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Analysis with Specific Polyclonal Antiserum Indicates that the E1A-Associated 300-kDa Product Is a Stable Nuclear Phosphoprotein That Undergoes Cell Cycle Phase-Specific Modification

PETER YACIUK AND ELIZABETH MORAN*

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

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Binding of a 300-kDa host cell protein (p300) is tightly correlated with the ability of the adenovirus E1A products to induce quiescent baby rat kidney cells to proliferate. We have generated rabbit polyclonal antibodies against p300 to characterize this protein further. We have found p300 to be a nuclear phosphoprotein that is actively synthesized in both quiescent and proliferating baby rat kidney cells. In partially purified mitotic cell populations, we observe a form of p300 with decreased electrophoretic mobility in sodium dodecyl sulfate-polyacrylamide gels that shares a nearly identical partial proteolytic digest pattern with p300. The slower-migrating form of p300 is greatly reduced by treating immune complexes with potato acid phosphatase. The relative stability and presence of p300 even in resting cells suggests that p300 has a basal cell function, but the appearance of differentially modified forms during the cell cycle suggests the possibility that p300 function is modulated specifically in growing cells.

Quiescent primary rodent cells can be stimulated to grow without restriction by the biological activities of the adenovirus type 5 E1A products. Full morphological transformation is attained when E1A functions cooperate with functions of second oncogene products, such as the adenovirus type 5 E1B or activated H-*ras* protein (14, 29). At least two cell growth-regulating activities, each of which is required for induction of even a single round of cell division, have been identified in the E1A products (15, 25, 43). These activities have been localized to two distinct regions of the protein (24). One activity is localized to a protein region designated conserved region 2 and is correlated with the binding of the product of the retinoblastoma tumor suppressor gene, p105RB, and other host cell proteins (6, 8, 19, 22, 26, 39, 40). A second transforming activity is localized to the extreme N terminus and is tightly linked with the binding of an unidentified 300-kDa host cell protein, p300 (6, 40). Disruption of p300 binding by mutation of extreme N-terminal E1A sequences correlates with complete loss of mitogenic activity, loss of certain E1A-mediated enhancer repressor functions, and partial impairment of the ability of E1A to induce cellular and viral DNA synthesis (6, 15–17, 20, 25, 28, 30, 31, 33, 34, 37, 39, 40). The p300 binding function is also linked with the ability of E1A to repress certain mitogen-inducible genes and to alter the activity of members of the AP-1 transcription factor family (27, 35, 36). Moreover, in the absence of E1A functional regions other than the regions associated with p300 binding, the E1A products retain the ability to induce DNA synthesis and wild-type levels of expression of specific cellular G_1 genes in quiescent cells (15, 38, 43). The integrity of the p300 binding site is therefore closely linked with E1A-mediated cell growth-regulating activities and with both positive and negative regulation of gene expression.

While the mitogenic activity of E1A requires that both transforming domains be functionally intact, the ability to induce G_1 gene expression and cellular DNA synthesis appears to be a redundant E1A function that is a property of either the N-terminal or domain 2 active site. Only double mutants, combining mutations in both the N terminus and domain 2, are devoid of these activities (15, 38). The degree of independence that exists between the two active sites is emphasized by the demonstration that E1A mutants, deficient in activities associated with the N terminus, can cooperate in *trans* with E1A mutants devoid of domain 2-associated activities to achieve cellular transformation (25, 33). Interestingly, although physical association with p300 has not been reported, large T antigen has a biological activity that can complement the p300-associated activity of E1A (41).

Since transformation by the E1A products requires a biological activity that is tightly correlated with the binding of p300, we have generated antibodies against p300 to characterize this protein further. We have also used these antibodies to characterize a cell cycle phase-specific form of p300.

MATERIALS AND METHODS

Virus and cell culture. Monolayer cultures of 293 (9) and HeLa cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 100 μg of penicillin per ml, and 100 μg of streptomycin per ml. Primary baby rat kidney (BRK) cells were prepared as described previously (23). HeLa spinner cells were grown in F13 medium (Flow Laboratories) supplemented with 5% calf serum and 100 μg of both penicillin and streptomycin per ml. Low-passage WI-38 cells obtained from the American Type Culture Collection (ATCC CCL75) were grown in DMEM supplemented with 10% fetal bovine serum (FBS). The 12S wild-type (12S.WT) virus was described previously (23) and was used at a multiplicity of infection of 10 PFU per cell.

Immunoprecipitations. Cells were metabolically labeled as described previously (41). Radioactively labeled cells were lysed in (1 ml/10-cm monolayer tissue culture plate) either (i)

* Corresponding author.

a low-detergent buffer (p300 lysis buffer) containing 0.1% Nonidet P-40, 250 mM sodium chloride, 20 mM sodium phosphate (pH 7.0), 30 mM sodium pyrophosphate, 5 mM EDTA, and 10 mM sodium fluoride supplemented with 5 mM dithiothreitol and protease and phosphatase inhibitors (0.1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride [PMSF], 100 kIU of aprotinin, 1 μg of leupeptin per ml, and 1 μg of pepstatin per ml) or (ii) a high-detergent buffer (RIPA buffer) containing 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM sodium chloride, and 50 mM Tris (pH 7.4) supplemented with 5 mM dithiothreitol and the phosphatase and protease inhibitors mentioned above. Proteins were immunoprecipitated and analyzed by polyacrylamide gel electrophoresis (PAGE) as described previously (41). In ^{35}S-labeled rodent cells, normal rabbit serum nonspecifically precipitates a cellular product which migrates close to authentic p300 in SDS-polyacrylamide gels. To avoid the complication of this background band, ^{35}S-labeled rodent cell lysates (see Fig. 4) were precleared with 20 μl of normal rabbit serum and 100 μl of a 10% slurry of *Staphylococcus aureus* per ml of cell lysate prior to immunoprecipitation of p300 with rabbit serum. E1A-specific monoclonal antibodies M73 and M1 and the simian virus 40 large T-antigen-specific monoclonal antibody 419 have been described previously (10, 11).

