Adenovirus Early Region 1B 58,000-Dalton Tumor Antigen Is Physically Associated with an Early Region 4 25,000-Dalton Protein in Productively Infected Cells

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In soluble protein extracts obtained from adenovirus productively infected cells, monoclonal antibodies directed against the early region 1B 58,000-dalton (E1B-58K) protein immunoprecipitated, in addition to this protein, a polypeptide of 25,000 molecular weight. An analysis of tryptic peptides derived from this 25K protein demonstrated that it was unrelated to the E1B-58K protein. The tryptic peptide maps of the 25K protein produced in adenovirus 5 (Ad5)-infected HeLa cells and BHK cells were identical, whereas Ad3-infected HeLa cells produced a different 25K protein. The viral origin of this 25K protein was confirmed by an amino acid sequence determination of five methionine residues in two Ad2 tryptic peptides derived from the 25K protein. The positions of these methionine residues in the 25K protein were compared with the nucleotide sequence of Ad2 and uniquely mapped the gene for this protein to early region 4, subregion 6 of the viral genome. A mutant of Ad5 was obtained (Ad5 *dl342*) which failed to produce detectable levels of the E1B-58K protein. In HeLa cells infected with this mutant, monoclonal antibodies directed against the E1B-58K protein failed to detect the associated 25K protein. In 293 cells infected with Ad5 *dl342*, which contain an E1B-58K protein encoded by the integrated adenovirus genome, the mutant produced an E4-25K protein which associated with the E1B-58K protein derived from the integrated genome. Extracts of labeled Ad5 *dl342*-infected HeLa cells (E1B-58K⁻) were mixed in vitro with extracts of unlabeled Ad5 wild type-infected HeLa cells or 293 cells (E1B-58K⁺). When the mixed extracts were incubated with the E1B-58K monoclonal antibody, a labeled E4-25K protein was coimmunoprecipitated. When extracts of Ad5 *dl342*-infected HeLa cells and uninfected HeLa cells (both E1B-58K⁻) were mixed, the E1B-58K monoclonal antibody failed to immunoselect the E4-25K protein. These data provide evidence that the E1B-58K antigen is physically associated with an E4-25K protein in productively infected cells. This is the same E1B-58K protein that was previously shown to be associated with the cellular p53 antigen in adenovirus-transformed cells.

At early times after adenovirus infection of human cells, five regions of the adenovirus genome are transcribed and produce cytoplasmic messenger RNAs (8, 28, 34, 36). These RNAs have been mapped to several discrete early regions of the genome termed E1, E2, E3, E4, and L1 (8, 36). The E1 transcription unit has been subdivided into two parts, E1A and E1B, that are genetically (12, 15, 16) and transcriptionally distinct (3, 6). E1A gene products are required for the positive transcriptional regulation of the other early region genes (2, 15, 26). E1B contains the genetic information for two proteins, E1B-58,000-dalton (58K) and E1B-19K antigens (20, 29, 30). Functions encoded in both the E1A and E1B genes appear to be involved in cellular transformation (11, 16). E2 is also subdivided into two parts. The E2A transcript encodes the adenovirus DNA binding protein (37, 38), whereas the E2B transcripts (9) encode a protein covalently bound to the 5' ends of adenovirus DNA (35). E3 genes and gene products are not required for efficient virus replication in cell culture (14). The nucleotide sequence of E4 was determined (13) and a number of putative proteins

encoded in this region were identified based upon this sequence and the known E4 transcripts (22). To date, a single E4 protein of 11,000 molecular weight (MW) has been identified in virus-infected cells (7, 31) and mapped to E4, subregion 3 in adenovirus 2 (Ad2).

This communication reports the identification and mapping of a second E4 adenovirus protein of 25,000 MW. The 25K protein was identified by virtue of its physical association, in a protein-protein complex, with the E1B-58K protein. Monoclonal antibodies specific for the E1B-58K protein coimmunoprecipitated the E4-25K protein in extracts of infected cells. Amino acid sequence analysis was used to determine the position of five methionine residues in two different tryptic peptides derived from the 25K protein. The 25K protein could be uniquely mapped to E4, subregion 6 by a comparison of the amino acid residues with the nucleotide sequence of Ad2 (13). A mutant was obtained which failed to synthesize the E1B-58K protein in HeLa cells but produced the E4-25K protein (detected by an in vitro association of E1B-58K and E4-25K proteins). Monoclonal antibodies directed against the E1B-58K protein coimmunoprecipitated the E4-25K protein in mutant-infected 293 cells but not in mutant-infected HeLa cells. These data indicate that the E1B-58K monoclonal antibodies do not cross-react with the E4-25K protein but detect it by virtue of its association in a complex with the E1B-58K protein. The E1B-58K-E4-25K protein complex found in productively infected cells may be

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related to an E1B-58K-p53 cellular protein complex previously detected in adenovirus-transformed rodent cells (32).

