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Structural Analysis of the Adenovirus Type 5 E1B 55-Kilodalton–E4orf6 Protein Complex

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The adenovirus type 5 (Ad5) early 1B (E1B) 55-kDa (E1B-55kDa)–E4orf6 protein complex has been implicated in the selective modulation of nucleocytoplasmic mRNA transport at late times after infection. Using a combined immunoprecipitation-immunoblotting assay, we mapped the domains in E1B-55kDa required for the interaction with the E4orf6 protein in lytically infected A549 cells. Several domains in the 496-residue 55-kDa polypeptide contributed to a stable association with the E4orf6 protein in E1B mutant virus-infected cells. Linker insertion mutations at amino acids 180 and 224 caused reduced binding of the E4orf6 protein, whereas linker insertion mutations at amino acid 143 and in the central domain of E1B-55kDa eliminated the binding of the E4orf6 protein. Earlier work showing that the central domain of E1B-55kDa is required for binding to p53 and the recent observation that the E4orf6 protein also interacts with the tumor suppressor protein led us to suspect that p53 might play a role in the E1B-E4 protein interaction. However, coimmunoprecipitation assays with extracts prepared from infected p53-negative H1299 cells established that p53 is not needed for the E1B-E4 protein interaction in adenovirus-infected cells. Using two different protein-protein interaction assays, we also mapped the region in the E4orf6 protein required for E1B-55kDa interaction to the amino-terminal 55 amino acid residues. Interestingly, both binding assays established that the same region in the E4orf6/7 protein can potentially interact with E1B-55kDa. Our results demonstrate that two distinct segments in the 55-kDa protein encoding the transformation and late lytic functions independently interact with p53 and the E4orf6 protein in vivo and provide further insight by which the multifunctional 55-kDa E1B protein can exert its multiple activities in lytically infected cells and in adenovirus transformation.

The adenovirus early 1B (E1B) 55-kDa protein (E1B-55kDa protein) is required for efficient viral DNA replication and transformation of primary cells in culture (5, 45). The 496-residue polypeptide from adenovirus 5 (Ad5) is a multifunctional phosphoprotein that exhibits a complex cellular distribution in infected and transformed cells (26, 40, 43, 54). This variety of cellular localizations may result in part from 55-kDa oligomerization (15) and specific interactions with the cellular tumor suppressor protein p53 (4, 8, 42, 54) and the Ad 34-kDa protein encoded by open reading frame 6 (orf6) (9) of the E4 transcription unit (42).

Genetic and biochemical studies demonstrate that the 55-kDa E1B polypeptide contains at least two functional domains encoding the transformation and late lytic functions (2, 7, 21, 46, 52). The transformation functions are located in the central and carboxy-terminal region of E1B-55kDa (51, 52). These regions have been shown to be responsible for p53 binding (21) and inhibition of p53 transactivation (46, 51, 53). Moreover, the carboxy-terminal domain seems to act as a direct transcriptional repressor in transient-transfection assays (53).

The most essential region required for late viral functions maps between amino acids 180 and 354 (52). Previous work by a number of investigators has demonstrated that the lytic functions encoded by E1B-55kDa regulate the accumulation of viral mRNAs, the control of late viral gene expression, and the shutoff of most host mRNAs at the level of mRNA transport (1, 2, 38, 50). The E1B-55kDa protein seems to modulate viral and cellular RNA metabolism in complex with the E4orf6 protein after transcription but prior to translocation of mRNAs through the nuclear pore complex (23). Remarkably, the selective transport is not dependent on the identity of individual mRNAs. Cellular mRNAs transcribed from recombinant viral chromosomes are transported to the cytoplasm late after infection, even at a time when the endogenous cellular transcript accumulated in the nucleus (11, 18). Consistent with this function in late-Ad-infected cells is the recent finding that Ad5 E1B-55kDa bearing a nuclear localization signal inhibits the export of mRNA in *Saccharomyces cerevisiae* (24).

Similar to E1B-55kDa, the E4orf6 protein encodes functions required for viral DNA synthesis, late viral gene expression, and host cell shutoff (9, 17, 19, 38, 48). It has therefore been suggested that the functional moiety of the E1B-E4 protein complex is responsible for the selective cytoplasmic accumulation of viral mRNAs during the late phase of productive infection (2, 7, 23, 38). Approximately 50% of total E4orf6 protein in late infected cells is found complexed to E1B-55kDa (9). The noncomplexed form of the E4orf6 protein seems to encode additional functions required for enhancement of nuclear RNA accumulation that are not dependent on the coexpression of E1B-55kDa (35). It appears that the stimulatory effect of the E4orf6 protein on late viral RNA accumulation is already detectable in the nucleus and requires wild-type 5′ and 3′ splice sites, suggesting that the E4orf6 protein is directly or indirectly involved in splice site recognition (32, 33, 35). Interestingly, we have recently found that the E4orf6 protein also forms a physical complex with p53 (10). The E4orf6 protein binds to the carboxy-terminal domain of the tumor suppressor protein and inhibits p53-mediated transcriptional activation.

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Because the E1B-55kDa and E4orf6 proteins bind to different domains on p53, they might bind to p53 simultaneously and cooperate to antagonize p53 function.

The E1B-E4 complex is located in the nucleus in association with viral inclusion bodies that are believed to be the site of viral DNA replication and transcription (37). In the absence of a functional E4orf6 protein, the 55-kDa polypeptide fails to associate with the viral inclusion bodies, suggesting that the E4orf6 protein targets the 55-kDa polypeptide to the centers of viral replication and transcription (37). Based on these observations, Ornelles and Shenk (37) have proposed a model by which the E1B-E4 protein complex facilitates transport and accumulation of viral mRNAs late after infection while blocking the same processes for most host mRNAs. According to their proposal, the E1B-E4 complex relocalizes a cellular factor required for nucleocytoplasmic transport of mRNAs from the sites of host transcription and processing to the viral inclusion bodies. However, the identity of the putative transport factor and the molecular mechanism by which the E1B-E4 protein complex modulates viral and cellular mRNA transport are still unknown.

In this study, we have investigated the structural requirements of the Ad5 E1B-55kDa–E4orf6 protein interaction in Ad-infected cells and in vitro. We demonstrate that two distinct segments in the 55-kDa polypeptide which partly overlap regions responsible for p53 binding are required for complex formation with the E4orf6 protein and that this protein interaction does not require p53. In addition, we provide evidence that E1B-55kDa can bind to the amino-terminal domain of the E4orf6 and E4orf6/7 proteins. Together with our recent observation showing that E4orf6 also binds to p53 (10), our data strongly support the idea that multiple protein interactions between E1B-55kDa, E4orf6, and p53 exist in Ad-infected cells.

MATERIALS AND METHODS

Plasmids. The Ad5 E1B-55kDa-encoding *Bss*HII fragment (Ad5 nucleotides [nt] 1974 to 4107) from plasmid pE1B (25) was blunted with T4 DNA polymerase. *Xba*I linkers (8-mer) were added, and the fragment was cloned into the *Xba*I site of the pGEM11Zf(+) polylinker (Promega). This construct (p11/55.X) was sequenced through the polylinker region and beyond the *Kpn*I site in the Ad5 E1B-55kDa sequence (Ad5 sequence 2048). To generate pbE1B-55kDa, the *Xba*I fragment was subsequently cloned into the *Nhe*I site of pBlueBac (InVitrogen).