Purification of p300 for antibody production. Large-scale affinity purification of p300 was accomplished by coimmunoprecipitation using E1A-specific monoclonal antibody M73 (11), which was covalently linked to protein A-Sepharose CL-4B beads (M73/protein A beads) with dimethylpimelimidate as described previously (12). One hundred sixty confluent 10-cm plates of 293 cells (cells which constitutively express E1A proteins) were lysed with 1 ml of p300 lysis buffer per plate. Plates were rocked at 4°C for 20 min, and equal volumes of lysates were transferred to six 35-ml polyallomer tubes (Beckman). Lysates were precleared of cell debris by centrifugation at 20,000 × *g* for 30 min in a Sorvall AH 627 swinging-bucket rotor. Approximately 150 ml of supernatant was transferred to three 50-ml conical polypropylene tubes, and roughly 20 μl (wet beads) of M73/protein A beads per mg of total cell protein (this corresponds to approximately 4 ml of wet beads per 150 ml of lysate) was used to immunoprecipitate E1A and its associated proteins. These tubes were rotated at 4°C for 1 h. M73/protein A beads were pelleted by centrifugation in a Sorvall RC-3B centrifuge at 1,000 rpm for 5 min with the break in the off position. Supernatants were removed, and beads were transferred to two 15-ml conical polypropylene tubes. Beads were washed six times with 10 ml of p300 lysis buffer. Then, a disposable 20-ml polypropylene column (Bio-Rad) was packed with the M73/protein A beads containing the immunocomplexes. Proteins were eluted in fractions from the column with RIPA buffer. Portions of each fraction were analyzed on silver-stained SDS-polyacrylamide gels. Fractions enriched for p300 were combined and dialyzed against a buffer containing 10 mM Tris (pH 7.4), 1 mM EDTA, and 1 mM dithiothreitol and concentrated to 250 μl by lyophilization prior to injection into a New Zealand White female rabbit. Prior to injection, the rabbit was bled for preimmune serum. The primary immunization was performed with a volume of Freund's complete adjuvant, mixed 1:1 with the antigen. Subsequent injections of adjuvant-antigen complex were made at approximately 14-day intervals with incomplete adjuvant. Injections were made either subcutaneously or into the right popliteal lymph node. The rabbit was bled 1 month after the initial injection and approximately every 2 weeks thereafter.

Potato acid phosphatase treatment. Proteins were coimmunoprecipitated as described above, using anti-E1A monoclonal antibody M73. Immunocomplexes bound to protein A-Sepharose were then washed with 1 ml of 100 mM MES (2-[*N*-morpholino]ethanesulfonic acid; pH 6.0) containing 1 mM PMSF, divided into two equal portions, and pelleted by centrifugation. A volume containing 0.5 U of an ammonium sulfate suspension of potato acid phosphatase (0.5 U; Boehringer Mannheim) was centrifuged to pellet the phosphatase. The supernatant was removed, and the phosphatase pellet was dissolved in 30 μl of 100 mM MES containing 1 mM PMSF and then transferred to one of the two immunocomplex portions. The other immunocomplex portion was resuspended in 100 mM MES containing 1 mM PMSF. Samples were incubated for 15 min at 37°C. Reactions were stopped by the addition of 30 μl of 2× Laemmli loading buffer (18) and heating to 100°C for 5 min. Proteins were then analyzed by electrophoresis.

Cleveland digests. Cleveland digests were performed as described by Cleveland et al. (4), using *S. aureus* V8 protease (Boehringer Mannheim) on proteins excised from dried gels.

Cell cycle phase analysis and synchronization. HeLa spinner cultures were separated by centrifugal elutriation with a Beckman JE-10X elutriation rotor, and cells were analyzed by flow cytometry as described previously (2). Monolayer cultures of HeLa and 293 cells were synchronized into the DNA synthesis phase or mitosis by 20-h treatments with 10 μM hydroxyurea or 20 ng of nocodazole per ml, respectively.

Determination of protein half-life. Subconfluent cultures of either WI-38 or 293 cells were labeled with 2.5 ml of methionine-free DMEM containing 100 μCi Tran-^{35}S-label per ml for a 1-h pulse. After the pulse, the radioactive medium was removed and cells were washed with complete DMEM containing 10% calf serum transferred from a sister culture and then fed with medium transferred from a second sister culture of the respective cell line for various chase periods. Cell lysates were normalized to total cell protein content. Proteins were analyzed by immunoprecipitation (using low-detergent lysis conditions), electrophoresis, and silver staining as described above. The amount of silver-stained p300 was constant for each time point. Dried gels were subjected to autoradiography and quantitated by using a Molecular Dynamics Phosphoimager.

Indirect immunofluorescence. HeLa cells, grown on coverslips at low density, were washed with phosphate-buffered saline (PBS) supplemented with 1% FBS (PBS + FBS), fixed in methanol for 10 min at −20°C, and then washed in PBS + FBS. The coverslips were incubated for 45 min at 37°C in preimmune or immune serum diluted 1:50 in PBS + FBS or in PBS + FBS alone. After washing in PBS + FBS, a 1:250 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (IgG) (Antibodies Inc.) was added, and the coverslips were incubated for an additional 45 min at 37°C. The coverslips were then washed in PBS + FBS and mounted. Fluorescence was observed with a Nikon Microphot FXA fluorescence microscope at a magnification of ×62.5 with oil immersion.

