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Ability of p53 and the Adenovirus E1b 58-Kilodalton Protein To Form a Complex Is Determined by p53

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We have investigated p53-E1b 58-kilodalton (kDa) protein complex formation during permissive and semipermissive infections with adenovirus type 5 (Ad5) *dl*309. While metabolic labeling studies easily detected p53-E1b 58-kDa protein complexes in transformed rat cells (*Xho*I-C), the same methods have not revealed complexes during infection of either human osteosarcoma cells (permissive) or normal rat kidney cells (semipermissive). Complexes were not detectable at any stage during the replicative cycle of Ad5 *dl*309 in osteosarcoma cells, and they could not be stabilized by using an in vivo cross-linking agent. In addition, using the E4-defective mutant Ad5 *dl*355, no complexes were observed either. Thus, the lack of p53-E1b 58-kDa protein complex formation during infection is not due to competition from the E4 34-kDa protein. In vitro association experiments showed that in vitro-translated mouse and human p53 could form complexes with E1b 58-kDa antigen expressed during infection. Thus, such E1b proteins are competent to form complexes. The converse experiment, in which in vitro-translated E1b 58-kDa protein was mixed with lysates of osteosarcoma cells, showed little or no p53-E1b 58-kDa protein association, even though the in vitro E1b 58-kDa protein could associate stably with p53 from cells containing endogenous p53-E1b 58-kDa protein complex. These data suggest that competence to form p53-E1b 58-kDa protein complexes resides in some property of p53.

p53 is a cellular phosphoprotein which has been implicated in a regulatory role in the G_0-to-G_1 transition in normal cells (6, 27, 28, 33). In tumor and transformed cell lines, p53 is often present at elevated levels (7, 24, 41), and this is associated with a marked increase in p53 stability (29, 34, 35). Direct evidence for a causative role for p53 in multistage carcinogenesis has been demonstrated by the observation that p53 cDNA constructs immortalize primary rodent cells in culture (18) and cooperate with an activated *ras* oncogene to cause complete transformation (9, 18, 30). Mutations generated in p53 which render the encoded gene product more stable than the wild-type protein have also been shown to stimulate colony outgrowth in immortalization assays (17). Thus p53 can itself be activated by mutation to cause transformation.

One of the best-characterized features of p53 is its ability to associate with other proteins. p53 was first identified in a complex with the simian virus 40 (SV40) large T antigen (23, 24) but has since been found to associate with the adenovirus type 5 (Ad5) E1b 58-kilodalton (kDa) tumor antigen in transformed rodent cells (37, 43), although not with the Ad12 E1b tumor antigen (25, 43) and with cellular heat-shock proteins (hsp 72-73) (32, 39). In addition, p53 self-complexes to form high-molecular-weight homo-oligomeric structures (5).

The significance of the ability of p53 to form complexes with different proteins of apparently dissimilar function is not clear. However, recent experiments from this laboratory have shown that SV40 T-antigen-binding p53 mutants restrict SV40 origin-dependent DNA replication, whereas non-T-antigen-binding mutants do not (4). These data might point the way towards an understanding of the function of one kind of complex.

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The adenovirus E1b large tumor antigen (58 kDa in Ad5; 54 kDa in Ad12) is one of two major proteins encoded in the E1b gene. Mutant viruses which lack a functional E1b 58-kDa product show impaired production of late viral mRNA (1, 31), which results in lowered late-protein synthesis and virus titer (as described above). The E1b 58-kDa protein gene mutations do not affect early-region protein synthesis or DNA replication but appear to be specifically involved in regulating the levels of late viral mRNA available for translation. Mutations in the Ad12 E1b 54-kDa protein gene have produced a similar set of results (10).

During lytic infection of HeLa cells with Ad5, the E1b 58-kDa protein was found in a complex with another 34-kDa viral protein encoded in early region 4 (E4) (8, 36). The function of this complex is unknown, although mutant E4 viruses that do not form such a complex are moderately impaired for lytic growth (13). No p53-E1b 58-kDa protein complexes have been detected during lytic infection of HeLa cells (37).

To learn more about the E1b 58-kDa protein function, we examined the ability of the E1b 58-kDa protein to associate with p53 in Ad5-transformed rodent cells, in permissive (human) and nonpermissive (rat) host cells for adenovirus replication, and in vitro. We show here that p53-E1b 58-kDa protein complexes are not detectable during permissive and nonpermissive infections, and we provide evidence that the ability to form such a complex is determined by a property of p53.

MATERIALS AND METHODS

Cells and viruses. Human osteosarcoma (HOS; TE85 clone 5 ATCC CRL 1543) (40) cells and monolayer HeLa cells were used as permissive hosts in Ad5 infection experiments, and normal rat kidney cells (NRK49F, passages 15 to 22) were used as a semipermissive host. 293 cells (11) and *Xho*I-C cells (43) were used in some experiments as sources of p53 and E1b 58-kDa protein from transformed cells. Ad5 *dl*309 (from C. Goding, Marie Curie Research Institute),

which contains only one *Xba*I site at base pair 1339, served as wild-type Ad5 in these studies (20). Ad5 *dl*355 (8, 13) was used as an early region 4 (E4)-defective mutant (inoculum provided by A. Bellett, John Curtin School, Canberra, Australia). Ad5 *dl*355 contains a 14-base-pair deletion in open reading frame 6 of Ad5 (8, 13). Both viruses were propagated in monolayer HeLa cells and were titrated as previously described (3).

