infected cells. Immunolocalization of E1B-55K and MRE11 in A549 cells infected with mutant H354in at 24 p.i. (A) or with S380in at 16 and 24 h p.i. (B).

of MRE11 to aggresomes in virus-infected cells. The expression of E4orf3 alone in HeLa cells transfected with an E4orf3 expression vector causes the accumulation of MRN subunits in cytoplasmic bodies that resemble the E1B-55K aggresomes reported here (56). However, E4orf3 is not sufficient for the nuclear export of MRE11 in adenovirus-infected cells, since little MRE11 was exported in cells infected with the E1B-55K deletion mutant *dl*1520 (2) (Fig. 7C) or a virus containing the H354in E1B-55K mutation that prevents binding to MRN (Fig. 8A). These observations indicate that MRE11 first associates with E4orf3 and PML in the PML nuclear domains of altered morphology observed earlier (10, 16, 19, 56, 57). E1B-55K subsequently joins these complexes before MRE11, E1B-55K, and E4orf3 are exported to aggresomes.

We also analyzed E1B-55K and MRE11 subcellular localization and the kinetics of MRE11 degradation in A549 cells infected with mutant E4*in*ORF3 defective in the E4orf3 protein only (29). The percentage of cells exhibiting an E1B-55K aggresome was only modestly decreased compared to cells infected at the same time with wt Ad5, 75% ± 3% for E4*in*ORF3 compared to 85% ± 5% for Ad5 at 24 h p.i. Treatment with MG132 to inhibit MRE11 proteosomal degradation revealed that MRE11 colocalized with these E1B-55K aggresomes in E4*in*ORF3-infected cells (data not shown). Western blotting of cell extracts prepared every 4 h from 4 to 24 h p.i. indicated that the rate of MRE11 degradation was not significantly different in E4*in*ORF3-infected cells than in wt Ad5-infected cells. Since E1B-55K aggresome formation and MRE11 nuclear export to aggresomes were greatly decreased in cells infected with E4*in*ORF3/*dl*355* mutant in both E4orf3 and E4orf6 (Fig. 6G) but were not greatly decreased in cells infected with mutants in only E4orf3 or only E4orf6, it appears

that either of these E4 proteins can function to stimulate E1B-55K aggresome formation and MRE11 export to these aggresomes in adenovirus-infected cells.

Stracker et al. (57) recently reported that when an E4orf3 expression vector was transfected into HeLa cells constitutively expressing E1B-55K from a retroviral vector, E1B-55K was relocalized from cytoplasmic structures that are probably similar to the E1B-55K aggresomes reported here into PML nuclear domains of altered morphology containing E4orf3. This raised the possibility that degradation of MRE11 in 293 cells following transfection of an E4orf3 expression vector occurs primarily in such nuclear structures formed from the transport of E1B-55K/MRN complexes from cytoplasmic aggresomes back into the nucleus. However, we observed that MRE11 remained associated with aggresomes that also contained E4orf3 and Cullin5 in such transfected 293 cells (Fig. 3A and B). Further evidence that MRE11 and p53 degradation occurs in cytoplasmic aggresomes came from analysis of E1B-55K and MRE11 localization in cells infected with the E1B-55K insertion mutant S380in. This mutant replicates in HeLa cells as well as wt Ad5 (64). However, unlike wt E1B-55K, it was localized exclusively in juxtanuclear cytoplasmic bodies throughout infection from the time it was first detected at 12 h p.i. (Fig. 8B, data from 16 and 24 h p.i.). It is unlikely that failure to observe nuclear E1B-55KS380in was due to a fixation artifact, since 10 other E1B-55K insertion mutants described previously (64) (with the exception of H354 and R443, in addition to S380) were observed in both the nucleus and cytoplasm during a viral infection using the same fixation method (data not shown). Western blotting showed that MRE11 and p53 were degraded with a time course similar to that of wt Ad5-infected cells (data not shown), so that no MRE11 was observed by immunofluorescence at 24 h p.i. (Fig. 8B).