The Ad5 E4orf6-expressing constructs were made by PCR from pXbaC (17) with primers orf6fw (5′-GGTCTAGAGGATCCATGACTACGTCCGG-3′) and orf6rev (5′-GGTCTAGAGGATCCCTACATGGGGGT-3′) containing *Xba*I cloning sites. The PCR fragment was inserted into the *Xba*I site of pGEM4Z polylinker (Promega). To generate the wild-type E4orf6-expressing plasmids pGEXE4orf6 and pCMVE4orf6, the *Bam*HI fragment from p11/34.X was cloned into the *Bam*HI sites of pGEX-2Tk (20) and pCMV/neo (22), respectively. The C-terminal deletion constructs were made by PCR with primers orf6fw, 461rev (5′-CGCGAATTCGTCGACACCTCTGATTAAACATGG-3′), 677rev (5′-CGCGAATTCGTCGAC-AATCAGCACAGTAACTGC-3′), and 861rev (5′-CGCGAATTCGTCGACGCGCGAATAAACTGCTGC-3′) containing the *Bam*HI and *Eco*RI cloning sites. The PCR products were cloned into the *Bam*HI/*Eco*RI sites of pGEX-4T1 and pcDNA3 (InVitrogen) to generate pE4$^{dlA152\text{-}M294}$, pE4$^{dlC224\text{-}M294}$, and pE4$^{dlR271\text{-}M294}$. The epitope-tagged E4orf6 deletion constructs were generated as follows. An 882-bp fragment corresponding to nt 1861 to 2743 in pXbaC (17) was generated by PCR using primers orf6fw and orf6* (5′-GTGAGATCTAGTCCACGCCGCCATGG-3′). Primer orf6* replaced the stop codon TAG in the E4orf6 cDNA with GCG and introduced a unique *Bgl*II site at the 3′ end. The *Bam*HI/*Bgl*II fragment was cloned into the *Bgl*II site of pVP16orf1 (47). A double-stranded oligonucleotide (5′-GATCTGCTTCTAGCTATCCTTATGACGTGCCTGACTATGCCAGCCTGGGAGGACCTTCTG-3′ and 5′-GATCCAGAAGGTCCTCCCAGGCTGGCATAGTCAGGCACGTCATAAGGATAGCTAGAAGCA-3′) encoding the 12CA5 epitope amino acid sequence YPYDVPDYA was then inserted into the *Bgl*II site of pE4VP16, and the *Eco*RI/*Eco*RV fragment (pXbaC nt 1861 to 2182) was cloned between the *Eco*RI/*Eco*RV sites of p11/34.X to generate pE4orf6-flu. This construct served as the template for the PCRs to construct the N-terminal deletions with primers flufix (5′-GCGGAATTCGTCGACAGATCCAGAAGGTCCTCCC-3′), 172fw (5′-CGCGGATCCATGCACAACGTGAGTTACGTG-3′), 331fw (5′-CGCGGATCCATGACGAGCATGATGATCC-3′), and 616fw (5′-CGCGGATCCATGAGCTTTGGATACAGCGCC-3′), which introduce a *Bam*HI site at the 5′ end and a *Sal*I/*Eco*RI site at the 3′ end, respectively. All reaction products were subsequently cloned into the *Bam*HI/*Eco*RI sites of pGEX-4T1 and pcDNA3.

The E4orf6/7-expressing plasmids pGEXE4orf6/7 and pCMVE4orf6/7 were made by cloning the *Bam*HI/*Sal*I fragment from pE4-17khis (27) into the *Bam*HI/*Sal*I sites of pGEX-4T1 and pCMV/neo, respectively.

Cells and viruses. A549 cells (12), 293 cells (13), W162 cells (49), and the p53-negative cell line H1299 (29) were grown as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (GIBCO BRL).

H5*wt*300 served as the wild-type Ad5 parent in these studies. The following mutant viruses were used in this study. H5*dl*338 (38) carries a 524-bp deletion in the E1B-coding region located between nt 2805 and 3329. The linker insertion mutations H17, A143, H180, H224, A262, R309, H326, H354, S380, R443, and F484 in the E1B-55kDa gene have been described previously (52). The mutants containing these mutations are recombinants of Ad2 and Ad5 and have been named first by a letter representing the restriction site at the insertion and then by a number representing the amino acid residue at or just preceding the 4-residue insertion. Mutant H5*in*3328(+) produces an E1B protein containing an 11-amino-acid insert between Ile-438 and Trp-439 (46). In the E1B mutant H5*pm*490A/491A, the codons for Ser-490 and Ser-491 have been changed to those for alanine (46). H5*dl*355 contains a 14-bp deletion between nt 2331 and 2346 in the E4orf6 gene, whereas mutant H5*dl*356 contains a 2-bp deletion between nt 2841 and 2844 in the E4orf6/7 gene (17). The two E4 mutants do not express the E4orf6 and E4orf6/7 proteins, respectively (9, 17). The details of the mutant viruses are summarized in Table 1. The Ad5 wild-type virus was propagated on A549 cells. E1B and E4 mutant viruses were propagated on monolayer cultures of 293 or W162 cells, respectively. Plaque assays were performed on A549 monolayers in minimal essential medium overlay containing 5% fetal calf serum and 0.5% $NaHCO_3$ (GIBCO BRL). Virions purified by cesium chloride equilibrium density centrifugation were used for all infections.

Spodoptera frugiperda Sf-9 cells were maintained in TNM-FH medium (GIBCO BRL) supplemented with 10% fetal calf serum and 0.2% F-68 pluronic acid (GIBCO BRL) at 28°C with three changes of medium weekly. For DNA transfections 2×10^6 cells were seeded in a 25-cm^2 flask. After 2 h, the cells were transfected with 1 μg of wild-type *Autographa californica* nuclear polyhedrosis virus DNA and 5 μg of the bE1B-55kDa construct by the calcium precipitation procedure as described previously (36). Briefly, the medium was aspirated and replaced with 0.75 ml of TNM-FH medium plus 0.75 ml of the premixed DNA solutions. After 4 h, the inoculum was removed and the cells were refed with supplemented TNM-FH medium. The virus-containing medium was collected 4 days after transfection.

To screen for recombinant virus, 2×10^6 cells were seeded in a 60- by 15-mm tissue culture plate and allowed to attach for 2 h. The cells were infected for 4 h with dilutions (10^{-1} to 10^{-6}) of the virus-containing supernatant. The medium was aspirated, and the recombinant viruses were assessed by standard plaque assay with 600 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) added to the overlay. At 5 days postinfection, recombinant plaques were identified by their occlusion (Occ$^-$) morphology and their ability to produce blue

TABLE 1. Mutant Ad strains used in this study

Virus	Mutated gene	Phenotypes[a]		
		Viral replication	Expression of late viral proteins	Host cell shutoff
H5*dl*338	E1B-55kDa	+	+	+
H17	E1B-19 and 55kDa	+	+++	+++
A143	E1B-55kDa	++	++	++
H180	E1B-55kDa	+	+	+
H224	E1B-55kDa	++++	++	++
A262	E1B-55kDa	+	+	+
R309	E1B-55kDa	+	+	+
H326	E1B-55kDa	+	++	++
H354	E1B-55kDa	++	++	++
S380	E1B-55kDa	++++	++++	++++
H5*in*3328(+)	E1B-55kDa	+	ND	ND
R443	E1B-55kDa	+	+++	+++
F484	E1B-55kDa	++	+++	++
H5*pm*490A/491A	E1B-55kDa	+	+++	+++
H5*dl*355	E4orf6	+++	++	++
H5*dl*356	E4orf6/7	++++	++++	++++

[a] Determined on HeLa cells. Symbols: ++++, same as wild-type virus; +++, modestly reduced; ++, reduced; +, severely reduced; ND, not determined.