MATERIALS AND METHODS

Cell lines and cell culture. The HeLa cell line and BHK cells used in this study were obtained from the American Type Culture Collection. Monoclonal cultures were grown in Dulbecco modified Eagle medium (GIBCO Laboratories) and 10% fetal calf serum (29, 30).

Viruses and infections. Plaque-purified Ad2, Ad3, and Ad5 were employed as the wild-type viruses. Infections were performed as described previously (31).

The mutant *d1342* was originally identified as a spontaneous Ad5 E1B-58K mutant after screening plaque isolates by immunoprecipitation with Ad2 serum. The phenotype of the mutant was determined by the failure to immunoprecipitate the E1B-58K antigen and by the appearance of a new immunoprecipitable 30K protein. Mutant *d1342* was a plaque isolate from a reconstruction of E1A mutations into virus as described previously (31).

Antisera. Preparation of the Ad2 serum has been described in detail previously (29, 30). 2A6 is a monoclonal antibody directed against Ad5 E1B-58K tumor antigen (33).

Preparation of cell extracts and immunoprecipitation. Infected cells were labeled from 12 to 16 h postinfection with [³⁵S]methionine (100 Ci/ml) (New England Nuclear Corp.) in Dulbecco modified Eagle medium lacking methionine. Cells were harvested by decanting the radioactive medium, followed by washing with phosphate-buffered saline. The cells were then scraped off the plate with a rubber policeman and pelleted by low-speed centrifugation. At this point the pellet was either frozen at -20°C or used immediately. Cells were lysed by adding 1 ml of lysis buffer (50 mM Tris-hydrochloride [pH 8.0], 5 mM EDTA, 150 mM NaCl, 0.15% Nonidet P-40 [vol/vol]). The lysate was sonicated twice with a Branson sonifier for 10 s, and the insoluble debris were pelleted at 12,000 × *g* at 4°C. The supernatant was the source of the antigens.

The lysate was preadsorbed with 50 µl of *Staphylococcus aureus* Cowan (17) for 15 min at 0°C. The bacteria were pelleted, antiserum was added to the supernatant, and the extracts were incubated at 0°C for 30 min. The antigen-antibody complexes were collected by the addition of *S. aureus* bacteria (10 min at 0°C) and pelleted. The pellet was washed three times with 50 mM Tris-hydrochloride (pH 7.6)-0.5 M NaCl-5 mM EDTA-1% Nonidet P-40-5% sucrose (wt/vol). The pellet was then washed with 10 mM Tris-hydrochloride (pH 7.4)-1 mM EDTA and resuspended in sodium dodecyl sulfate (SDS) sample buffer (19). The samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (19). The gels were fluorographed (24) and exposed to an X-ray film (Eastman Kodak Co.).

Reverse-phase HPLC analysis of radioactively labeled proteins. Radioactively labeled proteins were immunoprecipitated and separated in the SDS-PAGE system of Laemmli (19). After completion of the electrophoresis, the gel was marked with radioactive ink and exposed to X-ray film. The developed film was aligned with the gel by the radioactive ink spots, and the protein bands of choice were cut out of the gel. The elution of the proteins from the gel slices was performed in a tube elution gel system as described by Maltzman et al. (24). Tryptic peptides of radioactively labeled proteins were prepared and analyzed by reverse-phase high-pressure liquid chromatography (HPLC) as described by Oosterom-Dragon and Anderson (27).

Protein and nucleotide sequence analysis. Protein sequencing was performed in a spinning-cup Beckman 890C sequencer. This procedure is described by Anderson (1) and Oosterom-Dragon and Anderson (27). The nucleotide sequence of the mutation in *d1342* was determined by the procedure of Maxam and Gilbert (25).

