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# Simian Virus 40 Large T Antigen Stably Complexes with a 185-Kilodalton Host Protein

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**Stable interactions between simian virus 40 large T antigen and host proteins are believed to play a major role in the ability of the viral protein to transform cells in culture and induce tumors in vivo. Two of these host proteins, the retinoblastoma susceptibility protein (pRB) and p53, are products of tumor suppressor genes, suggesting that T antigen exerts at least a portion of its transforming activity by complexing with and inactivating the function of these proteins. While analyzing T antigen-host protein complexes in mouse cells, we noted a protein of 185 kDa (p185) which specifically coimmunoprecipitates with T antigen. Coimmunoprecipitation results from the formation of stable complexes between T antigen and p185. Complex formation is independent of the interactions of T antigen with pRB, p120, and p53. Furthermore, analysis of T-antigen mutants suggests that T antigen-p185 complex formation may be important in transformation by simian virus 40.**

Simian virus 40 (SV40) is a DNA tumor virus which can induce immortalization and transformation of cells in culture and generate tumors in vivo (39). The major oncoprotein of SV40, large T antigen, is a multifunctional protein of 708 amino acids whose expression is required for both initiation and maintenance of transformation (33). In addition to its role in the deregulation of cellular proliferation, large T antigen plays a critical role in the regulation of viral DNA replication and gene expression (3, 49).

Extensive studies indicate that T antigen interacts with host cell functions in order to carry out its multiple activities. The most direct evidence of these interactions is the stable association of T antigen with a number of host cell proteins. At least six host proteins complex with T antigen: DNA polymerase alpha (pol alpha) (18, 52); hsp73, a heat shock protein (50); AP-2, a transcription factor (38); p53 and the retinoblastoma susceptibility protein (pRB), two proteins which exhibit tumor suppressor activity (9, 30, 32); and p107/120, a protein of 107 kDa in monkey cells and 115 to 120 kDa in rodent cells which is structurally related to pRB (13–15) (for clarity, this protein will hereafter be referred to as p120). Complex formation with three of these proteins, p53, pRB, and p120, is important for T-antigen-mediated transformation, as mutations in T antigen which disrupt their binding usually diminish transforming activity (9, 14, 44, 60).

As part of our study of SV40 transformation-resistant cell lines (48) and large-T-antigen mutants (46, 47), we were interested in analyzing complexes between T antigen and these host proteins. While optimizing conditions for detection of T antigen-pRB complexes in rodent cells, we noted a protein of 185 kDa which consistently coimmunoprecipitated with T antigen. In this report, we describe our characterization of the complexes between T antigen and this host protein, p185. We find that these complexes are specific; that is, the presence of T antigen is required for the coimmunoprecipitation of p185 by anti-T-antigen monoclonal antibodies. The ability of T antigen to complex with p185 is independent of T antigen's ability to interact with p53, pRB, and p120. Of possible functional significance, an N-terminal fragment of T antigen is sufficient for p185 complex formation; previous studies have indicated the importance of this region in both transformation and transcriptional transactivation.

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## MATERIALS AND METHODS

**Reagents.** Phenylmethylsulfonyl fluoride, aprotinin, *Staphylococcus aureus* V8 protease, and bovine pancreatic β-chymotrypsin were obtained from Sigma; leupeptin was obtained from Boehringer Mannheim. Eagle's minimal essential medium (EMEM) lacking L-methionine and L-cysteine was prepared by using the GIBCO Select-Amine kit. Tran$^{35}$S-label (ICN Biomedicals) is a mixture of L-[$^{35}$S]methionine and L-[$^{35}$S]cysteine. Bio-Rad high-molecular-weight standards were used for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

**Cells and tissue culture.** B2-1 is a thymidine kinase-negative subclone of BALB/c 3T3 cells (24). A16 was derived from B2-1 cells by cotransfection with SV40 DNA and the herpes simplex virus (HSV) thymidine kinase gene (48). All cell lines were grown in EMEM supplemented with 10% fetal bovine serum (FBS; HyClone), 1 mM glutamine, 0.2% sodium bicarbonate, 100 U of penicillin per ml, and 100 μg of streptomycin per ml at 37°C in a 5% $CO_2$ atmosphere.