RESULTS

Purification of p300. p300 was affinity purified by coimmunoprecipitation with E1A products by using E1A-specific

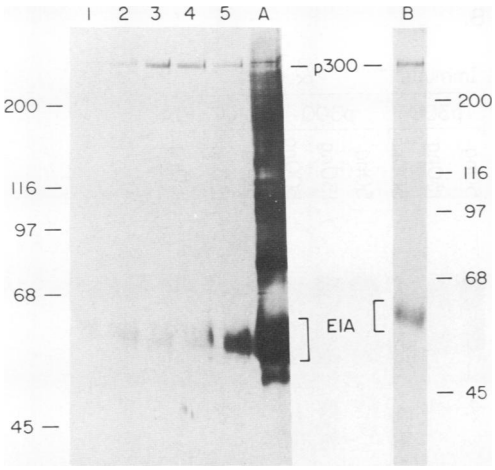

FIG. 1. Purification of p300 from E1A immunocomplexes. p300 was affinity purified from 293 cell lysates by using M73/protein A beads and elution under high-detergent-concentration conditions; 1% of each fraction was analyzed on a 10% SDS-polyacrylamide gel (lanes 1 to 5). Lane A represents 1% of the protein remaining on the beads after elution. Fractions 3 and 4 were combined, dialyzed, and concentrated; a 1% portion of the concentrated sample is shown in lane B. Sizes are indicated in kilodaltons.

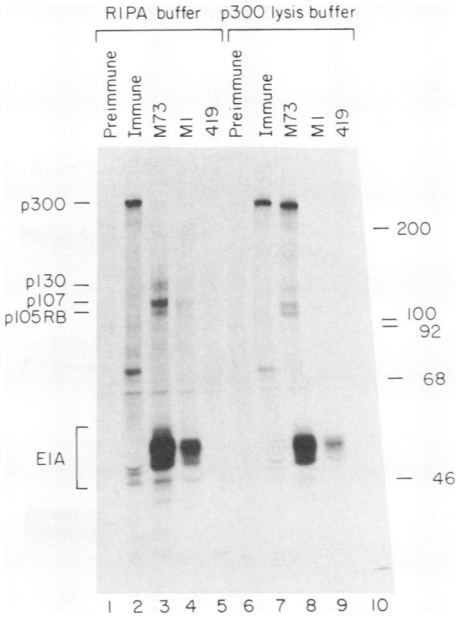

FIG. 2. Immunoprecipitation of p300 from 293 cell lysates. 293 cells were labeled with Tran-^{35}S-label for 2 h prior to lysis with either RIPA buffer (lanes 1 to 5) or p300 lysis buffer (lanes 6 to 10). Proteins were immunoprecipitated with either rabbit immune serum (Immune), anti-E1A monoclonal antibody M73 or M1, preimmune rabbit serum (Preimmune), or control antibody 419. Positions of E1A-associated proteins are indicated at the left, and the sizes (in kilodaltons) of protein molecular weight standards are indicated at the right.

monoclonal antibody M73 (11) under low-detergent conditions. We have found that p300 association with E1A is unstable in high-detergent conditions (see, for example, Fig. 2, lane 3 versus lane 8) and have exploited this observation to elute p300 preferentially from E1A immunocomplexes. For large-scale preparation of p300 (see Materials and Methods), we used M73/protein A beads to immunoprecipitate E1A and its associated proteins from lysates of 293 cells, cells which constitutively express E1A proteins (9). M73/protein A beads containing immunocomplexes were used to pack a column, and fractions were eluted from this column under high-detergent conditions. One percent of each protein fraction eluted from the column was analyzed on silver-stained SDS-polyacrylamide gels to identify fractions enriched for p300. Fractions from a typical preparation are shown in Fig. 1, lanes 1 through 5. One percent of beads from the column, after elution, were run in lane A to determine relative amounts of proteins still bound to M73/protein A beads. Fractions 3 and 4 show substantial amounts of p300 and relatively small amounts of IgG, E1A, and other associated proteins (compare lanes 3 and 4 with lane A). (The silver-staining species seen in the E1A region of the gel probably represents mostly IgG heavy chain.) Lane B shows 1% of fractions 3 and 4 after they were combined, dialyzed, and concentrated by lyophilization. The remaining sample was injected into a rabbit as described in Materials and Methods. Comparing the intensities of silver staining of p300 and of known amounts of proteins run on the same gel, we estimate that between 10 and 20 μg of p300 was introduced into the rabbit with each injection.

Anti-p300 antibody characterization. Test serum, withdrawn from the rabbit 2 weeks subsequent to a second injection, was analyzed in immunoprecipitation assays to determine whether antibodies recognizing p300 had developed. Proteins were immunoprecipitated from extracts of 293 cells that had been labeled with Tran-^{35}S-label and lysed under either high- or low-detergent conditions. Lysates were immunoprecipitated with either rabbit preimmune serum (10 μl), rabbit immune serum (10 μl), E1A-specific monoclonal antibody M73 or M1, or control monoclonal antibody 419, directed against simian virus 40 large T antigen. Immunoprecipitated proteins were separated on a 7.5% SDS-polyacrylamide gel and visualized by autoradiography. In low-detergent (p300 lysis buffer) conditions, the M73 antibody coprecipitated p300 (Fig. 2, lane 8). p300 was not observed when the anti-E1A M1 antibody was used (lane 9), as previously shown (13), presumably because M1 binding blocks sequences required for p300 binding to the E1A products. The immune serum (lane 7) precipitated a prominently labeled protein species that comigrated with the p300 species that coprecipitated with E1A. The relationship between the immune serum-precipitated p300 species and E1A-associated p300 was verified by peptide digest analysis, described below. Under high-detergent conditions, while detectable amounts of E1A-associated proteins p130, p107, and p105 were clearly visible in M73 immunoprecipitates, binding of p300 was greatly reduced (lane 3). High-detergent conditions, however, did not appear to interfere with the interaction between p300 and the immune serum antibodies (lane 2). The p300 protein immunoprecipitated by rabbit serum was not observed in rabbit preimmune serum immunoprecipitates (lanes 1 and 6) or in immunoprecipitates obtained by using serum from an independent nonimmunized rabbit (data not shown). Increasing amounts of immune serum did not precipitate appreciably more of the p300 species in either buffer (data not shown). These results suggest that the majority of detectable p300 is associated with the E1A products in 293 cell lysates under nonstringent conditions. The immune serum did not precipitate detectable amounts of E1A proteins (lanes 2 and 7), suggesting that the