DISCUSSION

Adenovirus exploits the cellular aggresome response. These results reveal that adenovirus exploits the cellular aggresome pathway to rapidly inactivate and then degrade the MRN complex following infection. Expression of E1B-55K alone stimulated cells to sequester the viral protein into an aggresome, as observed in transient-transfection assays with an E1B-55K expression vector (Fig. 2A to C). The cytoplasmic localization of E1B-55K expressed in the absence of other viral proteins has been observed previously (4, 8, 15, 22, 35, 43, 61). In Ad5-transformed cells expressing the E1A and E1B proteins only, E1B-55K is found primarily in large juxtanuclear cytoplasmic bodies (66). These juxtanuclear bodies containing E1B-55K satisfy the definition of an aggresome because they are located at the MTOC (Fig. 2B); are surrounded by a vimentin cage (Fig. 2C); are associated with a high concentration of HSP70, as well as vimentin (Fig. 1A); and are dependent for their formation and integrity on the microtubule cytoskeleton, as revealed by their sensitivity to nocodazole treatment (Fig. 2D and E); and their formation requires the function of a dynein motor complex, as shown by their sensitivity to overexpression of dynamitin, which dissociates dynein motor proteins from their cargo (17) (Fig. 2F and G).

Aggresome formation requires functional E1B-55K, because four insertion mutations in the protein that inactivate all of its known biological functions and protein-protein interactions also inhibit aggresome formation. Instead, the mutant proteins become evenly distributed through both the nucleus and cytoplasm (Fig. 3C). E1B-55K in aggresomes is functional because it binds MRN, p53, E4orf6, and Cullin5, and the E1B-55K/E4orf6/elongin BC/Cullin5/Rbx1 ubiquitin ligase functions in aggresomes to induce the degradation of p53 and MRE11 in 293 cells transfected with an E4orf6 expression vector.

E1B-55K aggresomes were also observed in adenovirus-infected cells beginning 12 to 16 h p.i., early in the late phase of infection, when E1B-55K functions are required for high levels of viral late-gene expression (2, 24, 47). In wt Ad5-infected cells, most E1B-55K was observed in nuclei, as opposed to 293 cells and cells transfected with an E1B-55K expression vector, where most E1B-55K was observed in aggresomes (compare Fig. 1 and 2 with 4 and 5). This may have obscured the functional significance of cytoplasmic E1B-55K in earlier studies of virus-infected cells. However, the juxtanuclear aggresomes in infected cells that were noted earlier (37, 44) became obvious when cells infected with *dl*355 (E4orf6 mutant) were costained for E1B-55K and MRE11 (Fig. 4B). In such cells, where p53 and MRN were not degraded because of the absence of E4orf6, MRE11, the marker for the MRN complex, was completely sequestered into E1B-55K aggresomes in ~90% of cells by 20 h p.i., clearly distinguishing the aggresome in a merged image of the E1B-55K and MRE11 antibody stains (Fig. 4B). In contrast, only a small fraction of p53 was exported from the nucleus to the aggresome, even though p53 is also bound by E1B-55K and degraded by the E1B-55K/E4orf6 viral ubiquitin ligase (Fig. 4C). Interestingly, following infection with the E1B-55K mutant S380in, MRE11 and p53 are degraded with a time course similar to that observed in wt Ad5-infected cells, while the mutant E1B-55K was observed only in juxtanuclear cytoplasmic bodies throughout the infection (Fig. 8B). This result indicates that the high level of nuclear E1B-55K detectable by immunofluorescence in wt Ad5-infected cells is not required for MRE11 and p53 degradation.

MRE11 was also exported to the aggresome in wt Ad5-infected cells, although, since it was degraded by proteosomes after ubiquitination by the viral ubiquitin ligase, it did not accumulate in most aggresomes as it did in cells infected with the E4orf6 mutant that cannot assemble the viral ubiquitin ligase (Fig. 5B and C). However, transport of the MRN complex to aggresomes in wt Ad5-infected cells could be visualized by treatment with the proteosome inhibitor MG132 to prevent degradation of MRE11 in aggresomes (Fig. 6C and D). Nuclear export of both E1B-55K and the MRN complex was blocked by leptomycin B (6A and B), a highly specific inhibitor of the CRM1 exportin that interacts with an E1B-55K nuclear export signal (15). Consequently, the MRN complex is probably exported from the nucleus as part of a larger complex with E1B-55K.