Antibodies. The mouse monoclonal anti-p53 antibody pAb421 (14) was used to immunoprecipitate p53 from both human and rat cells in all experiments. To precipitate the E1b 58-kDa protein, a polyclonal serum from tumor-bearing hamsters, designated anti-Ad5 58-kDa protein serum (H146) (from E. Blair, Leeds University, England), was used. To precipitate hexon, a polyclonal antihexon antiserum (Hex) was used (from E. Blair).

Infections. Unless otherwise indicated, 9-cm plastic dishes (Nunc, Roskilde, Denmark) were seeded with 3×10^6 to 4×10^6 cells per dish in Dulbecco modified Eagle medium (Flow Laboratories, Inc.) supplemented with 10% fetal calf serum (GIBCO Laboratories) and L-glutamine. The cells were incubated overnight and were then infected with 50 to 100 infectious units of virus as previously described (3).

Labeling of cells and preparation of cell lysates. At indicated times after infection, or the day after seeding for uninfected cells, cells were starved for 1 h with methionine-free medium (Flow Laboratories, no. 16-222-49) and were then labeled for 2 h with 200 μCi of [^{35}S]methionine (Amersham Corp., SJ235; specific activity, >800 Ci mmol^{-1}) in 3 ml of the medium described above. Cells were washed twice with phosphate-buffered saline and were then lysed in RIPA buffer (10 mM Tris hydrochloride [pH 8.0]–150 mM NaCl–1 mM EDTA–1% Nonidet P-40–1% sodium deoxycholate–0.25-mg ml^{-1} phenylmethylsulfonyl fluoride–30-μg ml^{-1} aprotinin without sodium dodecyl sulfate [SDS]). Extracts were cleared by two spins for 5 min each at 15,000 × *g* in a bench-top centrifuge (MSE Microcentaur) at 4°C. Extracts were stored at −70°C. For in vitro association, lysates were prepared by using RIPA buffer plus 0.1% SDS, and the extracts were cleared by centrifugation at 120,000 × *g* for 20 min.

In vivo cross-linking. After labeling, but before lysis of cells, monolayers were briefly treated with hypotonic buffer containing 12.5 mM deoxyglucose to swell cells and were then incubated for 20 min with dimethyl-3,3′-dithiobispropionimidate dihydrochloride (1 mg ml^{-1}) (Pierce Chemical Co., Rockford, Ill.)–phenylmethylsulfonyl fluoride (2 mg ml^{-1}). Cells were then washed with phosphate-buffered saline containing 200 mM glycine–12.5 mM deoxyglucose for 20 min and were lysed, and immunoprecipitates were prepared as described herein.

Protein analysis. Approximately one-third of each 9-cm dish was used for each immunoprecipitation. Proteins were immunoprecipitated with specific antibodies by using the *Staphylococcus aureus* procedure (21). Briefly, lysates were preabsorbed for 2 h with nonimmune normal rabbit serum (NRS) at 4°C and were then immunoprecipitated with appropriate polyclonal serum or monoclonal antibody for 2 h or overnight. Antigen-antibody complexes were collected by the addition of *S. aureus* and were pelleted. After three washes in RIPA buffer with or without 0.1% SDS, depending on the experiment, the pellet was suspended in 30 μl of loading buffer (60 mM Tris hydrochloride [pH 6.8]–1% SDS–10% sucrose–0.5% β-mercaptoethanol–0.05% bromophenol blue). Samples were analyzed by using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (22). Gels were fluorographed and then exposed to XAR-5 (Eastman Kodak Co.) high-intensity X-ray film. Most gels were exposed for 16 to 40 h.

Western blotting. Unlabeled proteins were immunoprecipitated and analyzed by SDS-PAGE as described above. Following electrophoresis, the separated proteins were transferred to a nitrocellulose membrane overnight as described by Simanis and Lane (38). The proteins were immunoprecipitated with pAb421 and labeled with ^{125}I-protein A, also by the procedure described above.

In vitro transcription-translation reactions. Transcription-translation reactions were carried out as described by Jenkins et al. (19), and the reaction products were analyzed by SDS-PAGE and autoradiography.