(Gal^+) plaques. After two rounds of plaque purification, the expression of the recombinant E1B-55kDa protein was confirmed by electrophoresis in an 10% polyacrylamide gel and Western blot analysis of whole-cell extracts from infected cells with anti-55-kDa monoclonal antibody 2A6 (43). E1B-55kDa-expressing recombinant viruses *bac*E1B-55kDa were amplified (P1 and P2 stocks) and subjected to titer determination as described previously (36).

Preparation and purification of GST and fusion proteins. Expression of glutathione *S*-transferase (GST) fusion proteins was induced as described previously (20). Overnight cultures of *Escherichia coli* TOPP6 (Stratagene), transformed with the expression plasmids, were diluted 1:10 in Terrific Broth medium with ampicillin (100 μg/ml). After 1 h at 37°C, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and incubation was continued for an additional 4 h. Cells were harvested by centrifugation and suspended in 1/50 volume of NETN buffer (20 mM Tris-chloride [pH 7.5], 100 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40). Cells were lysed by mild sonification, and the cellular debris was removed by centrifugation at 14,000 × *g* for 30 min at 4°C. Glutathione-Sepharose 4B beads (Pharmacia) were washed with NETN buffer containing 0.5% nonfat dry milk. Then 200 μl of beads (1:1 in NETN) was incubated with 10 ml of lysate for 1 h at 4°C. The beads were washed four times with buffer A (10 mM HEPES/KOH [pH 7.9], 1.5 mM $MgCl_2$, 1 M KCl, 0.5 mM dithiothreitol), equilibrated in buffer B (10 mM HEPES/KOH [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.05% nonfat dry milk, 7% glycerol), and stored at −70°C. For analysis of bound fusion proteins, the beads were boiled in Laemmli sample buffer and loaded onto a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel. After electrophoresis, fusion proteins were visualized by Coomassie blue staining.

Analysis of polypeptides and immunoprecipitations. The following monoclonal antibodies were used in this study. 2A6 (43) is specific for the E1B-55kDa protein. The epitope site for 2A6 maps to the amino-terminal 180 amino acids of the 55-kDa protein (21). RSA3 recognizes the amino terminus of the E4orf6 and E4orf6/7 proteins (27). Monoclonal antibody 12CA5 (Boehringer) has been described elsewhere (31).

Subconfluent A549 cells or H1299 cells were infected with wild-type or mutant Ad at an infectivity of 20 PFU per cell. At different times after infection, cells were washed twice in phosphate-buffered saline (PBS) and lysed by adding 0.8 ml of lysis buffer (50 mM Tris-chloride [pH 8.0], 5 mM EDTA, 150 mM NaCl, 0.15% [vol/vol] Nonidet P-40, 1 mM dithiothreitol, 0.05 mM phenylmethylsulfonyl fluoride PMSF) per 90-mm dish. After 1 h on ice, the lysate was sonicated, and the insoluble debris was pelleted at 10,000 × *g* at 4°C.

Subconfluent 293 cells in 90-mm-diameter tissue culture dishes were transfected with 10 μg of the corresponding expression plasmids by the calcium phosphate protocol (14) or with Lipofectamine (GIBCO BRL) as specified by the manufacturer. At 36 h after transfection, total-cell extracts were prepared and subjected to immunoprecipitation followed by immunoblotting as described below.

For immunoprecipitations, protein A-Sepharose (30 mg/ml) was incubated with 1 ml of hybridoma supernatant 2A6 for 2 h at room temperature and washed twice in lysis buffer. The antibodies bound to protein A-Sepharose were added to the protein A-Sepharose precleared extracts and rotated overnight at 4°C. The immune complexes were washed five times with lysis buffer, resuspended in SDS sample buffer, separated on SDS–10 to 12.5% polyacrylamide gels, and blotted onto nitrocellulose membranes by using an electroblotting transfer system. The filters were incubated overnight in PBS–5% nonfat dry milk and then for 2 h in PBS–3% nonfat dry milk containing antibody. The filters were washed three times in PBS–3% nonfat dry milk–0.1% Tween 20, incubated with a secondary antibody linked to horseradish peroxidase (Amersham) in PBS–3% nonfat dry milk, and washed three times in PBS–0.1% Tween 20. The bands were visualized by enhanced chemiluminescence as recommended by the manufacturer (Amersham) on X-ray films (Kodak XAR-5 or Kodak BioMax MR-1).

Autoradiograms were scanned and cropped with Adobe Photoshop, and figures were prepared with Adobe FreeHand software on an Apple Macintosh computer. If necessary, the proteins were quantitated from a TIFF file with the Analyze Particles program (NIH Image 1.52) on an Apple Macintosh.

Whole-cell extracts from mock- or adenovirus-infected cells were prepared as described above, separated on SDS–10% polyacrylamide gels, and transferred to nitrocellulose membranes. For detection of viral and cellular proteins, the filters were processed as described above.

Analysis of viral DNA synthesis. Ad DNA replication was determined by PCR. At the indicated time points, 6×10^3 infected cells were harvested as described above and lysed in 50 μl of 1 × PCR buffer supplemented with 0.5% Tween 20 and 100 μg of proteinase K (Sigma) per ml. Then 20 cycles of PCR (30 s at 95°C, 1 min at 55°C, and 2 min at 72°C) were performed with 20 μl of lysate and 2.5 U of *Taq* polymerase in a 50-μl reaction volume. Two synthetic oligonucleotides, 5′-ATGGAGCGAAGAAACCCATCTGAGC-3′ and 5′-CGGTGTCTGGTCA TTAAGCTAAAA-3′, complementary to the Ad5 E1B-55kDa gene (Ad5 nt 2019 to 2043 and 2378 to 2401) served as specific primers. Plasmid p11/55.X was used as a positive control. The reaction products (382 bp) were analyzed on a 1% agarose gel containing 0.66 μg of ethidium bromide per ml. A TIFF file was generated, and the reaction products were quantitated with the Analyze Particles program on an Apple Macintosh computer.

In vitro binding assays. For protein blot experiments, purified GST fusion proteins were separated in an SDS–10% polyacrylamide gel and transferred to nitrocellulose membranes in a buffer containing 192 mM glycine, 25 mM Tris-chloride (pH 8.8), 0.1% SDS, and 20% methanol. The membranes were incubated for 12 h at 4°C in buffer Z (20 mM HEPES/KOH [pH 7.9], 60 mM KCl, 6 mM $MgCl_2$, 0.6 mM EDTA, 2 mM dithiothreitol, 10 μM $ZnCl_2$, 10% glycerol, 5% fetal calf serum) on a rocking platform. The membranes were then incubated under constant agitation for 3 h at 37°C with a 0.25-ml/cm^2 volume of buffer Z that contained 5 μg of whole-cell extract/ml of buffer Z from *bac*E1B-55kDa virus-infected *Sf*-9 cells. The filters were washed once in RIPA buffer (50 mM Tris-chloride [pH 8.0], 150 mM NaCl, 5 mM EDTA, 1% [vol/vol] Nonidet P-40, 0.1% Triton X-100, 0.1% SDS) and then twice in PBS. Bound E1B-55kDa proteins were visualized by incubating the membranes with antibody 2A6 and then with horseradish peroxidase-coupled goat-anti-mouse antibody and using the enhanced chemiluminescence system (Amersham).