**Plasmids and recombinant DNA.** The following plasmids were used: pGEM3zf- (Promega); pSV2neo (53); pJYM (35); *dl*1135 and *dl*1137 (45); 5002Δt (43); *dl*423 (50); and 3213 and 3214 (47). All plasmids were propagated in and isolated from *Escherichia coli* DH5α, using standard techniques.

**Transfections. (i) Stable transfections.** Cells in 60-mm dishes were transfected with supercoiled plasmid DNA, including both the test construct and pSV2neo, by the method of Chen and Okayama (5), using *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES)-buffered saline (BBS) (pH 6.8 to 7.0). Four-fifths of each transfection dish was split into a 10-cm focus assay dish and fed with EMEM plus 5% FBS. Foci were visualized by staining the dishes with Wright's solution after 4 weeks. One-fiftieth to one-tenth of each dish was split into another 60-mm dish and fed every 3 to 4 days with EMEM plus 10% FBS plus 450 μg of G418 (GIBCO) per ml. In certain transfections, subclones

were isolated by picking well-isolated G418$^r$ colonies from selection dishes and then expanded into lines in nonselective media.

**(ii) Transient transfections.** Cells in 10-cm dishes were transfected as previously described (28), with 15 μg of test construct plus 5 μg of pGEM3zf- as carrier. On day 2 or 3 after transfection, cells were labelled and lysates were harvested as detailed below.

**Monoclonal antibodies.** PAb414, PAb416, PAb419, PAb423, PAb430, PAb442 (25), PAb100, and PAb101 (22) are anti-T monoclonal antibodies. PAb421 is a p53-specific monoclonal antibody (25). C6 (anti-HSV gC-1; immunoglobulin G3 [IgG3] [11]), C2-5 (anti-HSV gC-2; IgG2a [11]), M73 (anti-E1A; IgG2a [26]), anti-pp60$^{v\text{-}src}$ (IgG1; NCI-BCB Repository/Quality Biotech Inc.), and 180.2E3 (antiphosphocholine; IgG1 [7]) were used as control monoclonal antibodies. ICT71 (a generous gift of Ellen Fanning) and SJK 132-20 (American Type Culture Collection) (59) are monoclonal antibodies against human pol alpha. Both of these antibodies cross-react with mouse pol alpha (15a, 61). PAb416, PAb419, PAb430, PAb442, C6, and M73 were used as ascites fluids, while all of the other antibodies were used as hybridoma tissue culture supernatants.

**Metabolic labelling.** Eighty to one hundred percent confluent 10-cm dishes were washed once with phosphate-buffered saline (PBS) and then incubated for 5 to 6 h at 37°C with 1 ml of labelling medium (EMEM, lacking L-methionine and L-cysteine, plus 10% FBS and 270 μCi of Tran$^{35}$S-label) with rocking every 30 min. The cells were then washed with ice-cold PBS and incubated with 1 ml of ice-cold lysis buffer (50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.0], 250 mM NaCl, 0.1% Nonidet P-40, 5 μg each of phenylmethylsulfonyl fluoride, aprotinin, and leupeptin per ml) for 30 min at 4°C. Lysates were transferred to 1.5-ml tubes, and cell debris was cleared by centrifugation at 16,000 × *g* for 2 min at 4°C. The cleared lysates were used immediately or stored at −80°C.