RESULTS

Detection of a 25K protein in adenovirus-infected cells. An experiment was designed to compare the adenovirus early proteins that were detected in infected cell extracts with either a polyclonal serum from tumor-bearing animals (29) or a monoclonal antibody directed against the E1B-58K antigen (33). HeLa cells were infected with Ad5 at a multiplicity of infection of 100 PFU per cell, and the cultures were labeled with [³⁵S]methionine from 12 to 16 h postinfection. Soluble cell extracts were prepared, and the viral proteins were immunoprecipitated (29, 30) with either polyclonal Ad2 tumor serum, a monoclonal antibody (2A6) directed against the E1B-58K tumor antigen (33), or normal serum. An autoradiogram of the adenovirus early proteins detected by these antibody preparations and analyzed on an SDS-polyacrylamide gel is presented in Fig. 1. The Ad2 tumor serum detected the E1B-58K protein, the E1B-19K protein, an E4-11K protein, and a 25K protein. The assignments of these proteins to adenovirus early region genes was previously documented (29, 30, 31). The monoclonal antibody 2A6 directed against the E1B-58K antigen immunoprecipitated that protein (33) and a second antigen of 25K. Normal serum failed to react with any of these virus-specific antigens. The

FIG. 1. Immunoprecipitation of early Ad5 antigens in infected HeLa cells. ad2, Polyclonal tumor serum; 2A6, anti-E1B-58K monoclonal antibody; ns, normal serum. The faint protein band migrating around 25K in the normal serum lane was a contaminant protein that reproducibly migrated more slowly than the 25K protein described in the text.

observation that the monoclonal antibody directed against the E1B-58K protein also immunoprecipitated a 25K protein could be explained in several ways. The 25K protein could be a common proteolytic breakdown product of the 58K antigen, as has been observed with the 72K-44K adenovirus DNA binding proteins (18, 21). So long as the 58K and 25K proteins shared a common antigenic determinant, one would obtain this result (Fig. 1). Alternatively, the 58K and 25K proteins could be distinct but contain cross-reacting determinants detected by the polyclonal and monoclonal antibodies. In this case, the 25K protein could be either viral or cellular in origin. Finally, the possibility exists that the E1B-58K protein is physically associated, in a protein complex, with the 25K protein. Thus, antibody directed against the 58K antigen would coimmunoprecipitate the 25K protein, as is the case with the simian virus 40 T antigen or the adenovirus E1B-58K antigen and the cellular protein p53 (23, 32.)

Characterization of the 25K protein. To determine the origin of the 25K protein detected in adenovirus-infected cell

extracts (Fig. 1), tryptic peptides from the E1B-58K and 25K proteins were compared. Ad5-infected HeLa cells were labeled with [35 S]methionine from 12 to 16 h postinfection. The soluble protein extracts prepared from these cells were incubated with the monoclonal antibody directed against the E1B-58K protein, and the immunoprecipitates were analyzed on SDS-polyacrylamide gels. The 58K and 25K proteins (as above for Fig. 1) were removed from the gel by electrophoretic elution, oxidized, and treated with trypsin. The [35 S]methionine-labeled tryptic peptides were separated by reverse-phase HPLC. Figure 2 presents the tryptic peptide elution pattern for those peptides derived from the 25K protein (A) and the 58K protein (B). These two proteins shared few, if any, common peptides, indicating that the 25K protein was not a proteolytic breakdown product of the 58K protein.

Reverse-phase HPLC analysis of the 25K methionine-labeled tryptic peptides was next employed to study the viral or cellular origin of this protein. Human HeLa cells were

FIG. 2. Reverse-phase HPLC of the [35 S]methionine-labeled tryptic peptides of the 25K and 58K proteins immunoprecipitated from infected HeLa cells by monoclonal antiserum specific for the Ad5 E1B-58K protein. Samples were prepared and analyzed as described in the text. A, Profile of [35 S]methionine tryptic peptides obtained from the 25K protein; 29,300 cpm were applied to the column. B, Comparable profile for the 58K protein; 176,500 cpm were applied to the column. The radioactivity in a 150- μ l portion of each fraction has been plotted without background subtraction. Inspection of the 215-nm adsorption profile for each sample separation indicated that they are matched to within ± 1 fraction. The theoretical acetonitrile gradient profile used to develop the column is shown.

infected with either Ad5 or Ad3 serotypes of the virus, and BHK cells were similarly infected with Ad5. These cells were labeled with [35 S]methionine, soluble extracts were incubated with polyclonal Ad2 serum, and the 25K proteins were obtained as in the previous experiments. The tryptic peptides produced by the 25K protein from Ad5-infected HeLa cells and Ad5-infected BHK cells were identical (Fig. 3A and B), whereas the 25K protein from Ad3-infected HeLa cells was distinct from the Ad5-derived proteins produced in HeLa cells (C). These data indicate that the Ad5-25K protein produced in human and BHK cells is likely viral encoded and independent of the host cell species.