In vitro association experiments. Lysates of unlabeled cells to be used for in vitro association were prepared as described above, using RIPA buffer plus 0.1% SDS and high-speed preclearing. Lysate (1.0 ml) was added to the ^{35}S-labeled in vitro translation mixture, and the proteins were left on ice to associate for 2 to 4 h. After this incubation period, the mixture was precleared with nonimmune serum and was then sequentially immunoprecipitated with the appropriate specific antibody. The immunoprecipitates were then analyzed by SDS-PAGE and fluorography as described above. When 293-, *Xho*I-C-, or *dl*309-infected HeLa cells were used as the source of nonradioactive protein, half of a near-confluent 9-cm dish was used for each immunoprecipitation. However, when infected or uninfected HOS cells were used as a source of p53, one entire 15-cm dish was used for each immunoprecipitation. These amounts of lysate gave approximately similar levels of p53 (see Fig. 6B). The in vitro association was carried out under reducing conditions (5 mM dithiothreitol) in which no E1b 58-kDa protein self-association occurs (12).

Fluorographs were scanned by laser densitometry (LKB Instruments, Inc.) and computer-assisted integration analysis of detected radioactive peaks.

Plasmids. For mouse p53, full-length cDNA (19) was cloned into pSp65 (*Eco*RI-*Bam*HI), a Riboprobe system vector. For human p53, full-length cDNA containing the mouse noncoding sequence 5′ to the first *Nco*I site was cloned into the *Eco*RI site of pSp65. For the E1b 58-kDa protein, an Ad2 *Pst*I (base pair 1831)-*Sma*I (base pair 3931) fragment was cloned into the *Eco*RI site of pSp64.

RESULTS

p53-E1b 58-kDa protein complex is restricted to adenovirus-transformed cells. A previous report (37) showing that p53 forms a stable complex with the Ad5 E1b 58-kDa protein in Ad5-transformed mouse cells also indicated that such a complex does not form during lytic infection of HeLa cells. HeLa cells, however, do not express detectable p53 (2, 41), although translation-competent p53 mRNA has been demonstrated (26). Here we asked whether p53-E1b 58-kDa protein complexes are detectable in infected normal rat kidney (NRK) cells and in infected HOS cells. Both cell lines express p53 clearly detectable with the anti-p53 monoclonal antibody pAb421 (14).

First, to demonstrate that p53-E1b 58-kDa protein complexes are detectable under our conditions, the Ad5 *Xho*I C fragment-transformed baby rat kidney cells (referred to here as *Xho*I-C) were analyzed (Fig. 1). These cells had previously been found to contain p53-E1b 58-kDa protein complexes (43). *Xho*I-C cells were labeled with [^{35}S]methionine, lysates were prepared, and sequential immunoprecipitations

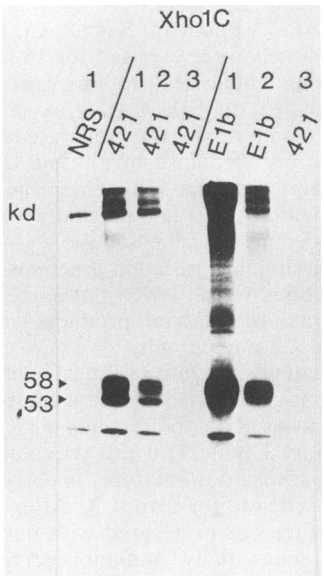

FIG. 1. p53-E1b 58-kDa protein complexes were detected in Ad5-transformed rat cells. Rat cells transformed with the *Xho*I C fragment of Ad5 DNA (*Xho*I-C) were labeled with [^{35}S]methionine and lysed in RIPA buffer plus 0.1% SDS as described in Materials and Methods. Lysates were then incubated with appropriate antibodies to immunoprecipitate p53 and E1b 58-kDa proteins. Results were analyzed by SDS-PAGE. *Xho*I-C cells were treated with NRS (lane 1), were treated sequentially with the anti-p53 monoclonal antibody pAb421 (421 lanes 1, 2, and 3), or were treated sequentially with the anti-Ad5 tumor serum (E1b) to precipitate the E1b 58-kDa protein and then with pAb421 (E1b and 421 lanes 1, 2, and 3). The positions of the p53 and E1b 58-kDa proteins are indicated at the left.

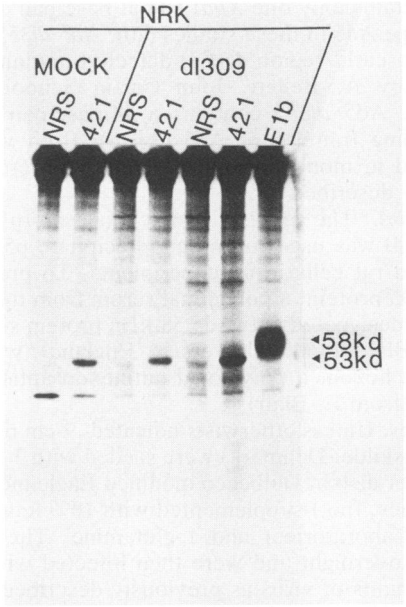

FIG. 2. p53-E1b 58-kDa protein complexes were not detected during adenovirus infection. NRK cells were infected with wild-type Ad5 *dl*309 or were mock infected and were subsequently labeled for 2 h with [^{35}S]methionine (see text). Radioactive lysates were immunoprecipitated with NRS, pAb421, or anti-Ad5 tumor serum (E1b) and were analyzed by SDS-PAGE. The positions of the E1b 58-kDa and p53 proteins are indicated at the right.