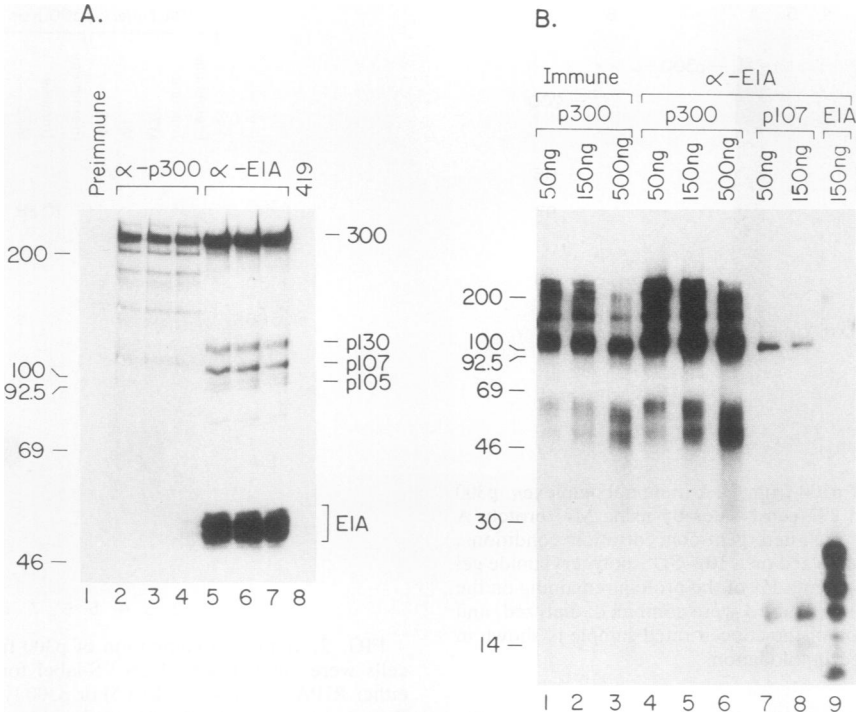

FIG. 3. Immunoprecipitation and V8 protease digestion of p300 proteins. (A) 293 cells were labeled with $^{32}P_i$ for 2 h prior to lysis in p300 lysis buffer. p300 protein species were immunoprecipitated with preimmune rabbit serum (lane 1), p300 rabbit antiserum (α-p300; lanes 2 to 4), E1A-specific monoclonal antibody M73 (α-E1A; lanes 5 to 7), or control monoclonal antibody 419 (lane 8). Positions of E1A-associated proteins are indicated at the right. (B) p300 proteins immunoprecipitated with rabbit serum or anti-E1A monoclonal antibody M73 were excised from the dried gel shown in panel A and treated with 50, 150, or 500 μg of *S. aureus* V8 protease as described previously (4). As controls, p107 proteins (panel A, lanes 7 and 8) and E1A proteins (panel A, lane 8) were also treated with V8 protease in panel B (lanes 7 to 9). Sizes (in kilodaltons) of protein molecular weight standards are indicated at the left of both panels.

relatively small amounts of E1A proteins that might have been injected into the rabbit were not sufficient to induce an antigenic response in this rabbit (Fig. 1). In addition, the immune serum did not coprecipitate the relatively large amounts of E1A proteins present in lysates of 293 cells (Fig. 2, lanes 2 and 7), possibly because binding of the polyclonal antibodies to p300 interferes with E1A binding. The antibodies appear to be of sufficient affinity to compete effectively with E1A to bind p300 even in low-detergent conditions.

Since E1A-associated p300 has been shown to be a phosphoprotein (42), we tested whether this comigrating species is phosphorylated (Fig. 3). Subconfluent cultures of 293 cells were labeled with $^{32}P_i$ for 2 h prior to lysis. Equal volumes of the same cell lysate were immunoprecipitated with rabbit preimmune serum (Fig. 3A, lane 1), rabbit immune serum (lanes 2 to 4), anti-E1A monoclonal antibody M73 (lanes 5 to 7), or the control monoclonal antibody 419 (lane 8). Rabbit immune serum and M73 immunoprecipitations were done in triplicate to provide material for peptide digest analysis. The immune serum recognizes a prominent phosphorylated protein species of approximately 300 kDa (lanes 2 to 4), consistent with the known properties of E1A-associated p300. This p300 phosphoprotein comigrates with the p300 seen in E1A affinity-based immunoprecipitates (lanes 5 to 7). The p300 protein species immunoprecipitated by M73 and rabbit immune serum were excised from the dried gel shown in Fig. 3A and digested with three different amounts of *S. aureus* V8 protease, as described previously (4). We observed indistinguishable V8 protease digestion patterns for both p300 protein species (Fig. 3B), indicating that these are the same or very closely related proteins. These protein digest patterns are clearly different from the V8 digest patterns of p107 or E1A protein excised from the same gel (Fig. 3B, lanes 7 to 9).