Mapping the gene for the 25K protein. To confirm the suggestion that the 25K protein was adenovirus encoded and to localize the viral gene for this protein, amino acid sequence determinations were carried out for the 25K protein. Because the complete nucleotide sequence of Ad2 is available for comparison, the 25K protein from Ad2 was employed. Ad2-infected HeLa cells were labeled from 12 to 16 h with [35 S]methionine, and the 25K protein was obtained as in the experiment described above. Tryptic peptides derived from the Ad2-25K protein were analyzed by reverse-

FIG. 3. Reverse-phase HPLC analysis of [35 S]methionine tryptic peptides of the 25K protein immunoprecipitated from Ad5-infected BHK or HeLa cells and Ad3-infected HeLa cells. The 25K protein was prepared and analyzed as described in the text, and a polyclonal Ad2 serum was used to precipitate the samples displayed in A (Ad5-BHK), B (Ad5-HeLa), and C (Ad3-HeLa). The total radioactivity applied to the reverse-phase column was: A, 3,250 cpm; B, 3,500 cpm; C, 3,600 cpm. The entire 1.0 ml fraction was assayed for radioactivity with 9 ml of Aquasol II (New England Nuclear) per fraction.

FIG. 4. Reverse-phase HPLC profile of the [35 S]methionine-labeled tryptic peptides from the Ad2 E4-25K protein. Soluble extracts were prepared and immunoprecipitated with the 2A6 anti-E1B-58K monoclonal antibody. Samples were prepared and analyzed as described in the text.

phase HPLC, and their elution profile is presented in Fig. 4. The patterns of methionine-containing tryptic peptides of the 25K protein derived from Ad2, Ad5, and Ad3 were all distinct (Fig. 3 and 4), once again indicating a viral origin for this protein. The tryptic peptides M3 and M5 (Fig. 4) were subjected to sequential automated Edman degradation to determine the positions of the methionine residues in these two peptides. The positions of methionine residues as determined from this analysis are presented in Fig. 5A for peptide M3 and 5B for peptide M5. Two methionine residues at positions 1 and 7 were found in peptide M3, and three methionine residues at positions 3, 5, and 10 were detected in peptide M5 (Fig. 5). A computer-assisted search of the nucleotide sequences from both strands of Ad2 DNA re-

FIG. 5. Amino terminal sequence analysis of [35 S]methionine-labeled tryptic peptides from the Ad2 E4-25K polypeptide. [35 S]methionine-labeled Ad2 E4-25K polypeptide was purified by SDS-PAGE of immunoprecipitated proteins derived from infected cell extracts. The electrophoretically eluted 25K polypeptide was oxidized with performic acid, digested with trypsin, and applied to a reverse-phase (C-18) HPLC column for separation of the tryptic peptides as described in the text. Appropriate fractions were pooled, lyophilized, and subjected to automated Edman degradation as previously described (31). The total radioactivity released after each Edman cycle is shown with two methionine-containing tryptic peptides designated M3 and M5 (Fig. 4). The radioactivity applied to the sequencer for each experiment was: A (peptide M3), 500 cpm; B (peptide M5), 680 cpm.

vealed only one possible coding location for these five methionine residues derived from peptides M3 and M5 in the same protein. This sequence was localized in E4, subregion 6, and the amino acid sequences expected for peptides M3 and M5 are given in Fig. 5 in the one-letter amino acid code. The nucleotide sequence of E4 in Ad2 (13) has an open reading frame for a 34,000-MW protein located between 95.2 and 92.4 map units, and peptides M3 and M5 are localized within this protein. The difference between the actual molecular weight calculated from the nucleotide sequence (34K) and the apparent molecular weight in SDS-PAGE (25K) could be due to the gel system employed or the 34K protein could be a precursor to the 25K protein. In either case, these experiments clearly map the gene for the 25K protein to E4, subregion 6.

Adenovirus mutant Ad5 *dl342* fails to produce the E1B-58K antigen. The viral origin of the 25K protein still leaves open the question of whether the 2A6 E1B-58K monoclonal antibody detects the E4-25K protein because E4-25K is in a protein complex with the E1B-58K antigen or because 2A6 cross-reacts with antigenic determinants in the E4-25K protein. Several experiments addressed this question. First, both the E1B-58K and the E4-25K proteins were found to copurify in a variety of chromatographic procedures (results not presented). Second, E1B-58K protein purified by SDS-PAGE was shown to be immunoprecipitable with the 2A6 E1B-58K monoclonal antibody, but gel-purified E4-25K protein failed to react with this 2A6 monoclonal antibody (results not presented). Although both of these observations are consistent with a putative E1B-58K-E4-25K protein complex, they do not prove such a complex exists.