were carried out with pAb421 and the polyclonal anti-Ad5 tumor serum (see Materials and Methods) which detects the E1b 58-kDa protein. The results are shown in Fig. 1. pAb421 was found to precipitate p53 and a slightly larger protein as was shown by Zantema et al. (43). This protein we take to be the E1b 58-kDa product. Sequential immunoprecipitation (Fig. 1, 421 lanes 1, 2, and 3) depleted all detectable p53 and complex, indicating that most p53 is associated with the E1b 58-kDa protein. Sequential immunoprecipitations with the Ad5 tumor serum (Fig. 1) precipitated first the E1b 58-kDa protein and a 53-kDa protein (E1b lane 1) and then only the E1b 58-kDa protein (E1b lane 2). Finally, the third precipitation with pAb421 shows that all p53 has been depleted (421 rightmost lane 3) with the Ad5 tumor serum. We conclude that, under our conditions, we can demonstrate a complex between the E1b 58-kDa protein and p53. Also, we noted that while most p53 appears to be complexed to the E1b 58-kDa protein, there is still much unbound E1b protein. Similar results were obtained with the E1a- and E1b-expressing human 293 cells (data not shown).

Having established conditions in which p53-E1b 58-kDa protein complexes are detectable, we next asked whether complexes could be detected in Ad5-infected NRK cells and in infected HOS cells. NRK and HOS cells were infected with Ad5 *dl*309 and labeled with [^{35}S]methionine, respectively, from 46 to 48 h postinfection (p.i.) and 23 to 25 h p.i. Cells were lysed and immunoprecipitated with nonimmune serum (NRS), pAb421, and the Ad5 tumor serum. The results for NRK cells are shown in Fig. 2. In contrast to the result obtained with the *Xho*I-C cells, neither immunoprecipitation of p53 with pAb421 nor immunoprecipitation of the E1b 58-kDa protein with the polyclonal tumor serum showed any convincing evidence of coprecipitation of an additional radiolabeled protein. The same result was obtained with the monoclonal anti-p53 antibody pAb607 (data not shown). For examples of the lack of p53-E1b 58-kDa protein complex formation in lysates of infected HOS cells, see Fig. 3 and 4.

We conclude that no p53-E1b 58-kDa protein complex is detectable during either permissive (HOS) or semipermissive (NRK) infections with Ad5 *dl*309. p53-E1b 58-kDa protein complexes appear, therefore, to be a feature associated with some Ad5-transformed cells.

p53-E1b 58-kDa protein complexes are undetectable throughout the lytic cycle. To determine whether complex formation depends on some modification of the Ad5 E1b 58-kDa protein which might occur during a specific period of the lytic cycle, the following experiment was done. HOS cells were infected with Ad5 *dl*309 or were mock infected, and the cells were labeled with [^{35}S]methionine for 2 h at intervals from 3 to 42 h p.i. Cells were lysed and immunoprecipitated with NRS, pAb421, Ad5 tumor serum, or a polyclonal serum against the Ad5 hexon protein (Hex). The monitoring of hexon production in this way serves as a marker for the infection time course. The results (Fig. 3) show that the E1b 58-kDa protein was first detectable between 11.5 and 13.5 h p.i., had increased to maximum synthetic levels by 23 to 25 h p.i., and had declined substantially by 40 to 42 h p.i. Hexon was detectable first at 15 to 17 h p.i., had increased by 23 to 25 h p.i., and was still high at 40 to 42 h p.i. Levels of synthetic p53 detectable with pAb421 remained essentially unchanged throughout the course of infection and were similar to those of mock-infected cells. In no case was a