**Immunoprecipitation analysis.** Cleared lysates (0.5 ml) were incubated with primary antibodies for 30 to 60 min on ice, a secondary rabbit anti-mouse IgG antibody (Cappel) was added, and the lysates were incubated on ice for an additional 30 min. Immune complexes were precipitated by addition of 125 μl of 3% Sepharose–protein A (Pharmacia) suspended in lysis buffer and then incubated for 60 min at 4°C on a rocking device. The bound Sepharose beads were washed five times with lysis buffer and then resuspended in gel loading buffer (2% SDS, 10% glycerol, 100 mM dithiothreitol, 60 mM Tris-Cl [pH 6.8], 0.001% bromophenol blue). After boiling for 3 min, the immune complexes were loaded onto discontinuous SDS-polyacrylamide gels and electrophoresed at 100 V (29). The gels were stained and destained according to standard protocols (27), treated with Amplify (Amersham), dried, and exposed to Kodak X-Omat AR film at −80°C with intensifying screens.

**In vitro mixing analysis.** Equal amounts of unlabelled A16 protein lysates were treated with an anti-T monoclonal antibody or a control monoclonal antibody, and immune complexes were precipitated by two sequential incubations with fixed *S. aureus* cells (Pansorbin; Calbiochem). Each treated lysate was mixed with equal amounts of labelled B2-1 protein lysate and incubated on ice for 2 h to allow complex formation; these mixed lysates were then subjected to immunoprecipitation analysis as detailed above.

**Peptide mapping.** Proteins were gel purified and treated with β-chymotrypsin or V8 protease during electrophoresis in 15% SDS-polyacrylamide gels according to the protocol of Harlow and Lane (27).

## RESULTS

**A 185-kDa protein coprecipitates with large T antigen.** Given the importance of T antigen-host protein complexes in SV40 transformation, we were interested in analyzing such complexes in a set of mouse cell lines which express T antigen after nonselective introduction of SV40 DNA (48). A16, a transformed line that expresses large T antigen, was metabolically labelled with [$^{35}$S]methionine and [$^{35}$S]cysteine. Protein lysates were prepared from the labelled cells and incubated with anti-T monoclonal antibody PAb430 or a control antibody. When the resulting immunoprecipitates were analyzed by SDS-PAGE, two proteins that had previously been shown to complex with T antigen, p120 and p53, were evident in the PAb430 immunoprecipitate (Fig. 1A, lane 5). In addition, PAb430 reproducibly coimmunoprecipitated another protein of 185 kDa. This protein, p185, was not precipitated by a control, isotype-matched monoclonal antibody, C6 (Fig. 1A, lane 4), suggesting that its presence in the PAb430 immunoprecipitate was due to a specific interaction.

Previous immunoprecipitation analyses have indicated that monoclonal antibodies reactive against T antigen also recognize various cellular proteins, presumably by interacting with epitopes that resemble those of T antigen (8, 25, 31). To rule out the possibility that p185 is being directly recognized by PAb430, we examined whether other anti-T monoclonal antibodies could immunoprecipitate p185. As seen in Fig. 1, six other anti-T monoclonal antibodies precipitate a protein that comigrates with the p185 precipitated by PAb430, while control antibodies fail to do so. These antibodies are directed against epitopes throughout T antigen, suggesting that p185 is being coimmunoprecipitated as a result of an interaction with T antigen and not by virtue of antibody cross-reaction.

PAb416 precipitates little or no p185 despite the presence of an equivalent amount of T antigen in the immune complex (Fig. 1A, lane 2). This finding could be explained by either of two possibilities. First, PAb416 may recognize a discrete subpopulation of T antigen which does not interact with p185. Indeed, previous studies have indicated that certain anti-T monoclonal antibodies recognize T-antigen molecules enriched for various biochemical activities, suggesting the existence of discrete large-T-antigen subpopulations (10, 22, 51, 56–58). Alternatively, PAb416 binding may disrupt the T antigen-p185 interaction by inducing a conformational change in T antigen or by competing with p185 for a common binding domain on T antigen. These alternatives were tested by performing double-antibody immunoprecipitations using PAb430 and PAb416. If PAb416 were simply recognizing a different population of T antigen, then the double immunoprecipitate should contain a level of p185 similar to that precipitated by PAb430 alone. If PAb416 were disruptive, however, the double immunoprecipitate should contain significantly less p185 than is seen with PAb430 alone. As shown in Fig. 2A, the double immunoprecipitate contains little or no p185, while a double immunoprecipitation using PAb430 plus a control antibody does not decrease the amount of p185 relative to the amount precipitated with PAb430 alone (compare lanes 3 and 4). A p53-specific monoclonal antibody, PAb421, also coimmunoprecipitates p185 with T antigen, indicating that p185 and p53 are in a common complex with T antigen (Fig. 2B, lane 3). PAb416-