The export of MRN to aggresomes facilitates MRN inactivation in at least two ways. First, in a significant fraction of cells infected with wt Ad5, the MRN complex accumulates in aggresomes before it is degraded (Fig. 5A, 20 h p.i., and 5B). Sequestration of MRN complexes into aggresomes before their degradation may occur in a high percentage of wt Ad5-infected cells, but it may be difficult to observe because of asynchrony of the process in different cells, since the MRN

complex is degraded rapidly once it is exported to an aggresome. Also, MRE11 export to the aggresome occurs before MRE11 degradation in cells infected with the S380in E1B-55K mutant (Fig. 8B). When MRN is exported to the aggresome, it is physically separated from viral DNA in the nucleus and is blocked from interacting with cellular DNA double-strand breaks in gamma-irradiated cells (Fig. 3F and G). Thus, export to aggresomes inhibits MRN complex interaction with viral DNA in advance of MRN degradation.

A second mechanism by which aggresome formation facilitates MRN inactivation is that it significantly increases the rate of its degradation by proteosomes, as revealed by the delay in MRE11 degradation when aggresome formation was blocked by leptomycin B or nocodazole (Fig. 6E and F). However, since these treatments affect the localization of other proteins in addition to E1B-55K, it is possible that other effects in addition to inhibition of E1B-55K aggresome formation contribute to the observed decrease in the rate of MRE11 degradation. It has been noted that multiple proteins involved in the ubiquitin-proteosome degradation process, including ubiquitin and proteosomes, are highly enriched in aggresomes (21, 33, 58), potentially accelerating the overall process of ubiquitination and degradation. These experiments with adenovirus show that proteosomal degradation of a protein is indeed significantly accelerated by its sequestration into an aggresome.

An interaction between E4orf3 and PML nuclear bodies is required for MRN nuclear export in virus-infected cells in the absence of E4orf6. At least one other viral protein in addition to E1B-55K participates in the MRN export process in cells infected with the *dl*355 mutant in E4orf6: E4orf3. E4orf3 is observed in aggresomes in *dl*355-infected cells (Fig. 7) and wt Ad5-infected cells (not shown) at 12 h p.i. and later. Its requirement for aggresome formation and MRN nuclear export during a viral infection in the absence of E4orf6 became apparent following infection with an E4orf3$^-$/E4orf6$^-$ double mutant, where E1B-55K aggresome formation was delayed and MRE11 was not depleted from nuclei by export to the aggresomes (Fig. 6G).

Interestingly, the association of MRN complexes with E4orf3 in PML nuclear domains observed earlier (10, 16, 19, 56, 57) occurred by 8 h p.i., before E1B-55K associated with this MRN-E4orf3-PML complex. The E1B-55K association with these nuclear complexes observed earlier (35) occurred at 12 h p.i., just in advance of the export of all these proteins except PML to cytoplasmic aggresomes (Fig. 7). Both E1B-55K (Fig. 3E and G) and E4orf3 (56) can cause the export of MRE11 to cytoplasmic bodies containing the associated viral protein when these viral proteins are expressed individually from expression vectors in transiently transfected cells. However, neither of these viral proteins is sufficient for MRE11 nuclear export in adenovirus-infected cells. MRE11 was not observed in aggresomes in cells infected with the E1B-55K deletion mutant *dl*1520 (Fig. 7C) or the E1B-55K insertion mutant H354in that bocks binding to MRE11 (Fig. 8A), even though both of these mutants express E4orf3 and E4orf6. Similarly, MRE11 was not observed in aggresomes in cells infected with a mutant defective in both E4orf3 and E4orf6 that expresses E1B-55K (Fig. 6G). Since E1B-55K aggresome formation and export of MRE11 to these aggresomes occurred in cells infected with a virus mutant in only E4orf3 or only E4orf6, it is apparent that either E4orf3 or E4orf6 can function in conjunction with E1B-55K to export MRE11 to aggresomes in virus-infected cells. This requirement for E4orf3 or E4orf6 for MRE11 nuclear export in virus-infected cells as opposed to cells transiently transfected with an E1B-55K expression vector or transformed cells can explain why 293 cells do not suppress the phenotype of an E4orf3/E4orf6 double mutant.