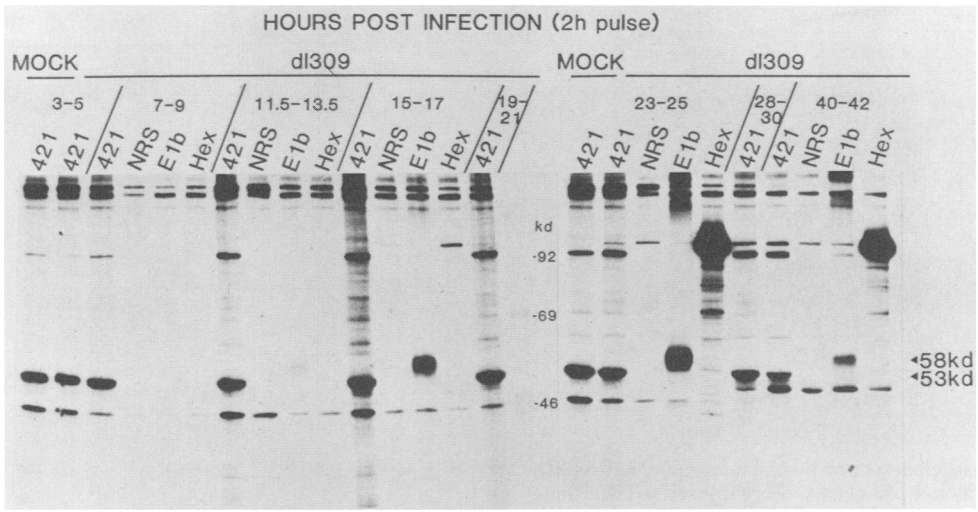

FIG. 3. p53 and E1b 58-kDa proteins did not associate at any stage during lytic infection. HOS cells were infected with Ad5 *dl*309 or were mock infected and were then labeled at indicated times for 2 h with [^{35}S]methionine. Cells were then lysed and immunoprecipitated with NRS, pAb421, E1b (anti-Ad5 tumor serum), or Hex (polyclonal antiserum against the structural hexon protein), and the products were analyzed by SDS-PAGE. The positions of the p53 and E1b 58-kDa proteins are indicated at the right.

complex between p53 and the E1b 58-kDa protein detectable. We conclude that neither p53 nor the E1b 58-kDa protein is modified during the adenovirus lytic cycle to allow complex formation to occur.

Ad5 E4 34-kDa protein did not inhibit p53-E1b 58-kDa protein complex formation. One explanation of why p53-E1b 58-kDa protein complexes do not occur during adenovirus infection is that another protein could be bound to the E1b 58-kDa protein and thereby prevent p53 association. The only reported candidate is the Ad5 E4 34-kDa protein, which has been found to associate with the E1b 58-kDa protein in infected HeLa cells (8, 36). This protein is encoded in open reading frame 6 from the E4 gene (15). We did not reproducibly detect this E4 protein under our conditions (two of five experiments) as a coprecipitate in E1b 58-kDa protein immunoprecipitations from infected HOS cell lysates (Fig. 2 and 3); nor did we reproducibly see it in immunoprecipitations of the E1b 58-kDa protein from Ad5 *dl*309-infected HeLa cells (unpublished results). The lack of clear detection of this protein on all our gels does not, however, preclude its involvement in interfering with p53-E1b 58-kDa protein complex formation. The lack of reproducible detection of the E4 34-kDa protein is likely to be due to experimental variations.

To determine whether the E4 34-kDa protein might block p53-E1b 58-kDa protein association, we infected HOS cells with the Ad5 deletion mutant *dl*355. Ad5 *dl*355 has a deletion in the carboxy-terminal region specific to the 34-kDa protein-coding sequences (13). In Ad5 *dl*355-infected HeLa cells, the E1b 58-kDa protein is clearly detectable, but no E4 34-kDa protein is present (8). In our experiments (Fig. 4), the Ad5 tumor serum immunoprecipitated comparable amounts of radiolabeled E1b 58-kDa protein from HOS cells infected with Ad5 *dl*309 and Ad5 *dl*355. In neither case was p53 coprecipitated. Comparable levels of synthetic p53 were also observed, and at the same time a complex between p53 and the E1b 58-kDa protein was detected from *Xho*I-C cell lysates by using pAb421 (Fig. 4, rightmost lane). As no p53-E1b 58-kDa protein complex was observed in the absence of expressed E4 34-kDa protein (*dl*355 result), we conclude that the E4 34-kDa protein does not prevent p53-E1b 58-kDa protein association.

p53-E1b 58-kDa protein complexes not detectable after in vivo cross-linking. It is possible that p53-E1b 58-kDa protein complexes do form during lytic infection but are so unstable

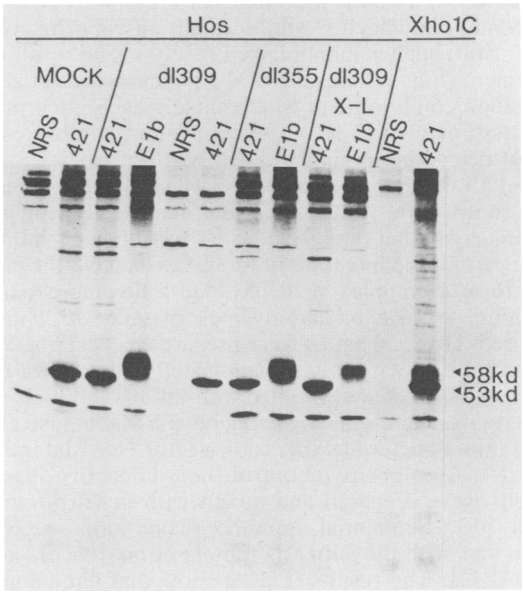

FIG. 4. Ad5 E4 34-kDa protein did not prevent the formation of p53-E1b 58-kDa protein complexes during infection. HOS cells were infected with Ad5 *dl*309 (as wild type) or Ad5 *dl*355 (E4-defective mutant) or were mock infected. Cells were labeled 23 to 25 h p.i. and proteins were analyzed as described in the legends to Fig. 1, 2, and 3. For lanes *dl*309 X-L, cells were infected with *dl*309 and labeled as usual, but just prior to lysis, cells were treated with an in vivo cross-linking agent (see text) and were then immunoprecipitated with NRS, pAb421, or E1b (anti-Ad5 tumor serum). *Xho*I-C cells were labeled and immunoprecipitated as a positive control for p53-E1b 58-kDa protein complexes. The positions of the p53 and E1b 58-kDa proteins are indicated at the right.