To this end, an Ad5 mutant, *dl342*, that failed to synthesize the E1B-58K protein was characterized. Ad5 *dl342*

replicated in HeLa cells to a titer about two orders of magnitude lower than that of Ad5 wild type. In 293 cells, however, Ad5 *dl342* and Ad5 wild type grew to the same titer (results not presented). These data suggested that the Ad5 *dl342* mutant contained a defect in the E1 region of the viral genome that was complemented by the Ad5 wild-type gene products produced in 293 cells. Nucleotide sequence analysis identified and localized this mutation as a deletion of one base pair (GC) at nucleotide 2179 in E1B (4). This deletion is located in the coding sequences of the E1B-19K protein and the E1B-58K protein. Figure 6 presents the map of the E1B transcripts and proteins with the nucleotide map numbers of Bos et al. (4).

The deletion in Ad5 *dl342* results in a frameshift mutation in the E1B-19K protein. The wild-type E1B-19K protein begins with an AUG at nucleotide 1714 and terminates with a UGA at nucleotide 2242, whereas the mutant protein reads through the termination codon in the -1 reading frame to nucleotide 2255. The E1B 13S mRNA is spliced at this site to an acceptor site at nucleotide 3595. The mutant 19K protein is translated through the splice and is fused in frame to protein IX. The size of such a fused protein would be predicted at 330 amino acids or about 30,000 MW. Evidence for the existence of this fused protein (Fig. 6) will be presented in the next sections. The 19K protein translated from the E1B 22S mRNA also reads out of frame from nucleotide 2179 and terminates at a TGA at nucleotide 2261. The deletion in Ad5 *dl342* also alters the E1B-58K protein. The wild-type E1B-58K protein is initiated at an AUG at nucleotide 2019 and terminates with a UGA at nucleotide 3507 (Fig. 6). The Ad5 *dl342* deletion creates a frameshift 53 amino acids into the protein, resulting in termination out of phase 22 amino acids later. An E1B-58K protein fragment of

FIG. 6. A map of the E1B DNA, transcripts, and the Ad5 *dl342* mutant. A, Ad5 nucleotide map and numbering system for E1B with the position of the Ad5 *dl342* mutation, a deletion of a single G residue at nucleotide 2179. The 22S and 13S transcripts, their splice junctions, initiation sites, and polyadenylation sites are given. B, Coding regions of the Ad5 wild-type E1B-58K, E1B-19K, and protein IX antigens with the nucleotide numbers for the AUG start and termination codons given. C, Proteins produced by the Ad5 *dl342* mutant: the predicted 7,500-MW E1B-58K protein fragment, the E1B-19K frameshift mutation, and the new E1B-19K protein IX fusion product predicted at 30K and detected in these experiments.

about 7,500 MW would be produced. Thus the single-base-pair deletion in Ad5 *dl342* would be expected to disrupt both the E1B-19K and E1B-58K proteins and create a new fusion protein between the E1B-19K antigen and protein IX.

In addition to the nucleotide sequence of Ad5 *dl342*, the evidence for these interpretations derives from examining the E1B proteins produced in HeLa cells infected with this mutant. The [³⁵S]methionine-labeled soluble proteins from HeLa cells infected with Ad5 wild type or Ad5 *dl342* were incubated with the Ad2 tumor serum, the E1B-58K 2A6 monoclonal antibody, or normal serum. The immunoprecipitates were collected and analyzed on SDS-polyacrylamide gels, and an autoradiogram of this gel is presented in Fig. 7. The 2A6 monoclonal antibody detected the E1B-58K and E4-25K proteins in Ad5 wild-type infected cells but not in Ad5 *dl342*-infected cell extracts. The Ad2 tumor serum immunoprecipitated the E1B-58K, E4-25K, E1b-19K, and E4-11K proteins from extracts of Ad5 wild type-infected cells but only detected the E4-11K proteins and a new 30K protein in Ad5 *dl342*-infected cell extracts (Fig. 7). The protein band migrating at 58K in this gel (Ad2 serum, Ad5 *dl342* infection) was a contaminant protein and not the E1B-

FIG. 7. Immunoprecipitation of early adenovirus antigens: Ad5 wild type- or mutant Ad5 *dl342*-infected HeLa cells. Extracts of soluble proteins from Ad5 wild type- or Ad5 *dl342*-infected cells immunoprecipitated and analyzed as described in the text. Ad2, Serum from hamsters bearing Ad2-induced tumors. 2A6, A monoclonal antibody directed against the E1B-58K tumor antigen. ns, Normal hamster serum.