Biological characterization of p300. The availability of immune serum allows us to monitor p300 expression in resting cells rather than only in E1A-expressing cells, which are not generally subject to growth arrest. Since binding of p300 to the adenovirus E1A products is tightly correlated with E1A cell growth-regulating activities assayed in primary BRK cells, we first tested whether p300 is synthesized in quiescent BRK cells. We assayed expression in either quiescent BRK cells or BRK cells induced to enter the cell cycle by infection with an adenovirus expressing 12S.WT cDNA, described previously (23). p300 synthesis was assayed in quiescent cells labeled with Tran-^{35}S-label for 2 h. The rate of p300 synthesis in quiescent cells was compared with the rate of synthesis in stimulated cells labeled 22 to 24 h postinfection. Lysates were normalized to trichloroacetic acid-precipitable radioactivity. The results (Fig. 4) show that p300 is actively synthesized even in quiescent cells. The rates of p300 synthesis in quiescent (lane 2) and stimulated (lane 3) cells are similar, although a small increase in the rate of p300 synthesis is observed in stimulated cells relative to that in quiescent cells. The E1A-associated p300 was coprecipitated and run as a marker (lane 4). We have done similar experiments in serum-depleted and density-arrested WI-38 and NIH 3T3 cells, and in these experiments also we find similar rates of p300 synthesis in either quiescent or restimulated cells (not shown).

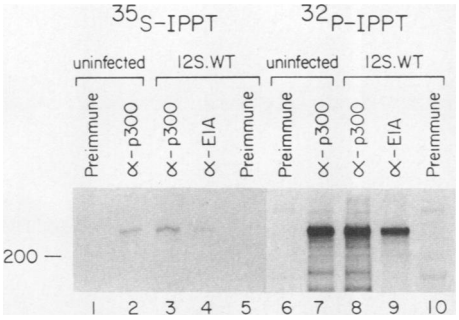

FIG. 4. Immunoprecipitation of p300 from quiescent or infected BRK cells. BRK cells were infected with 12S.WT virus or mock infected, as indicated. Cells were labeled for 2 h with either Tran-^{35}S-label (lanes 1 to 5) or $^{32}P_i$ (lanes 6 to 10) prior to lysis in p300 lysis buffer. For infected cells, this was 22- to 24-h postinfection. Proteins were immunoprecipitated with rabbit preimmune serum, p300 rabbit antiserum (α-p300), or α-E1A monoclonal antibody M73 (α-E1A). In this experiment, cell lysates were precleared with rabbit preimmune serum prior to immunoprecipitation as described in Materials and Methods. The position of the 200-kDa protein molecular weight marker is indicated on the left.

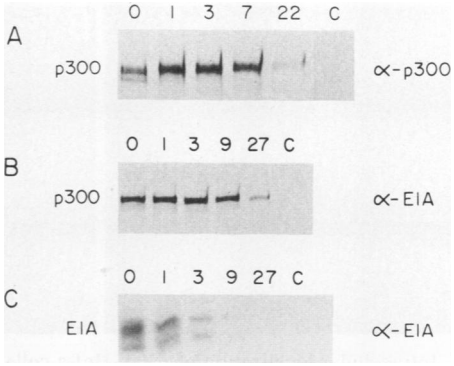

FIG. 5. Determination of p300 apparent protein half-life. WI-38 and 293 cells were metabolically pulse-labeled for 1 h with Tran-^{35}S-label and chased with nonradioactive medium for the number of hours shown above each lane as described in Materials and Methods. Immunoprecipitated proteins from cell lysates of WI-38 (A) or 293 (B and C) cells are indicated to the left of each panel; the antibodies used were directed against p300 (A) and E1A (B and C).

We have also used this immune reagent to determine the p300 phosphorylation state in quiescent cells. To address this question, we isolated p300 from quiescent and infected BRK cells that were labeled for 2 h with $^{32}P_i$ prior to lysis in the conditions described above. Lysates were normalized to total cell protein. The results (Fig. 4) show that p300 isolated from quiescent cells (lane 7) is phosphorylated to a similar degree as p300 isolated from infected cells (lane 8). As above, p300 was coprecipitated with E1A proteins from lysates of infected cells and run as a marker (lane 9). The p300 proteins were not observed with preimmune serum immunoprecipitations in quiescent (lanes 1 and 6) or infected (lanes 5 and 10) cell lysates. This experiment also demonstrates that the immune serum raised against human p300 recognizes rat p300 as well.

We measured the stability of p300 in normal human cells by pulse-labeling WI-38 cells for 1 h with Tran-^{35}S-label and then chasing with nonradioactive medium for various lengths of time (Fig. 5A). p300 was precipitated with the p300-specific antiserum. The amount of labeled p300 remaining at the end of each time interval was determined by scanning the gels with a Phosphoimager. Data from two independent experiments were analyzed by linear regression analysis to calculate the apparent p300 half-life. The results indicate that the half-life of p300 in normal proliferating human cells is approximately 9 h.

The stability of p300 in association with the E1A proteins was determined in similar experiments done in 293 cells. In this case, p300 was affinity precipitated with E1A-specific monoclonal antibody M73. The apparent half-life of E1A-associated p300 (Fig. 5B), determined from three experiments, was approximately 16 h, a value similar to that observed in WI-38 cells, given the limitations of this type of experiment. The half-life of the E1A products was also apparent in the 293 cell experiment and is shown in Fig. 5C for comparison. The E1A half-life in 293 cells has been reported in two previous independent studies to be either approximately 45 to 75 min (1) or approximately 2 h (32). Our results (2 h) are consistent with the longer reported value. Although a number of factors, including the growth rate of the cells and the turnover of intracellular pools of radioactive precursors, can affect the estimation of protein turnover time, results such as those shown in Fig. 5B and C enable us to determine the relative half-lives of two different proteins in the same conditions. This experiment indicates that the E1A products turn over much more rapidly than p300.