RESULTS

Analysis of the E1B-55kDa–E4orf6 protein complex in wild-type-Ad5-infected A549 cells. To analyze the E1B-E4 protein interaction, human A549 cells (12) were used as the permissive host in Ad infection experiments. Our decision to use A549 cells instead of HeLa monolayers was based mainly on the previous observation demonstrating that the E4orf6 mutant virus *dl*355 is severely defective when grown on HeLa cells (9, 17). The *dl*355 mutation results in significant impairment of synthesis of viral proteins, including the 55-kDa protein, during the late phase of infection in HeLa cells, compared to A549 cells, in which this defect is less severe (17, 44). In addition, A549 cells express clearly detectable levels of wild-type p53 (16) compared to HeLa cells, which contain very low levels of endogenous p53 (28). Since both viral proteins interact with p53 (10, 42), A549 cells seemed (additionally) to be more suitable for the analysis of the E1B-E4 protein complex in Ad-infected cells.

Previous work on the E1B-E4 protein interaction was performed mainly with infected HeLa cells (9, 37, 42, 44). Therefore, it was necessary to determine the rate of synthesis and physical association of both viral proteins during the early and late phases of wild-type-infected A549 cells (Fig. 1). Immunoblotting experiments with total-cell extracts from infected cells established that expression of the E4orf6 protein and E1B-55kDa (Fig. 1A) was detectable before the onset of viral DNA replication (Fig. 1B) 10 and 14 h-postinfection, respectively (Fig. 1A). The rate of synthesis of both viral proteins increased until 24 h postinfection (Fig. 1A), when viral DNA accumulated to high levels in wild-type-infected cells (Fig. 1B). We also monitored the expression of the viral E4orf6/7 protein (Fig. 1A) encoded in the E4 transcription unit open reading frames 6 and 7 (E4orf6/7) (17), since monoclonal antibody RSA3 recognizes the amino termini of both E4orf6 and E4orf6/7 proteins (27). The mutant viruses *dl*338, *dl*355, and *dl*356 were used as controls to verify the identity of the detected E1B-55kDa, E4orf6, and E4orf6/7 proteins, respectively. Almost identical patterns of viral protein expression were observed in *wt*300 virus-infected HeLa cells, although E1B-55kDa expression reproducibly peaked around 20 h postinfection (data not shown).

To analyze the E1B-E4 complex in lytically infected A549 cells, the same extracts were subjected to immunoprecipitation by using the E1B-55kDa-specific monoclonal antibody 2A6 (43). The E4orf6 protein was efficiently coimmunoprecipitated from *wt*300- and *dl*356-infected cells (Fig. 1C) but was absent in *dl*338- and *dl*355-infected cells, due to their mutations in the E1B-55kDa and E4orf6 genes. The level of coprecipitated E4orf6 protein increased from 12 to 24 h postinfection, consistent with its rate of synthesis during the late phase of infection (Fig. 1A). We were not able to specifically coimmunoprecipitate E1B-55kDa with the E4orf6 protein (data not shown).

In summary, our data demonstrate that wild-type-Ad5-in-

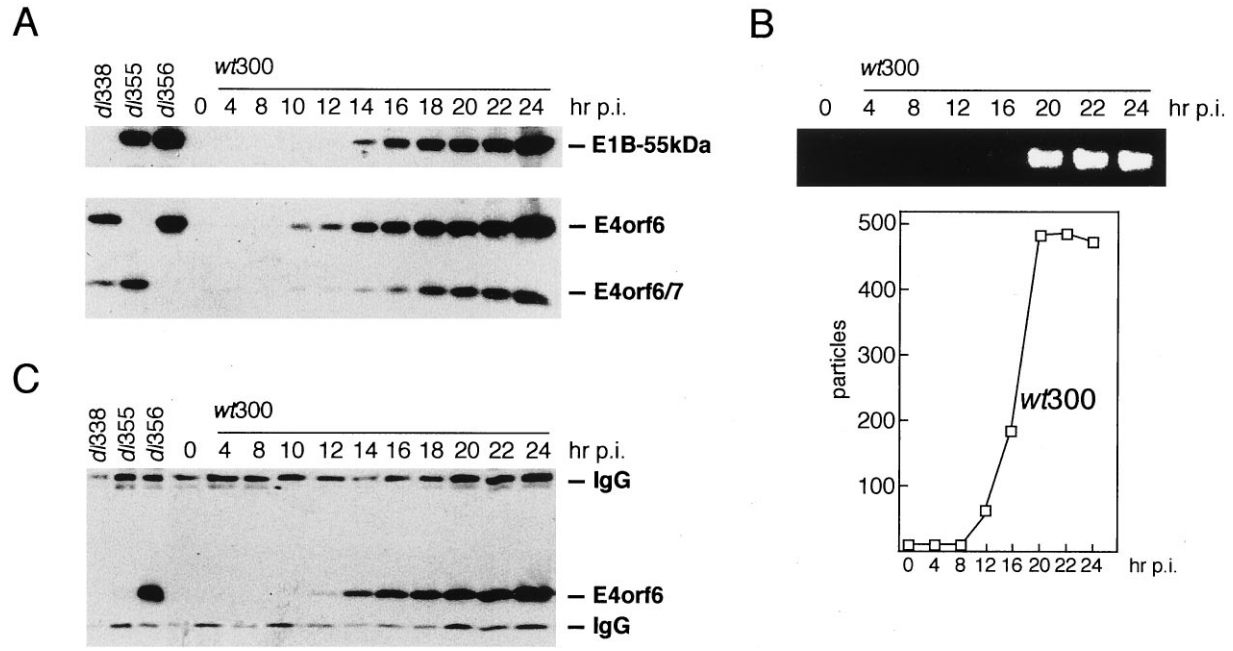

FIG. 1. Time course analysis of the E1B-55kDa/E4orf6 protein complex in *wt*300-infected A549 cells. (A) Expression of E1B-55kDa, E4orf6, and E4orf6/7 proteins. A549 cells were infected with *wt*300 virus and harvested at the indicated times. *dl*338-, *dl*355-, and *dl*356-infected cells were harvested at 24 h postinfection (p.i.). Proteins (40-μg samples) from each time point were separated on SDS–10% polyacrylamide gels, transferred to nitrocellulose filters, and probed with anti-E1B-55kDa (2A6) and anti-E4orf6/E4orf6/7 (RSA3) hybridoma supernatant. The bands representing the viral proteins are indicated on the right. (B) Analysis of viral DNA replication by PCR. At the indicated time points, total-cell extracts from infected cells were subjected to 20 cycles of PCR with opposing primers complementary to the Ad5 E1B-55kDa gene as described in Materials and Methods. The PCR products were analyzed on a 1% agarose gel and quantitated with the Analyze Particles program from the generated TIFF file on a Macintosh computer. (C) Coimmunoprecipitation of E4orf6 with E1B-55kDa. E4orf6 bound to the 55-kDa proteins made by the wild-type and mutant Ad5 was coprecipitated with 2A6 antibody from the same whole-cell extracts, resolved on SDS–12.5% polyacrylamide gels, and visualized by Western immunoblot analysis with the RSA3 anti-E4orf6 monoclonal antibody. The bands representing the immunoglobulin G (IgG) and E4orf6 proteins are indicated on the right.

fected A549 cells display almost the same pattern of expression of E1B-55kDa, E4orf6, and E4orf6/7 proteins as that previously described for infected HeLa cells (9, 39, 44, 52). Having established the experimental conditions needed to analyze the E1B-E4 protein interaction in infected A549 cells, we next mapped the domains in E1B-55kDa required for binding to the E4orf6 protein in vivo.