FIG. 8. Immunoprecipitation of early adenovirus antigens detected in virus-infected 293 cells. 293 cells were infected with Ad5 wild type or Ad5 *dl342*, and samples were processed as described in the text. Lane 1, Immunoprecipitates from Ad5 *dl342*-infected 293 cells. Lane 2, Immunoprecipitates obtained from Ad5 wild type-infected 293 cells. Lane 3, Immunoprecipitates from mock-infected 293 cells. Ad2 serum was used in these experiments.

58K protein. This contaminant was not reproducibly observed (see Fig. 10), and the monoclonal antibody 2A6 directed against the E1B-58K protein (Fig. 7 and see Fig. 10) did not detect it.

Coincident with the failure to synthesize the E1B-58K protein in Ad5 *dl342*-infected HeLa cells is the failure to detect the E4-25K protein with the 2A6 monoclonal antibody or the Ad2 tumor serum. This is consistent with the proposal that these antibodies, directed against the E1B-58K protein, coimmunoprecipitate the E4-25K protein because it is associated in an oligomeric complex with the E1B-58K antigen. It should be noted that the E4-11K protein is produced in normal levels by Ad5 *dl342*, demonstrating that this mutation has no abnormal effects upon this E4 protein. To prove, however, that the mutant Ad5 *dl342* does synthesize the E4-25K protein, Ad5 *dl342* was employed to infect 293 cells where the integrated adenovirus genome will supply the E1B-58K antigen that may then complex with the Ad5 *dl342* E4-25K protein. [³⁵S]methionine-labeled, uninfected 293 cell protein extracts incubated with Ad2 tumor serum contain only the E1B-58K antigen as detected in the autoradiogram presented in Fig. 8, lane 3. Ad5 wild type-infected 293 cell extracts immunoprecipitated with this same antiserum contained both the E1B-58K and E4-25K proteins (Fig. 8, lane 2), whereas Ad5 *dl342*-infected cell extracts contained the E1B-58K protein, the E4-25K protein, and the 30,000-MW species peculiar to this mutant (Fig. 8, lane 1). It is clear from this analysis that the Ad5 *dl342* mutant, like the wild-type virus, can produce the E4-25K protein in normal amounts. The failure of both the Ad2 polyclonal serum and the 2A6 E1B-58K monoclonal serum to detect the E4-25K protein in Ad5 *dl342*-infected HeLa cells is because these antibodies do not react with this protein. Instead these antibodies detect the E1B-58K antigen and coimmunoprecipitate the E4-25K protein by virtue of its physical associa-

tion in a protein complex with the E1B-58K antigen (in Ad5 wild type-infected cells or in Ad5 *dl342*-infected 293 cells).

In vitro association of the E1B-58K and E4-25K proteins. The experiments described in the previous section suggest that the E1B-58K monoclonal antibody coimmunoprecipitates the E4-25K protein by virtue of its association, in a complex, with the E1B-58K antigen. It remains possible, however, that the E1B-58K protein (in HeLa cells) is required for the production (synthesis or processing) of the E4-25K protein in virus-infected cells and that the monoclonal antibody cross-reacts with the E4-25K protein. This is because no formal proof has been presented that Ad5 *dl342*-infected HeLa cells contain the E4-25K protein. This protein was only observed in Ad5 *dl342*-infected 293 cells. To address this problem, an in vitro association of the E1B-58K and E4-25K proteins was attempted. Ad5 *dl342*-infected HeLa cells were labeled with [³⁵S]methionine as in the previous experiments, and soluble protein extracts were prepared. These extracts were mixed with unlabeled cell extracts from either uninfected HeLa cells, Ad5 wild type-infected HeLa cells, or 293 cells. The mixed extracts were incubated and then treated with the E1B-58K monoclonal antibody, 2A6. The immunoprecipitates were collected and analyzed on an SDS-polyacrylamide gel. Figure 9 presents an autoradiogram of this gel. Extracts from Ad5 wild type-infected HeLa cells treated with the 2A6 monoclonal antibody immunoselected the E1B-58K and E4-25K proteins as detected previously (Fig. 9, lane 1), whereas extracts from