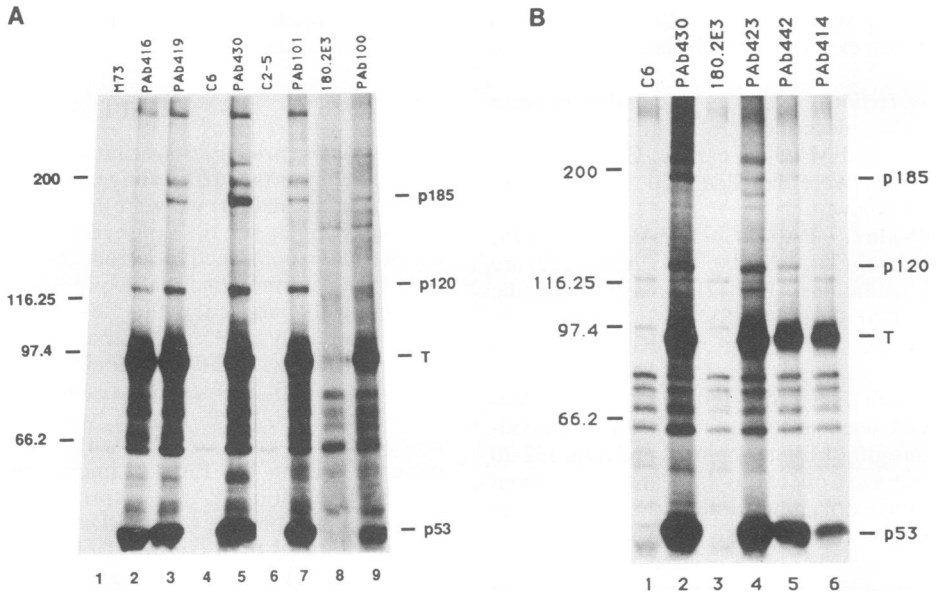


FIG. 1. Coimmunoprecipitation of p185 by anti-T monoclonal antibodies. Metabolically labelled A16 protein lysates were treated with a panel of anti-T monoclonal antibodies (A, lanes 2, 3, 5, 7, and 9; B, lanes 2, 4, 5, and 6) or with control antibodies (A, lanes 1, 4, 6, and 8; B, lanes 1 and 3), and the immunoprecipitates were analyzed as detailed in Materials and Methods. In panel A, lanes 8 and 9 are from an extended exposure of the same gel shown in lanes 1 through 7. The positions (in kilodaltons) of molecular weight standards are shown at the left in all figures. Epitope localization of anti-T antibodies (amino acid boundaries): PAb414 (367 to 699), PAb419 (1 to 82), PAb423 (682 to 708), PAb430 (1 to 82), and PAb442 (115 to 271) (25); PAb100 (277 to 699) and PAb101 (512 to 699) (23); PAb416 (82 to 130) (1).

PAb421 double immunoprecipitates show a similar decrease in the amount of p185 relative to the control precipitate (Fig. 2B; compare lanes 5 and 6). The lack of p185 in double immunoprecipitates containing PAb416 is not due to a higher affinity of PAb416 for the protein A beads, as similar results are obtained with and without the use of a rabbit polyclonal antibody specific for mouse IgG as a secondary reagent (data not shown). In addition, the presence of PAb416 in anti-p53 immunoprecipitates does not affect the ability of PAb421 to precipitate a cross-reacting cellular protein of 165 kDa (Fig. 2B, lanes 5 and 6). We conclude, therefore, that PAb416 disrupts the interaction of T antigen with p185. Disruption by