We have also used the immune serum to analyze the subcellular localization of p300. HeLa cells, plated at low density, were fixed and stained for indirect immunofluorescence as described in Materials and Methods. The immune serum showed a specific diffuse fluorescence pattern (Fig. 6) which was predominantly nuclear, although there may also be a low level of cytoplasmic staining visible above the nonspecific background seen with preimmune serum (Fig. 6B). Nuclear localization is consistent with expectations for p300 as an E1A-associated protein, since the E1A products themselves are predominantly nuclear localized (7). It should also be noted that the immune serum precipitates, in addition to the p300 species, several bands which are not seen with preimmune serum, most notably a protein that runs with an apparent molecular mass of about 70 kDa (visible in Fig. 2). Thus, it is possible that the fluorescence reaction seen with the antiserum is not due solely to p300, although the predominantly nuclear reaction suggests that all of the major protein species recognized by the immune serum are nuclear localized. We are in the process of raising monoclonal antibodies which will enable us to define the subcellular localization of p300 more definitively.

p300 is modified differentially during the cell cycle. While the results described above indicate that p300 is actively phosphorylated in both resting and growing cells, it is possible that specific phosphorylation events are associated with specific phases of the cell cycle. We tested whether we could detect any modifications of p300 in cell cycle phase-enriched cell populations by blocking cells in mitosis with nocodazole treatment or by blocking cells in S phase with hydroxyurea treatment. Treated 293 cells were labeled with $^{32}P_i$ for 2 h prior to lysis in the continued presence of drugs. Lysates were immunoprecipitated with either M73 or control monoclonal antibody 419, and immunoprecipitated proteins were analyzed by electrophoresis. While p300 immunoprecipitated from lysates of cells treated with hydroxyurea comigrated with p300 from unsynchronized cells, we detected the appearance of a p300 protein species migrating

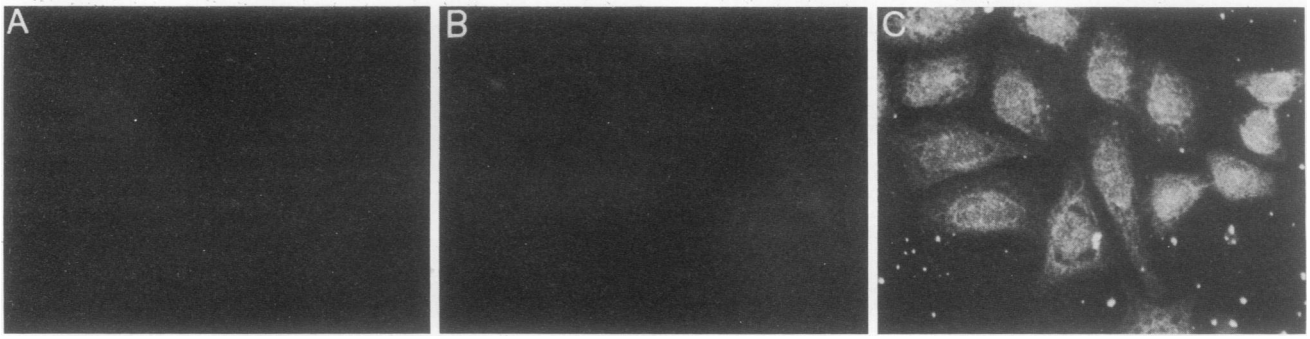

FIG. 6. Intracellular localization of p300. HeLa cells on coverslips were fixed in methanol at −20°C and incubated with preimmune (B) or immune (C) serum diluted 1:50 in PBS + FBS. Additional control cells (A) were incubated in PBS + FBS alone. All cells were then stained with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG and observed for immunofluorescence with a Nikon Microphot FXA fluorescence microscope at a magnification of ×62.5 with oil immersion.

with slightly retarded electrophoretic mobility in lysates from cells blocked in mitosis (Fig. 7).

The slower-migrating ^{32}P-labeled form of p300 observed in lysates from nocodazole-blocked cells is resolved better in the gel shown in Fig. 8. E1A-affinity precipitated p300, prepared as described above, was analyzed by electrophoresis through a 5.0% SDS-polyacrylamide gel run for twice the normal length of time (Fig. 8A). The arrows at the left indicate the positions of the major 300-kDa protein species observed in either unsynchronized or nocodazole-blocked cells. Samples were prepared in triplicate to provide protein for peptide digest analysis. p300 proteins from unsynchronized cells (indicated by the lower arrow in Fig. 8A) were excised along with the specific slower-migrating p300 species (indicated by the upper arrow of Fig. 8A) observed in cells

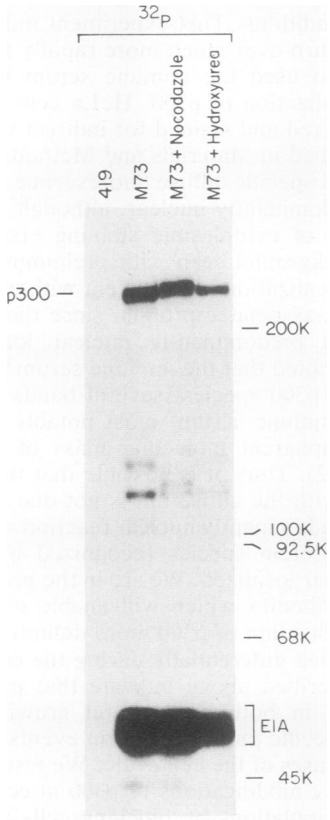

FIG. 7. Coimmunoprecipitation of E1A-associated proteins from 293 cells blocked at mitosis and S phases of the cell cycle. 293 cells were either untreated or treated with nocodazole or hydroxyurea for 20 h. Cells were labeled with $^{32}P_i$ in the continued presence of drugs for 2 h prior to lysis. Proteins were immunoprecipitated with E1A-specific monoclonal antibody M73 or with control antibody 419. Proteins were analysed on a 7.5% SDS-polyacrylamide gel. Positions of E1A and associated proteins are indicated at the left, and sizes of protein molecular weight markers are indicated in kilodaltons (K) at the right.