FIG. 9. In vitro association of the E1B-58K and E4-25K proteins. HeLa cells were infected with either Ad5 wild type or Ad5 *dl342*, and one set of cell cultures was labeled with [³⁵S]methionine, whereas a second set remained unlabeled. Soluble protein extracts were obtained as described in the text and mixed as follows. Lane 1, [³⁵S]methionine-labeled, Ad5 wild type (wt)-infected cell extract treated with the 2A6 monoclonal antibody directed against the E1B-58K antigen; 2, [³⁵S]methionine-labeled Ad5 *dl342*-infected cell extract treated with 2A6 antibody; 3, [³⁵S]methionine-labeled Ad5 *dl342*-infected cell extract mixed with unlabeled, uninfected HeLa cell extract and treated with 2A6 antibody; 4, [³⁵S]methionine-labeled Ad5 *dl342*-infected HeLa cell extract mixed with unlabeled Ad5 wild type-infected HeLa cell extract and treated with 2A6 antibody; 5, [³⁵S]methionine-labeled Ad5 *dl342*-infected HeLa cell extract mixed with an unlabeled 293 cell extract and treated with 2A6 monoclonal antibody.

Ad5 *dl342*-infected cells did not immunoprecipitate either of these proteins (Fig. 9, lane 2). The labeled Ad5 *dl342*-infected cell extract mixed with unlabeled, uninfected HeLa cell extracts also failed to detect the E4-25K protein as expected (Fig. 9, lane 3). [³⁵S]methionine-labeled Ad5 *dl342*-infected HeLa cell extract mixed with either an unlabeled Ad5 wild type-infected HeLa cell extract or 293 cell extract (which supplied unlabeled E1B-58K protein) (Fig. 9, lanes 4 and 5) detected a labeled E4-25K protein upon immunoprecipitation with the 2A6 (E1B-58K-specific) monoclonal antibody. This experiment (Fig. 9) demonstrated several points. The Ad5 *dl342* mutant synthesizes the E4-25K protein in infected HeLa cells. The 2A6 monoclonal antibody directed against the E1B-58K antigen fails to immunologically cross-react with E4-25K protein. And, the E1B-58K-E4-25K protein complex can be assembled in vitro by mixing crude extracts of these two proteins. Based upon the level of the E4-25K protein detected in extracts of Ad5 *dl342*-infected 293 cells (Fig. 8) and recovered by mixing Ad5 *dl342*-infected HeLa-293 cell extracts in vitro, the in vivo complex is formed more efficiently than is the E1B-58K-E4-25K complex assembled in vitro.

Ad5 *dl342* produces a fusion protein between the E1B-19K antigen and protein IX. Based upon the nucleotide sequence analysis of Ad5 *dl342* (summarized in Fig. 6) and the synthesis of a new protein of 30,000 MW in Ad5 *dl342*-infected cell extracts (Fig. 7 and 8), it has been suggested that this new protein is a fusion product between the N-terminal end of the E1B-19K protein and protein IX. This predicts that the 30,000-MW protein would contain both E1B-19K and protein IX antigenic determinants. To test this, [³⁵S]methionine-labeled extracts from Ad5 wild type- and Ad5 *dl342*-infected HeLa cells were prepared. These extracts were incubated with the Ad2 tumor serum, the 2A6 E1B-58K monoclonal antibody, a monoclonal antibody directed against protein IX (5), and normal serum. These immunoprecipitates were analyzed on SDS-polyacrylamide gels, and the autoradiogram of this gel is presented in Fig. 10. In the extracts from Ad5 wild type-infected cells, the Ad2 serum and the 2A6 E1B-58K monoclonal antibody immunoprecipitated the proteins as described above. The protein IX monoclonal antibody detected only the 12,000-MW protein IX, whereas normal serum detected only background bands (Fig. 10). In extracts derived from the Ad5 *dl342*-infected HeLa cells, the 30,000-MW protein made by this mutant was immunoprecipitated by the Ad2 tumor serum (in minor amounts) and the monoclonal antibody directed against protein IX (in major amounts). Normal-size protein IX was also made by this mutant, indicating that normal protein IX mRNA is also produced by this virus. These results demonstrate that the 30,000-MW protein made by Ad5 *dl342* contains protein IX and E1B-19K antigenic determinants in addition to eliminating the production of normal E1B-19K protein. Thus the origin of this 30,000-MW protein is from a fusion of the E1B-19K-protein IX products, as detailed in Fig. 6.