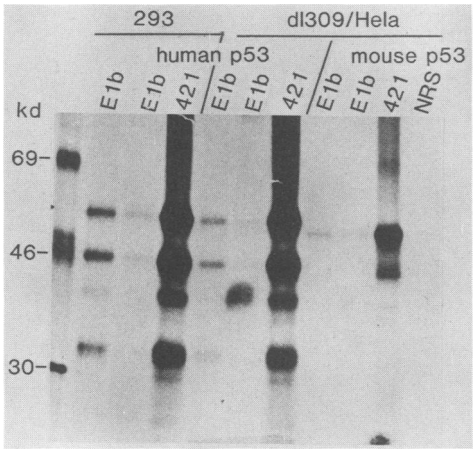

FIG. 5. In vitro association of p53 with endogenous E1b 58-kDa proteins. Human or mouse p53 cDNAs were transcribed and translated in vitro as described previously (19, 42). The radioactive protein mixture was added to lysates from 293 cells or from HeLa cells infected with Ad5 *dl*309 (harvested 18 to 20 h p.i.). After association for 2 h on ice, the reactions were immunoprecipitated sequentially with the anti-Ad5 tumor serum (E1b) followed by pAb421. Control nonimmune (NRS) precipitations were also done. Size markers (in kilodaltons) are shown at the left.

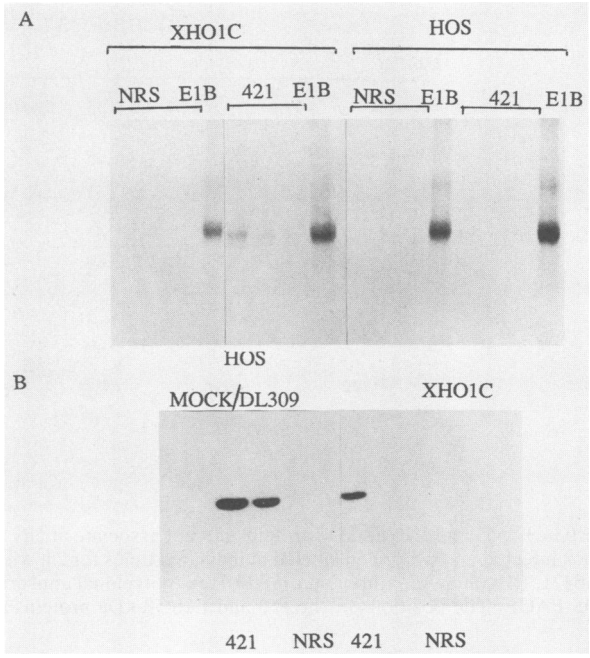

FIG. 6. p53 from HOS cells was incompetent to associate with in vitro-expressed E1b 58-kDa protein. Lysates were prepared from a 9-cm dish of *Xho*I-C cells and from 15-cm dishes of mock-infected and *dl*309-infected HOS cells. Lysis was done in RIPA buffer plus 0.1% SDS. These nonradioactive lysates were either (A) mixed with the radioactive in vitro-translated E1b 58-kDa protein and then sequentially immunoprecipitated three times with NRS or pAb421 before specific precipitation with anti-Ad5 tumor serum (E1b) or (B) directly immunoprecipitated with pAb421 or NRS and then Western blotted (see Materials and Methods) with pAb421 and ^{125}I-protein A.

that they dissociate under the lysis conditions of the experiments described. To test this, we incubated Ad5 *dl*309-infected HOS cells with the in vivo protein cross-linking agent dimethyl-3,3′-dithio-bispropionimidate dihydrochloride before lysis. Under these cross-linking conditions, monkey p53-SV40 T antigen complexes are stable even under lysis conditions which would normally disrupt the complex (H.-W. Stürzbecher, unpublished results). The result of this experiment (Fig. 4, lanes *dl*309 X-L) demonstrates that even under such conditions, in which closely associated proteins are cross-linked, p53-E1b 58-kDa protein complexes were still not detectable in infected cell lysates.