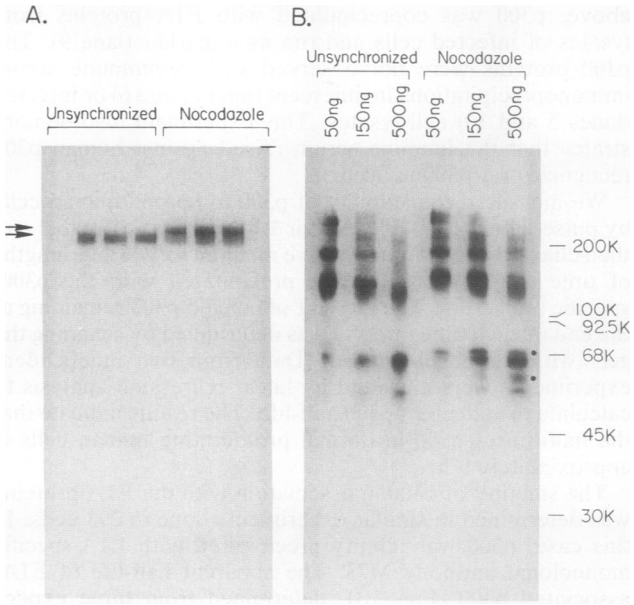

FIG. 8. Coimmunoprecipitation and V8 protease digestion of p300. (A) p300 was coimmunoprecipitated from cell lysates of 293 cells either unsynchronized or blocked in mitosis by nocodazole treatment as described in the legend to Fig. 6 and Materials and Methods except that proteins were analyzed on a 5% SDS-polyacrylamide gel run for twice the normal running time. (B) p300 from unsynchronized cells and only the slower-migrating p300 species from nocodazole-treated cells were excised from the dried gel shown in panel A and digested with the amount of *S. aureus* V8 protease shown above each lane. Positions (kilodaltons [K]) of protein molecular weight markers are indicated at the right.

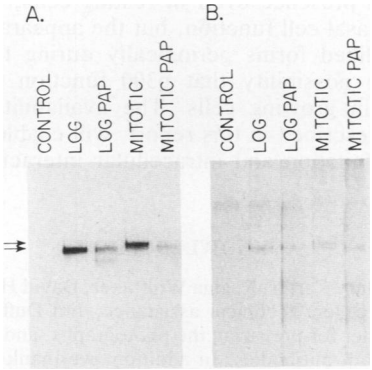

FIG. 9. Potato acid phosphatase treatment of E1A immunocomplexes. Proteins were coimmunoprecipitated with E1A and either mock treated or treated with potato acid phosphatase (PAP) as described in Materials and Methods. Samples were analyzed on a 6.5% SDS-polyacrylamide gel. The gel was silver stained, dried, and autoradiographed. (A) Autoradiograph; (B) silver-stained gel. Positions of p300 and the slower-migrating form of p300 are indicated at the left.

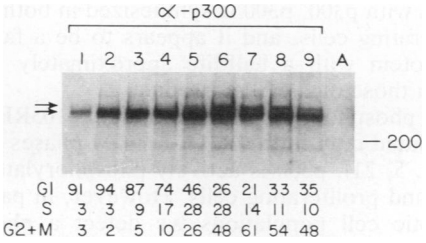

FIG. 10. Immunoprecipitation of p300 with p300-specific rabbit serum from cell cycle phase-enriched populations of HeLa cells. HeLa spinner cells were fractionated by centrifugal elutriation. A portion of each cell fraction was prepared for flow cytometric analysis to determine DNA content, used to estimate the proportion of cells in each phase (shown below the lanes). A total of 5×10^6 cells from each fraction were inoculated into phosphate-free DMEM containing $^{32}P_i$, and cells were incubated for 1 h prior to lysis with p300 lysis buffer. Proteins were immunoprecipitated from cell lysates by using either rabbit preimmune serum (lane A) or p300 rabbit antiserum (lanes 1 to 9). Positions of the p300 species (right) and the 200-kDa protein molecular weight marker (right) are indicated.

blocked in mitosis. The excised bands were treated with V8 protease at three different concentrations (Fig. 8B). We observed comigration of all p300 peptides from unsynchronized cells with p300 peptides from mitotic cells. We also observed two additional p300 peptides with relative molecular masses of approximately 70 and 60 kDa (indicated by the dots in Fig. 8B) that appear to be specific for the mitotic form of p300. The appearance of these peptide fragments in digests of the mitotic p300 form is reproducible (additional data not shown).

To test whether the amount of the slower-migrating form of p300 found in mitotic cells can be reduced by phosphatase treatment, we treated immunocomplexes with potato acid phosphatase prior to electrophoresis. ^{32}P-labeled p300 was coimmunoprecipitated with E1A products from unsynchronized logarithmically growing 293 cells or 293 cells blocked in mitosis. Immunocomplexes were divided into equal portions and either treated with potato acid phosphatase or left untreated. The ^{32}P-labeled proteins were then separated by electrophoresis, and the proteins were visualized by autoradiography (Fig. 9A) and silver staining (Fig. 9B). Potato acid phosphatase treatment reduced the amount of ^{32}P label in p300 from either unsynchronized or mitotic cells, as expected (Fig. 9A). The slower-migrating mitotic cell-specific p300 form was greatly reduced by phosphatase treatment. The appearance of the mitotic cell-specific form of p300 was better resolved by silver staining the same gel (Fig. 9B). Silver staining corresponds to the total amount of protein in each radioactive band. By this criterion, approximately half the p300 protein in mitotically blocked cells appears to be in the mitotic cell-specific form. Consistent with the autoradiography results, the mitotic cell-specific form of p300, visualized by silver staining, is greatly reduced upon phosphatase treatment. This appears to be a phosphatase-specific reaction, since we can preserve the appearance of the mitotic-specific ^{32}P-labeled p300 form in the presence of potato acid phosphatase by addition of the phosphatase inhibitor *p*-nitrophenyl phosphate or by incubating the reactions at pH 8.0 to specifically inhibit the acid phosphatase (data not shown). Phosphatase treatment does not appreciably reduce the total p300 protein level detected by silver staining, although incorporated ^{32}P label is greatly reduced. No silver-stained or ^{32}P-labeled p300 proteins are detected when lysates are immunoprecipitated with control antibody. Similar results were obtained with p300 immunoprecipitated with p300-specific antiserum. In addition, a similar mitotic cell-specific form of p300 is seen in mitotic HeLa cell populations, collected by mechanical shake-off in the absence of drugs (data not shown), indicating that the appearance of this form is not an artifact due to drug treatment.