DISCUSSION

There are several interesting observations and conclusions to be made by the studies presented here. First, these experiments have detected and mapped a new 25K adenovirus protein derived from E4, subregion 6. Second, this E4-25K protein is found to be in a protein-protein complex or in association with the E1B-58K viral antigen during productive infection of HeLa or 293 cells. This protein complex can

FIG. 10. Immunoprecipitation of early adenovirus proteins in Ad5 wild type- and Ad5 *dl342*-infected cells with Ad2 serum, anti-E1B-58K monoclonal serum, and antiprotein IX monoclonal serum. The experimental protocols were as presented in the text. Ad2, Polyclonal tumor serum; 2A6, anti-E1B-58K monoclonal antibody; IX, antiprotein IX monoclonal antibody; ns, normal hamster serum.

be assembled *in vitro*. In adenovirus-transformed cells, the E1B-58K antigen was shown to be in an oligomeric protein complex with a cellular protein, p53 (32). The E1B-58K-cellular p53 complex, however, was not detected in productively infected HeLa cells (32). Third, a mutant, Ad5 *dl342*, which contains a single-base-pair deletion in E1B is described. This mutation results in a reading frame shift near the N-terminal end (after 53 amino acids) of the E1B-58K protein, eliminating the production of this viral antigen. This mutation also results in the creation of a new 30,000-MW fused protein derived from the E1B-19K antigen and protein IX (Fig. 6). The fusion protein arises from translation in the 19K open reading frame from the E1B 13S mRNA (Fig. 6). This mutant is therefore an interesting example of how a single-base deletion can create a new protein that can then be acted upon for selective advantage or disadvantage over evolutionary time scales.

Several lines of evidence demonstrate that the 25K protein detected in this study is of viral origin. The peptide maps of this protein synthesized in Ad5-infected HeLa and BHK cells were identical, whereas the 25K proteins synthesized in Ad2-, Ad3-, and Ad5-infected HeLa cells were all distinct in their methionine-containing tryptic peptides. The positions of five methionine residues in two tryptic peptides from the 25K protein were localized in the amino acid sequence of these peptides. Based upon the positions of these amino acid residues, the 25K protein could be shown to be derived from the adenovirus nucleotide sequence found in E4, subregion 6. This E4 protein has a calculated 34,000 MW and maps between 92.4 and 95.2 map units of the genome. This is the second E4 viral protein to be detected in infected cell

extracts, the first being the E4-11K protein from E4, subregion 3 (7, 31).

Sera from animals bearing adenovirus-induced tumors always contain antibodies directed against the E1B-58K tumor antigen (10, 20, 29, 30). The Ad2 tumor serum employed in this study also contained antibodies reacting with the E1B-19K and E4-11K antigens (Fig. 1, 8, and 9). Both this Ad2 tumor serum as well as the 2A6 monoclonal antibody directed against the E1B-58K antigen detected the E4-25K protein (Fig. 1, 8, and 9) in these experiments. This could be due to either the physical association of the E4-25K protein with the E1B-58K antigen, resulting in the coimmunoprecipitation of the E4-25K protein or the cross-reaction of the E1B-58K antibody with this E4-25K protein. Soluble protein extracts of Ad5 *dl342*-infected HeLa cells, a mutant which failed to synthesize a normal E1B-58K antigen, contained no detectable E4-25K protein when the Ad2 polyclonal serum or 2A6 monoclonal antibodies were employed to immunoselect these proteins. Ad5 *dl342*-infected HeLa cells do, however, contain the E4-25K protein as demonstrated by its presence and association *in vitro* (in mixed extracts) with the E1B-58K protein from Ad5 wild type-infected cells or 293 cell extracts. Thus, the polyclonal Ad2 serum and the 2A6 monoclonal antibodies do not cross-react with the E4-25K protein. These experiments demonstrate therefore that an E1B-58K-E4-25K protein complex is formed (*in vivo* and *in vitro*) in virus-infected cells. Consistent with this observation is the fact that mutants in the E1B-58K and E4-25K genes appear to have similar phenotypes (J. Cutt, S. Pilder, D. Halbert, and T. Shenk, personal communication).

Given these conclusions, it is of some interest that the E1B-58K antigen has been shown to also form a protein-protein complex with a cellular antigen, p53, in adenovirus-transformed cells (32). The existence of two protein oligomeric complexes, E1B-58K-p53 and E1B-58K-E4-25K, that share one subunit in common may reflect some functional significance that will now need to be investigated.

ACKNOWLEDGMENTS

We thank A. K. Teresky, R. Pashley, and G. Urban for assistance. The gift from C. Cepko of monoclonal antibodies against adenovirus protein IX is gratefully acknowledged.

This work was supported by grants MV-45 and MV-47 from the American Cancer Society, P.H. and D.H. were supported by postdoctoral fellowships from the Jane Coffin Childs Memorial Fund for Medical Research (61562) and the National Institutes of Health (CA 07078), respectively.

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