Ad5 E1b 58-kDa proteins expressed during infection competent to associate with human and mouse p53 proteins. To determine whether any E1b 58-kDa proteins synthesized during permissive infection of human cells have the capability to form a complex with p53, the following experiment was done. Nonradioactive lysates of Ad5 *dl*309-infected HeLa cells (18 to 20 h p.i.) were prepared as described above and were mixed with in vitro-translated radiolabeled human or mouse p53 proteins. In vitro translations in rabbit reticulocyte lysates were done by using human and mouse cDNAs cloned into a bacterial expression vector (see Materials and Methods). As a positive control, nonradioactive lysates of 293 cells were prepared and mixed with in vitro-translated human p53. Sequential immunoprecipitations were performed first with the anti-Ad5 tumor serum (twice) and then with pAb421. The results (Fig. 5) show that immunoprecipitations of E1b from both 293 cells and Ad5 *dl*309-infected HeLa cells coprecipitated several radiolabeled p53 polypeptides. The additional proteins migrating below 53 kDa are due to translation initiating at internal AUG codons (19). Similar results were obtained irrespective of whether the in vitro-translated protein was of human or mouse origin. Laser densitometry analysis of the associated radiolabeled human p53 indicates that 10 to 20% of the input p53 coprecipitated with immunoprecipitated E1b 58-kDa protein from both 293 and *dl*309-infected HeLa cells. The final immunoprecipitation with pAb421 showed substantial in vitro-translated p53 remaining after precipitation of E1b with the tumor serum. That 80% or more of the in vitro-labeled product remained unassociated presumably indicates that only a subpopulation of the proteins present in the in vitro mixing reactions are competent to form stable complexes. We conclude from these experiments that the defect in p53-E1b 58-kDa protein complex formation during infection is likely not to be due to a difference in E1b 58-kDa proteins expressed during infection compared with those expressed in adenovirus-transformed cells.

p53 from HOS cells incompetent to associate with in vitro-synthesized E1b 58-kDa protein. To determine whether endogenous p53 from HOS cells is competent to bind the E1b 58-kDa protein, radiolabeled in vitro-translated E1b 58-kDa protein was mixed with lysates of uninfected HOS cells or HOS cells infected with Ad5 *dl*309, and sequential immunoprecipitations with pAb421 were carried out as described above. *Xho*I-C lysates were used as positive controls for association. The results (Fig. 6A) show that immunoprecipitation of p53 with pAb421 from *Xho*I-C cell lysates coprecipitated the radiolabeled in vitro-translated E1b 58-kDa product as expected. The amount coprecipitated in this experiment represents about 35% of the input radioactivity. In a series of four experiments (Table 1), the average association was around 20% of input, about sevenfold above the background with NRS. The association detected is not due to the association of the radiolabeled in vitro 58-kDa product with nonradioactive endogenous E1b 58-kDa protein already present in complex with p53, as the association was done under reducing conditions which are more than sufficient to prevent self-association (12).

TABLE 1. Association of radiolabeled E1b 58-kDa product with p53

p53 source	Antibody	Area (mm^2) of labeled E1b 58-kDa protein[a] in expt				Mean % association (amt associated/input radioactivity)
		1	2	3	4	
*Xho*I-C	NRS	0.15	0	0	0.83	2.7
	pAb421	1.16	3.54	1.84 3.37	8.27	20.6
	αE1b	1.92 2.13	13.08 18.78	18.9 23.43	20.29	
HOS	NRS	0.35	ND	0	0.81	2.9
	pAb421	0.43	ND	0	0.66	2.2
	αE1b	2.0 3.67	ND	24.4	28.28	

[a] Densitometrically determined by using a scanning laser densitometer (LKB) and computer-based assessment of integrated area of detected peaks. ND, Not determined. 0 = below detection limits. Where two values are shown for one antibody, the upper value refers to radioactivity remaining after sequential precipitation with NRS and the lower value indicates radioactivity remaining after sequential treatment with pAb421.

When p53 was immunoprecipitated from either mock-infected HOS cell extracts (Fig. 6A) or from *dl*309-infected extracts (data not shown), coprecipitation of in vitro E1b 58-kDa product was at background levels compared with NRS (Fig. 6A). Table 1 shows the values for three independent experiments, and the mean association of E1b 58-kDa protein with immunoprecipitated p53 was 2.2% of input; background coprecipitation with NRS was 2.9%. This lack of association of endogenous p53 with the in vitro E1b product is not due to insufficient p53 in HOS cells, as Western (immunoblot) analysis shows mock-infected HOS cells and *Xho*I-C cells to have similar amounts (Fig. 6B).

The results from these in vitro association experiments suggest an explanation for the lack of p53-E1b 58-kDa protein complexes in infected cells: p53 in the untransformed cells is in some way incompetent to form a complex with the E1b 58-kDa product.

DISCUSSION

We report here experiments that investigate the factors involved in determining whether the p53 oncoprotein and the Ad5 large tumor antigen (the E1b 58-kDa protein) can form a complex. We demonstrate that such complexes are detectable in Ad5-transformed rat (*Xho*I-C) cells (Fig. 1) as has been reported previously (43) and, although not shown here, in Ad5-transformed human (293) cells (41). However, we failed to detect complexes during infection of either permissive human (HOS) cells or semipermissive rat (NRK) cells (Fig. 2 and 3). Thus, host cell permissivity to adenovirus is not a determining factor in p53-E1b 58-kDa protein complex formation. We also examined the possibility that p53-E1b 58-kDa protein complexes might occur transiently during the adenovirus lytic cycle. No complexes were detectable, however, at any stage during the replicative cycle (Fig. 3).