To determine whether the slower-migrating form of p300 also occurs in the absence of E1A expression, HeLa cells were fractionated by centrifugal elutriation and then labeled for a 2-h pulse with $^{32}P_i$. p300 was immunoprecipitated with the p300-specific rabbit immune serum, and the immunocomplexes were separated by SDS-PAGE and visualized by autoradiography. The positions of the two predominant forms of p300 are indicated by the upper and lower arrows in Fig. 10. The slower-migrating form of p300 is not readily detectable in G_1-enriched cells (fractions 1 to 4). Only in populations of cells containing enriched amounts of cells in S, G_2, and M (fractions 5 to 9) do we observe a protein migrating slower than the major p300 band from unsynchronized cells. The appearance of the slower-migrating forms begins to decline as the proportion of G_1 cells begins to increase again in later fractions. The two forms of p300 had near identical V8 protease digestion patterns, and the appearance of peptide fragments specific for the slower-migrating forms (Fig. 8) was apparent in digests from this experiment as well (data not shown).

DISCUSSION

We have generated rabbit polyclonal antibodies against the 300-kDa host cell protein that associates with the adenovirus E1A proteins in order to characterize p300 further. Comparison of antibody-bound p300 with E1A-associated p300 (Fig. 2 and 4) indicates that in nonstringent conditions, the majority of detectable p300 is associated with E1A in both newly infected and stably transformed cells. p300 association with E1A is less stable than the association of p105RB, p107, and p130, at least in vitro, as judged by the resistance of these complexes to dissociation in high-detergent conditions. In addition, the binding of p105RB, p107, or p130 to E1A products does not appear to be dependent on

association with p300. p300 is synthesized in both quiescent and proliferating cells, and it appears to be a fairly stable cellular protein with a half-life approximately five times longer than those of the E1A products.

p300 is a phosphoprotein. In contrast to p105RB, which is phosphorylated only in the S, G_2, and M phases of the cell cycle (2, 3, 5, 21), p300 is actively phosphorylated in both quiescent and proliferating cells. However, in partially purified mitotic cell populations we detect a phosphatase-sensitive form of p300 with decreased electrophoretic mobility, suggesting that there is an additional modification that is specific to mitotic cells, or at least to cells in phases outside of G_1. This form occurs in both the presence and absence of E1A expression and appears to comprise at least half the p300 detected in mitotic-phase-enriched cells. Considering that relatively large changes in molecular mass would be necessary to resolve proteins of high molecular mass in the 300-kDa range of an SDS-polyacrylamide gel, the fact that we can clearly resolve this form of p300 is notable. Whether this shift in mobility is due to one or more phosphorylation events, to a change in protein conformation induced by a phosphorylation event directly, or to some additional mechanism or modification is not known. Since we do not detect the slower-migrating form of p300 in elutriated cell fractions highly enriched for G_1, we conclude that the slower-migrating form is removed by a mechanism such as dephosphorylation or proteolysis prior to the M/G_1 boundary at cell division. There appear to be multiple forms of p300 in addition to the two major forms most readily resolved by one-dimensional electrophoresis in mitotic phase-enriched cells. These are occasionally resolved in one-dimensional gels (for example, Fig. 5, lane 0, and Fig. 10, lane 1). Two-dimensional gel analysis shows at least four forms in unsynchronized cells, and as many as seven forms in cells treated with nocodazole (not shown), so it is possible that p300 undergoes successive modification steps through the cell cycle, which involve, at the least, some phosphorylation events. Significantly, we observe two peptide fragments that appear to be specific for the mitotic-phase-enriched form of p300, and we are continuing to investigate the nature of these peptides.

In addition to precipitating the p300 species, the immune serum precipitates several bands which are not seen with preimmune serum, most notably a protein which runs with an apparent molecular mass of about 70 kDa (visible in Fig. 2). We do not have sufficient evidence at this time to ascertain whether this is a cross-reacting species, a coprecipitating species, or a contaminating product which independently stimulated the generation of antibodies. Experiments are in progress to distinguish these possibilities. In particular, we are in the process of raising monoclonal antibodies which will be especially useful for this type of study.

The polyclonal serum shows p300 structure to be sufficiently conserved in human, rat (Fig. 2 and 4, respectively), monkey, mouse, and mink cells (data not shown) to be readily detectable by antiserum raised against human cell-derived p300. p300 also appears to have a fairly broad tissue distribution, as we have observed roughly similar amounts of synthesis in cervical carcinoma (HeLa) cells, adenovirus-transformed human embryonic kidney (293) cells, primary BRK cells, normal human embryonic lung (WI-38) fibroblasts, mink lung epithelial (Mv1Lu) cells, and human T-lymphocyte (Jurkat) cells (experiments reported here and additional data not shown). The conserved nature of p300 in different species and different tissues, along with its apparent stability and presence even in resting cells, suggests that p300 has a basal cell function, but the appearance of differentially modified forms periodically during the cell cycle suggests the possibility that p300 function is modulated specifically in growing cells. The availability of specific antiserum, described in this report, will enable us to investigate p300 function and intracellular interactions in more detail.

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