Domains in E1B-55kDa required for interaction with the E4orf6 protein. To determine the domains in the E1B-55kDa polypeptide required to interact with the E4orf6 protein in late-infected A549 cells, we used a series of mutant viruses (Table 1; Fig. 2A) carrying linker insertions in the 55-kDa gene (46, 52). In *pm*490A/491A, the codons for two serine residues (amino acids 490 and 491) which represent potential casein kinase II substrate sites have been changed to those for alanines (46). This mutant was found to be defective in viral replication and to transform primary cells at reduced efficiency (46).

Extracts of A549 cells were prepared at 24 h postinfection and analyzed with monoclonal antibodies 2A6 and RSA3 (Fig. 2B). The viral proteins we analyzed by densitometry as described in Materials and Methods, and the amount of protein for each sample was determined as a percentage of wild-type virus (data not shown). With the exception of *dl*338, all of the mutants expressed stable 55-kDa proteins, migrating at roughly the same molecular mass. The most dramatic shift in mobility was observed with H5*in*3328(+), containing an 11-amino-acid insertion in the carboxy-terminal region of the 55-kDa polypeptide (Fig. 2B). The differences observed with the other 55-kDa proteins are also most probably due to their chimeric nature (recombinants of Ad2 and Ad5) and to their inserted amino acids (52). The steady-state levels of the E1B-55kDa and E4orf6 proteins varied for different mutant viruses. Mutant F484 reproducibly expressed lower levels of E1B-55kDa protein, while mutants A262, R443, and *pm*490A/491A produced reduced amounts of E4orf6 protein, with levels reduced to 60 to 70% of wild-type virus levels (Fig. 2B). Interestingly, the E4orf6 protein from A262, R443, and F484 mutant virus-infected cells migrated as a clearly detectable doublet.

To analyze the domains required for the interaction between E1B-55kDa and E4orf6 proteins, the same extracts were subjected to immunoprecipitation and immunoblotting (Fig. 2C) and the coprecipitated E4orf6 proteins were analyzed by densitometry. Several mutations in the 55-kDa protein changed its ability to coimmunoprecipitate the E4orf6 protein. As expected, no E4orf6 protein was coprecipitated from *dl*338- and *dl*355-infected extracts. Insertions at amino acids 143, 262, 309, and 326 strongly interfered with the binding of the E4orf6 protein, whereas insertions at amino acids 17, 354, 380, and 438 [Fig. 2C, *in*3328(+)] had no effect on the interaction. Insertions at amino acids 180 and 224 clearly showed decreased binding of the E4orf6 protein. The reduction of coprecipitated E4orf6 protein in R443-, *pm*490A/491-, and F484-infected cells is most probably due to decreased expression of the E4orf6 (mutant virus R443 and *pm*490A/491) and E1B-55kDa (mutant virus F484) polypeptides. In extracts prepared from R443- and F484-infected cells the two previously noted forms of the E4orf6 protein coprecipitated with E1B-55kDa. Although the nature of the second faster-migrating E4orf6 species has not been defined yet, both polypeptides might represent different phosphorylated forms of the E4orf6 protein. Clearly, the fast-

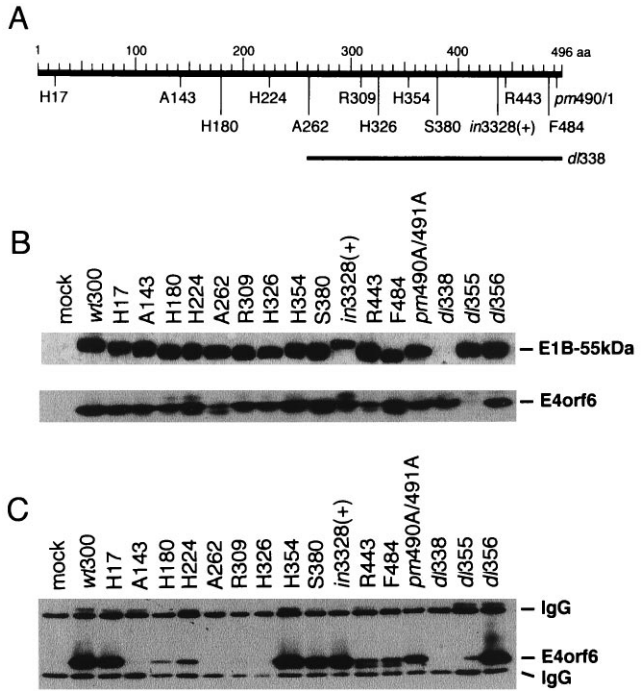

FIG. 2. Analysis of the E1B-55kDa/E4orf6 protein interaction in mutant and wild-type virus-infected A549 cells. (A) E1B-55kDa mutation sites. The thick black bar on top represents the 496 residues of the 55-kDa polypeptide. Number 496 denotes the last amino acid (aa). The insertion mutations and the point mutation *pm*490A/491A (*pm*490/1) are shown below in their relative positions along the E1B-55kDa polypeptide. The deletion in *dl*338 is denoted by a thin bar. (B) Expression of E1B-55kDa and E4orf6 in E1B and E4 mutant virus-infected cells. Whole-cell extracts used for the coimmunoprecipitation experiment containing 40 μg of protein were subjected to polyacrylamide gel electrophoresis followed by Western blotting with anti-E1B-55kDa (2A6) and anti-E4orf6 (RSA3) hybridoma supernatant. The bands representing the viral polypeptides are indicated. (C) Coimmunoprecipitation of E4orf6 with E1B-55kDa. Total-cell extracts from infected A549 cells were prepared 24 h postinfection. E4orf6 bound to the 55-kDa proteins made by the wild-type and mutant viruses was coprecipitated with anti-E1B-55kDa monoclonal antibody (2A6), resolved on SDS–12.5% polyacrylamide gels, and visualized by Western immunoblot analysis with the RSA3 anti-E4orf6 monoclonal antibody. The E1B insertion mutants are shown from left to the right according to their positions in the 55-kDa polypeptide from the amino terminus to the carboxy terminus. The bands representing the IgG and E4orf6 proteins are indicated at right.

er-migrating species is absent in *wt*300-infected A549 cells (Fig. 1 and 2), suggesting that this second form is related to the mutations in A262, R443, and F484 mutant viruses.