Next, the possibility of an additional viral protein bound to the E1b 58-kDa protein and preventing p53 association was considered. As outlined above, the only candidate to be reported (36) is the Ad5 E4 34-kDa product. However, we show (Fig. 4) that in HOS cells infected with the Ad5 mutant *dl*355, which does not express the 34-kDa product but does express wild-type levels of the E1b 58-kDa protein, still no p53-E1b 58-kDa protein complexes were detectable. Thus, the E4 34-kDa product does not block p53-E1b 58-kDa protein complex formation. This result does not exclude the possibility of other viral or cellular proteins associating competitively with the E1b 58-kDa protein to exclude p53 binding. However, we have not detected any such proteins coimmunoprecipitating with the E1b 58-kDa protein (Fig. 2, 3, and 4).

p53, however, may be complexed to a cellular protein to prevent E1b 58-kDa protein association. The only reported candidates for this are the cellular heat-shock proteins (hsp 72-73), which coimmunoprecipitate with p53 in some transformed rat cell lines (32). In some of our experiments, we have detected a 72- to 73-kDa doublet coimmunoprecipitating with pAb421-precipitated p53 from Ad5-infected HOS cells, although not reproducibly (cf. Fig. 3 and 4), but we also see this in *Xho*I-C cells, in which p53-E1b 58-kDa complexes are also detectable (Fig. 4, rightmost lane). Although not proof, these data suggest that the reason p53 and E1b 58-kDa proteins do not associate during infection is not due to competition from hsp 72-73.

The hypothesis that one or both of the p53 and E1b 58-kDa proteins expressed in infected cells might be incompetent to form a complex with the other protein was examined. This was addressed by mixing either radiolabeled in vitro-translated p53 with lysates of infected HeLa cells or 293 cells or the converse experiment in which in vitro-translated E1b 58-kDa protein was mixed with lysates from *Xho*I-C cells or from infected and uninfected HOS cells. The results show that immunoprecipitation of E1b 58-kDa proteins from either transformed or infected cells allowed coprecipitation of about 10 to 20% of input in vitro-translated human p53 (Fig. 5). Coprecipitation was also detected with in vitro-translated mouse p53 (Fig. 5). Thus, at least some E1b 58-kDa protein expressed during lytic infection is competent to bind to some in vitro-translated p53 product of either human or mouse origin.

In the converse experiment, around 35% of in vitro-translated E1b 58-kDa protein was coimmunoprecipitated with p53 from lysates of *Xho*I-C cells (Fig. 6A), and in successive experiments an average coprecipitation of about 20% was detected (Table 1). Similar levels of E1b 58-kDa protein association were found with 293 lysates (data not shown). However, there was no detectable E1b 58-kDa protein coimmunoprecipitated with p53 from mock-infected (Fig. 6A) and Ad5 *dl*309-infected (data not shown) HOS cells, even though comparable levels of p53 were present in the lysates (Fig. 6B). In three separate experiments, similar results were obtained. These results suggest that the majority of the p53 expressed in HOS cells is incompetent to form a complex with in vitro-translated E1b 58-kDa product. A similar finding was obtained with NRK cells (data not shown).

It seems possible that the properties of in vitro-translated proteins do not accurately reflect those expressed in vivo. However, for p53 at least, several parameters have been examined, including the expression of denaturation-sensitive epitopes (42), the ability to bind SV40 T antigen (16), and the ability to form oligomeric structures (H.-W. Stürzbecher and J. R. Jenkins, manuscript in preparation). These properties all appear to parallel the in vivo situation. Thus, notwithstanding the possible dangers of interpreting data from in vitro experiments, the available information suggests that in

vitro association experiments as presented in this article are a valid measure of the biochemical properties of proteins.

Given this argument, the ability of p53 to associate with the E1b 58-kDa protein appears to be dependent on the existence of a competent form of p53. Thus, in infected HOS cells in which no detectable complexes occur the majority of p53 exists in an incompetent form, whereas in *Xho*I-C cells the majority of p53 exists in the competent form. Transformation of Ad5 might in some way upregulate the (normally rare) competent form of p53 or select for a subpopulation of cells expressing more of this form of p53. Different forms of p53 could exist as a result of direct modification of the protein, alternate splicing, or mutation. However, as in vitro-translated wild-type p53 is competent to bind the E1b 58-kDa protein (Fig. 5), direct protein modification to generate the E1b-competent form seems most likely.

In summary, in this article we have studied the ability of p53 and E1b 58-kDa proteins to form complexes in vivo and in vitro. Our data show that p53 does not complex with the Ad5 E1b 58-kDa protein during lytic and nonlytic infections under a variety of conditions. We also show that p53 from some cells appears to be incapable of forming a complex, as determined by in vitro association with radiolabeled E1b 58-kDa product. The E1b 58-kDa product expressed in cells (in the form of an infecting virus) does, however, complex with in vitro-labeled p53. On the basis of these data, we suggest that the lack of p53-E1b 58-kDa protein complexes during infection is due to p53 incompetence.

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