Why is E4orf3 or E4orf6 required for MRE11 nuclear export to E1B-55K aggresomes in a virus-infected cell but not in cells transfected with an E1B-55K expression vector? The DNA damage response is induced in cells infected with an Ad5 E4 deletion mutant, leading to the phosphorylation of the MRN NBS1 subunit (56) and ATM (8), processes that occur in PML nuclear bodies (14). Perhaps E4orf3 or E4orf6 is required in virus-infected cells because posttranslational modifications of MRN induced by the DNA damage response in virus-infected cells inhibit MRN nuclear export by E1B-55K. These E4 proteins might function by blocking MRN modification in PML nuclear bodies.

Adenoviruses have evolved several mechanisms to inactivate the MRN complex during viral infection. The first is degradation of MRE11 and other MRN subunits by a mechanism that requires both E1B-55K and E4orf6 (8, 56, 57) and probably involves polyubiquitination of MRE11 by the E1B-55K/E4orf6/elongin BC/Cullin5/Rbx1 ubiquitin ligase (8, 25, 48). Second, the E4orf3 protein inhibits MRN function (56), probably by sequestering it away from viral DNA replication centers in PML nuclear domains with altered morphology compared to the structures in uninfected cells (10, 16, 19, 56, 57). The export of MRN subunits into cytoplasmic aggresomes following their association with E4orf3 in the nucleus and in advance of their complete degradation appears to be one more mechanism used by Ad5 to inhibit MRN function. In addition, E4orf6 alone recently has been shown to inhibit double-strand break repair (26), a process that may also function through inhibition of MRN. This redundancy of mechanisms to inhibit MRN is similar to the inhibition of p53 function by several redundant adenoviral functions (28).

The other known important target of the viral ubiquitin ligase is p53. Interestingly, while MRN is depleted from nuclei by export to the E1B-55K aggresome, only a small fraction of nuclear p53 is exported to the aggresome during viral infection (Fig. 4B and C). The reason for this difference between MRN and p53 is not clear. Nuclear export of p53 may not be as critical because of the multiple viral mechanisms that inhibit p53. E1B-55K alone can inhibit p53 function as a transcriptional activator by tethering a repression domain to it (38, 39, 63, 65). Other, as yet poorly characterized viral functions also inhibit p53 function in the absence of E1B-55K and E4orf6 (28).

Both E1B-55K and E4orf6 mutants are defective in nuclear export and translation of late viral mRNAs (23, 24, 45). This phenotype is mimicked by treatment of infected cells with a proteosome inhibitor (11), implying that efficient viral late mRNA export and translation require the ubiquitin ligase activity of the E1B-55K/E4orf6/elongin BC/Cullin5/Rbx1 complex. The important substrate for the viral ubiquitin ligase controlling late-gene expression is not p53, since the same phenotype is observed in many p53-minus cells (24, 45). It will be interesting to determine if the target of the viral ubiquitin

ligase involved in viral late mRNA nuclear export and translation is the MRN complex or another, as yet unknown, cellular protein.

Implications for aggresome function in degenerative diseases. The observation that the MRN complex is inactivated when it is sequestered into aggresomes is relevant to the debate about whether aggresomes are beneficial or deleterious in degenerative diseases. The acceleration of protein degradation by the aggresome pathway is likely to be beneficial when the substrates are misfolded aggregated proteins that are cytotoxic, because they interact with and inactivate other properly folded proteins. This is probably one of the reasons why the formation of inclusion bodies correlated with prolonged neuron survival in a model where huntingtin protein with a pathogenic polyglutamine expansion was expressed from a strong expression vector (1). However, juxtanuclear cytoplasmic inclusion bodies observed in tissue from diseased patients often contain high concentrations of multiple proteins, as found for the Lewy bodies observed in Parkinson's disease (42). It has been suggested that aggresomes may be cytopathic because they sequester and inactivate essential normal cellular proteins that are bound by an aggregation-prone protein that is sequestered into an aggresome (7). The finding that wt MRN complexes are inactivated when sequestered into an adenovirus E1B-55K-induced aggresome lends support to this hypothesis and calls for a reconsideration of this proposal.

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ADDENDUM IN PROOF

After the present paper was submitted, F. D. Araujo et al. (F. D. Araujo, T. H. Stracker, C. T. Carson, D. V. Lee, and M. D. Weitzman, J. Virol. **79:**11382–11391, 2005) also demonstrated that E4orf3 colocalizes with MRE11 in aggresomes.

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