From these experiments, we concluded that two regions located in the amino-terminal and central domains of the 55-kDa polypeptide are required for the binding to the E4orf6 protein. Our findings are supported by the previous observation showing that E1B mutant viruses A143, A262, R309, and H326 display severe lytic defects (52), which are similar to those described for the E4orf6 mutant *dl*355 (9, 17). According to our data, the phenotypes of these E1B mutant viruses are most probably due to the inability of the encoded 55-kDa polypeptides to form a functional complex with the E4orf6 proteins in productively infected cells.

p53 is not required for E1B-55kDa/E4orf6 complex formation. The characterization of the E1B-E4 interaction has been complicated in the past (21) by the difficulty in demonstrating protein association in vitro (9a). In contrast to the E1B-p53 interaction, high levels of expression in proteins are required to show binding between these two proteins, and E1B-55kDa and E4orf6 do not interact in the yeast two-hybrid assay (40a). These observations suggest either that modification of these proteins is required for their interaction or that a third protein component is essential for stabilization of the complex. Previous work has demonstrated that amino acids at position 180 and in the central region between amino acids 262 and 326 of the 55-kDa polypeptide are required for the interaction with p53 in vitro and in vivo (21, 51). These observations and our data from coimmunoprecipitation experiments raise the possibility that binding of the E4orf6 protein to the amino-terminal region of 55-kDa is mediated and/or stabilized through p53 bound to the central region of E1B-55kDa. Thus, in the absence of endogenous p53, the E4orf6 protein should not bind to E1B-55kDa. To address this question, we examined the E1B-E4 protein complex formation in human H1299 cells, which do not express endogenous p53 (29) (Fig. 3). Total-cell extracts from infected H1299 cells were prepared at 24 h postinfection and analyzed by immunoblotting (Fig. 3A) and coimmunoprecipitation experiments (Fig. 3B) as described above. The E1B-E4 protein complex was also evident in infected H1299 cells, demonstrating that p53 is not needed for the E1B-E4 interaction in Ad-infected cells.

The amino-terminal domains of E4orf6 and E4orf6/7 interact with E1B-55kDa. We next mapped the domain on the E4orf6 protein responsible for its interaction with E1B-55kDa (Fig. 4). Wild-type E4orf6, E4orf6/7, and segments of the E4orf6 protein were expressed as GST fusion proteins in *E. coli* (Fig. 4A), purified by affinity chromatography, and tested by immunoblotting with monoclonal antibody RSA3 (Fig. 4B). Monoclonal antibody 12CA5 was used to verify the expression of the epitope-tagged fusion proteins pGEXE4$^{dlM1\text{-}T55}$, pGEXE4$^{dlM1\text{-}I108}$ and pGEXE4$^{dlM1\text{-}A203}$ that do not contain the RSA3 epitope (Fig. 4B, lanes 4 to 6). The same amounts of full-length fusion proteins were then subjected to electrophoresis and transferred to nitrocellulose membranes. The bound proteins were reacted with E1B-55kDa from baculovirus-infected insect cells (*bac*E1B-55kDa) and monoclonal antibody 2A6 (Fig. 4C). All fusion proteins containing the amino-terminal region of the E4orf6 protein were able to bind E1B-55kDa efficiently (Fig. 4C, lanes 1 to 3), while the amino-terminal deletions of the E4orf6 protein failed to interact with the 55-kDa product (Fig. 4C, lanes 4 to 6). Remarkably, deletion of the amino-terminal 55-amino-acid residues of the E4orf6 protein (Fig. 4C, lane 4) was already sufficient to abolish 55-kDa binding. E1B-55kDa also bound to the E4orf6/7 protein (Fig. 4C, lane 7), indicating that the minimal domain required for 55-kDa binding in vitro is in fact located within the first 55 amino acid residues of both viral proteins.

To confirm the result from the protein blot assay, we per-

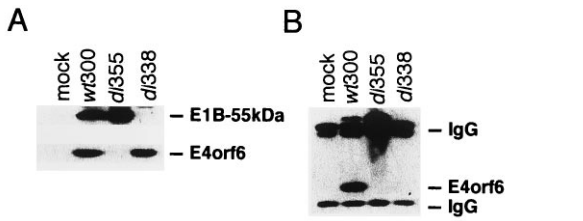

FIG. 3. p53 is not required for E1B-55kDa/E4orf6 complex formation. (A) Immunoblot of extracts used for the immunoprecipitation experiment with anti-55kDa and anti-E4orf6 hybridoma supernatant. (B) Coimmunoprecipitation of E4orf6 with E1B-55kDa. H1299 cells were infected with *wt*300, *dl*355 and *dl*338 virus. Total-cell extracts were prepared 24 h postinfection, and 50 μg of protein was subjected to immunoprecipitation with monoclonal antibody 2A6. The precipitates were resolved on SDS–12.5% polyacrylamide gels, and coprecipitated E4orf6 protein was visualized with RSA3 monoclonal antibody.

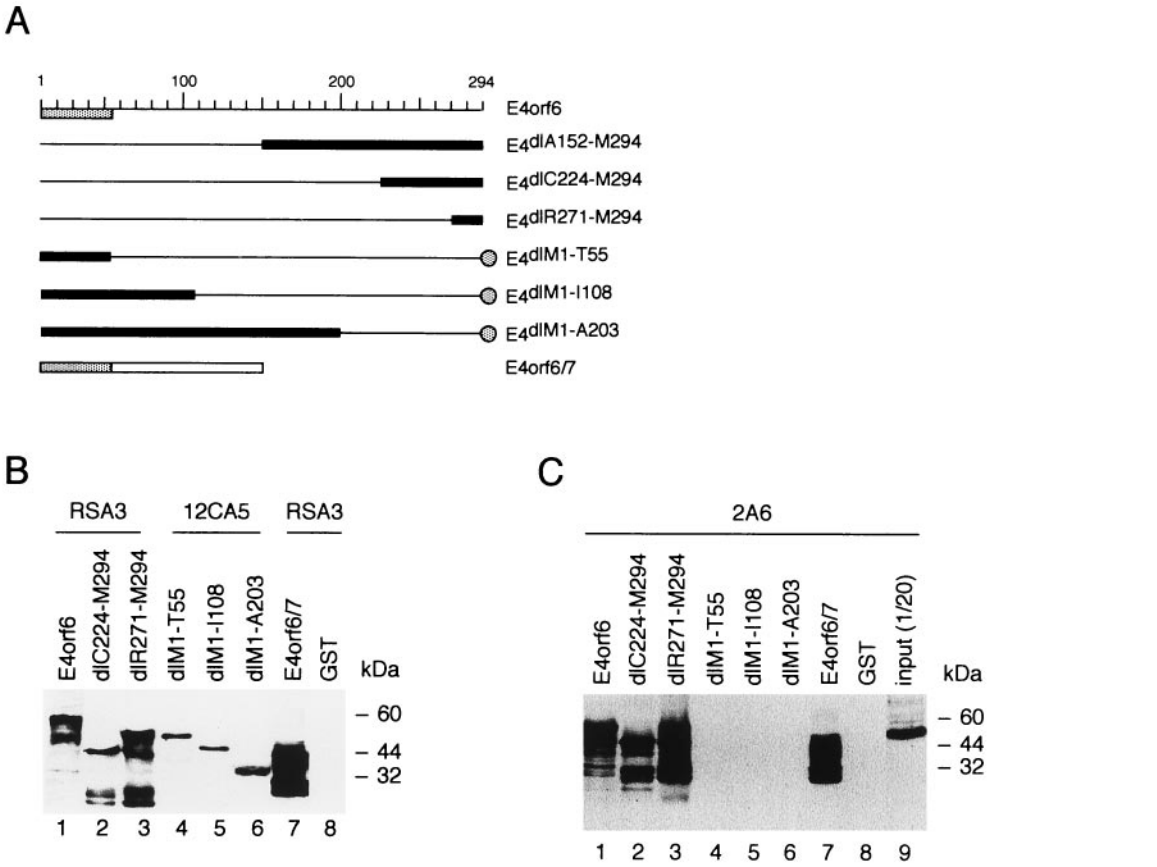

FIG. 4. The amino-terminal domain of E4orf6 and E4orf6/7 proteins interacts with E1B-55kDa in vitro. (A) E4orf6 deletion mutations. Diagram of the E4orf6 protein, with deleted domains designated by solid bars shown below. The deletion mutations (*dl*) have been named according to their first and last deleted amino acids. The open bar on the bottom represents the E4orf6/7 protein. The epitope tag at the carboxy terminus is indicated by shaded circles. Shaded bars indicate the identical 58 amino acids in the E4orf6 and E4orf6/7 proteins. (B) GST fusion proteins purified from *E. coli* TOPP6 were subjected to electrophoresis in an SDS–10% polyacrylamide gel and analyzed by Western blot analysis. Monoclonal antibody RSA3 was used to detect wild-type E4orf6/7, E4orf6, and E4orf6 carboxy-terminal deletion mutants pGEXE4$^{dlC224\text{-}M294}$ and pGEXE4$^{dlR271\text{-}M294}$ (lanes 1 to 3 and 7). Monoclonal antibody 12CA5 was used to detect the epitope-tagged pGEXE4$^{dlM1\text{-}T55}$, pGEXE4$^{dlM1\text{-}I108}$, and pGEXE4$^{dlM1\text{-}A203}$ (lanes 4 to 6). The positions of molecular mass markers are indicated in kilodaltons. (C) In vitro interaction of the amino-terminal domain of E4orf6 assayed by a protein blot experiment. E1B-55kDa from baculovirus-infected insect cells was reacted with immobilized E4 proteins. Bound E1B-55kDa proteins were then visualized with 2A6 monoclonal antibody. The amount of E1B-55kDa used in the binding reaction is shown in lane 9 (input).

formed coimmunoprecipitation experiments. 293 cells were transfected with plasmids expressing wild-type E4orf6, E4orf6/7, and deletions of the E4orf6 protein (Fig. 5). Total-cell extracts were prepared and analyzed by immunoblotting (Fig. 5A) and coimmunoprecipitation experiments (Fig. 5B). As predicted from the protein blot experiment, the coimmunoprecipitation assay did not detect a requirement for carboxy-terminal sequences for E1B-55kDa binding. Wild-type E4orf6/7, E4orf6, and E4orf6 containing carboxy-terminal deletions efficiently coprecipitated with E1B-55kDa (Fig. 5B, lanes 1 to 5) while the amino-terminal deletion mutants E4$^{dlM1\text{-}T55}$ and E4$^{dlM1\text{-}I108}$ failed to bind the 55-kDa polypeptide (lanes 6 and 7). On the basis of these results, we conclude that the E1B-E4 interaction occurs within amino acids 1 to 55 of E4orf6 and E4orf6/7 in vitro and in vivo.

DISCUSSION

Ad mutants that fail to express a functional E1B-55kDa protein display defects in viral DNA-replication (1–3, 38, 45). As shown by a number of investigators, these defects are most probably due to the inability of the corresponding mutants to efficiently promote late viral protein synthesis and shutoff of

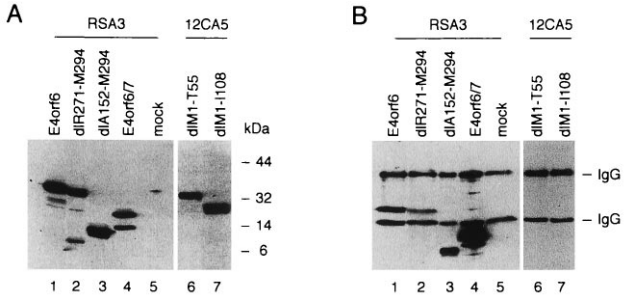

FIG. 5. E4orf6 and E4orf6/7 proteins specifically coprecipitate with E1B-55kDa from 293 cells. (A) Immunoblot of whole-cell extracts from transfected 293 cells. Subconfluent 293 cells grown on 90-mm culture dishes were transfected with 10 μg of plasmids pCMVE4orf6, pCMVE4orf6/7, pCMVE4$^{dlA152\text{-}M294}$, and pCMVE4$^{dlR271\text{-}M294}$ by calcium phosphate coprecipitation or by Lipofectamine (pCMVE4$^{dlM1\text{-}T55}$ and pCMVE4$^{dlM1\text{-}I108}$). At 36 h after transfection total cell extracts were prepared and analyzed by immunoblotting with RSA3 and 12CA5 hybridoma supernatants. The positions of molecular mass markers are indicated. (B) Coimmunoprecipitation of E4 proteins with E1B-55kDa. The same extracts were subjected to immunoprecipitation with monoclonal antibody 2A6. The precipitates were resolved on SDS–15% polyacrylamide gels, and coprecipitated E4 proteins were visualized with RSA3 (lanes 1 to 5) and 12CA5 (lanes 6 and 7) hybridoma supernatants. The bands representing the IgG proteins are indicated on the right.

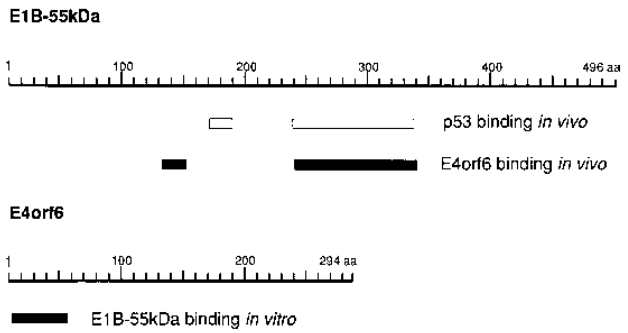

FIG. 6. Summary of protein interaction domains in Ad5 E1B-55kDa and E4orf6. The p53 interaction domains (open rectangles) are shown according to their positions along the 55-kDa polypeptide below the E1B-protein and were defined by Yew et al. (51) by double immunoprecipitations from Ad-infected primary BRK cells. The regions required for binding of the E4orf6 protein in vivo and in vitro are represented by black bars. The E1B-55kDa binding site in the E4orf6 protein is shown according to its position below the 294-amino-acid polypeptide.

host translation at the level of nucleocytoplasmic mRNA transport (1, 23, 38, 50). This E1B-dependent function also involves the Ad E4orf6 protein, since E4orf6 mutant viruses exhibit similar phenotypes at late times during infection (7, 17, 19, 41) and both proteins form a stable complex in lytically infected cells (42). However, the molecular mechanism by which the E1B-E4 protein complex modulates RNA transport in late-infected cells is still unknown.

This study was designed to gain further insight into the structural and functional requirements of the E1B-55kDa and E4orf6 protein interaction in Ad-infected cells and in vitro. The coprecipitation experiments from E1B mutant virus-infected A549 cells demonstrated that several regions in the 55-kDa polypeptide contributed to stable binding of the E4orf6 protein (Fig. 2). The most essential regions mapped to the amino terminus surrounding amino acid 143 and the central domain between amino acids 262 and 326, suggesting that two distinct segments are required for E4orf6 binding (Fig. 5 and 6).

As previously noted, mutations in the central region of the E1B-55kDa protein also interfere with the binding to p53 in vitro and in vivo (21, 51). We therefore tested the possibility that p53 was involved in the E1B-E4 complex formation. The coimmunoprecipitation experiments from wild-type and mutant virus-infected H1299 cell extracts demonstrated that p53 is not required for interaction with the E4orf6 protein (Fig. 3). Together, these results suggest that the E4orf6 protein and p53 independently interact with E1B-55kDa in virus-infected cells and share at least one binding domain in the 55-kDa polypeptide. At present, however, we cannot exclude the possibility that the insertions in the central region disrupt the tertiary structure of the 55-kDa protein, since these mutations also interfere with an apparently distinct function of E1B-55kDa (GAL4-55k) involved in the transcriptional repression of reporter constructs containing GAL4 binding sites (53). However, these mutations did not interfere with the binding of anti-55-kDa monoclonal antibody 2A6 to its amino-terminal epitope in immunoprecipitations (data not shown). These structural speculations are additionally complicated by the recent observation demonstrating that E4orf6 binds to the carboxy-terminal region of p53 (10). Thus, it seems likely that at least three separate protein complexes (E1B-E4, E1B-p53, and E4-p53) exist in Ad-infected cells. Because the E1B-55kDa and E4orf6 proteins bind to different domains on p53, it is tempting to speculate that some E4orf6 and E1B-55kDa molecules simultaneously bind to the tumor suppressor protein and cooperate to antagonize p53 function.

We also mapped the region in the E4orf6 protein responsible for the binding of E1B-55kDa to the amino-terminal 55 amino acid residues by a protein blot assay and coimmunoprecipitation experiments from transfected 293 cells (Fig. 4 to 6). At this time, we do not know whether the same region mediates the interaction with E1B-55kDa in Ad-infected cells. As described above, we were not able to coimmunoprecipitate the E1B-55kDa protein with E4orf6 from Ad-infected A549 cells with monoclonal antibody RSA3, which recognizes an epitope within the amino-terminal 55 amino acids of E4orf6 and E4orf6/7. Consistent with this result, Cutt et al. (9) have demonstrated that an E4orf6-E4orf6/7-specific antiserum recognizes only the free (noncomplexed) form of E4orf6 in Ad-infected HeLa cells and does not disrupt the E1B-E4 protein complex. These results indicate that E1B-55kDa binds to the amino-terminal region of the E4orf6 protein in Ad-infected cells, thereby masking the RSA3 epitope. The result from the in vitro binding assay also suggests that the E4orf6 protein directly interacts with E1B-55kDa. Interestingly, we were not able to detect the E1B-E4 protein interaction in vitro with either in vitro-translated proteins or proteins present in cellular extracts (9a). It seems possible that the properties of in vitro-translated proteins are different to those expressed in vivo. A similar situation has been described for the E1B-p53 interaction (6).

Both binding assays revealed that E1B-55kDa also interacts with the E4orf6/7 protein (Fig. 4 and 5), which shares the first 58 amino acids at the amino terminus with the E4orf6 polypeptide (17). This result was unexpected since we and others reproducibly failed to precipitate the E4orf6/7 protein with E1B-55kDa from Ad-infected cell extracts (9, 17) (Fig. 1 and 2). Apparently, the ability of both E4 proteins to associate with E1B-55kDa in Ad-infected cells is modulated by additional protein-protein interactions and/or posttranslational modifications. Immunoprecipitation experiments with Ad-infected MCF-7 cells indicate that both E4orf6 and E4orf6/7 proteins interact with p53, presumably through their identical amino termini (10). Furthermore, the E4orf6/7 protein transactivates the E2a promoter through its direct interaction with the cellular transcription factor E2F (30). This activity seems to involve E4orf6/7 dimerization and at least two cellular components required for E2F induction by the E4orf6/7 product (34). Although E2F binds to two segments within the carboxy-terminal region of the E4orf6/7 protein (34), these interactions could possibly interfere with the binding of E1B-55kDa to the amino-terminal region of the E4orf6/7 polypeptide in virus-infected cells.

Previous work by a number of investigators has indicated that the E4orf6 protein encodes at least two independent biological activities that regulate RNA accumulation from the major late transcription unit. The first E1B-independent function appears to regulate nuclear mRNA accumulation at the level of RNA splicing (32), whereas the second activity seems to mediate mRNA transport and/or cytoplasmic mRNA stability in conjunction with E1B-55kDa (7, 35). In addition to its suggested role in the regulation of late viral RNA metabolism, the E4orf6 protein seems to modulate transcriptional activation by the p53 tumor suppressor (10). These observations and the fact that only 50% of the E4orf6 protein are found complexed to E1B-55kDa in late-infected cells (9) suggest that different functional forms of the E4orf6 protein exist in virus-infected cells. In fact, the E4orf6 protein migrated as a doublet in A262-, R443-, and F484-infected A549 cells (Fig. 2B) that coprecipitated with E1B-55kDa (Fig. 2C). Most probably, both

polypeptides represent two different phosphorylated forms, since the E4orf6 protein contains several putative casein kinase II and protein kinase C substrate sites (unpublished data). Although the faster-migrating form was reproducibly not detectable in immunoblots from total-cell extracts and coimmunoprecipitations from *wt*300-infected A549 cells, two similarly migrating forms of the E4orf6 proteins are also expressed in wild-type-infected HeLa cells (9). Thus, the biochemical differences and biological functions of both E4orf6 forms need to be investigated in the future.

So far, we cannot say with certainty whether the amino-terminal and central region of E1B-55kDa encodes all of the functions required for the E1B-dependent modulation of nucleocytoplasmic RNA transport in late-infected cells. Mutant analysis of the E1B-55kDa product indicates that regions in the carboxy-terminal region of E1B-55kDa are also required for late functions (1, 38). Therefore, it seems possible that the amino-terminal and central domain of 55-kDa is responsible for the E4orf6-dependent localization to the viral centers of replication and transcription (37) whereas a different domain, located in the carboxy-terminal part, directly participates in the RNA transport process. Since p53 binding to the central region of E1B-55kDa is an integral way in which E1B-55kDa contributes to the transformation of primary cells in cooperation with E1A proteins (3, 51), it is tempting to speculate that 55-kDa late functions are mechanistically related to its transformation functions. Given its multifunctional nature, 55-kDa may exert its transforming activities by modulating viral and host cell RNA transport. Nevertheless, there is no evidence yet that p53 contributes to the function of the viral proteins and the E1B-E4 protein complex in late viral mRNA metabolism. The recent observation by Liang et al. (24) showing that Ad5 E1B-55kDa but not the E4orf6 protein encodes functions that directly interfere with mRNA export in *S. cerevisiae* could provide a powerful model system to further investigate the structural and functional requirements of E1B-55kDa involved in the modulation of RNA transport pathways.

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