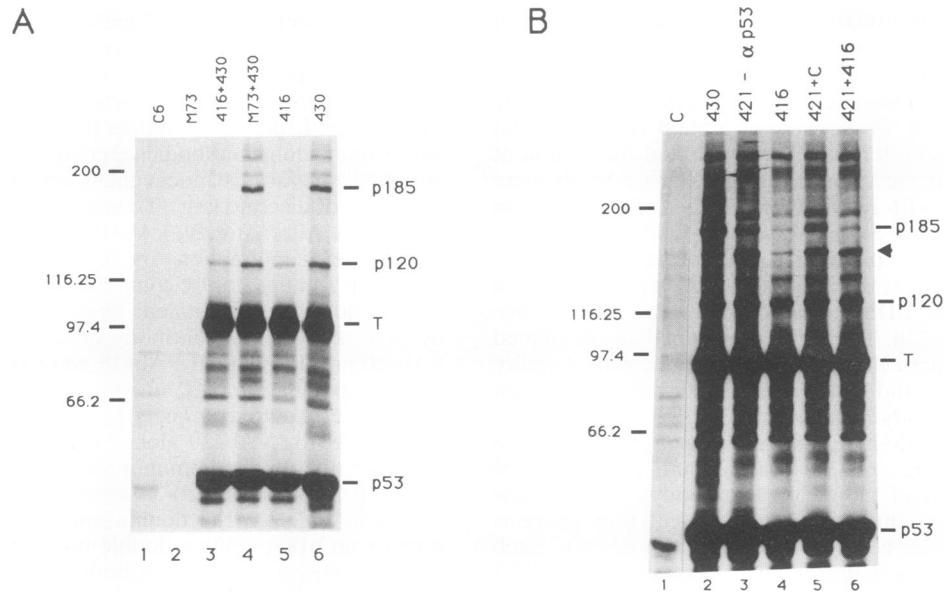


FIG. 2. PAb416 disruption of T antigen-p185 complexes. Immunoprecipitations of metabolically labelled A16 protein lysates were analyzed as for Fig. 1. (A) Lanes 3 and 4 are double-antibody immunoprecipitates (lysates were treated with two primary antibodies simultaneously). (B) Lanes 5 and 6 are also double-antibody immunoprecipitates. C in lane 1 is control antibody C6, while C in lane 5 is control antibody M73. The arrowhead at the right denotes a 165-kDa cellular protein that cross-reacts with PAb421 (unpublished observation).

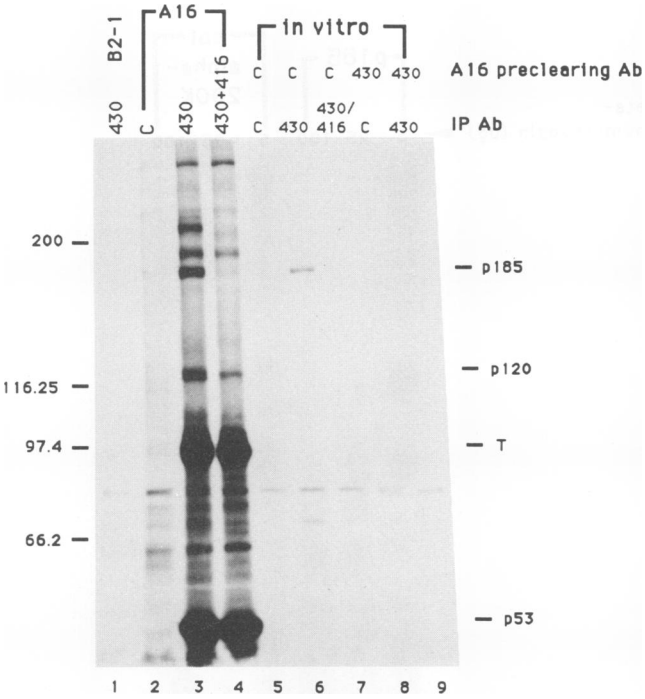


FIG. 3. Presence of p185 in T-antigen-negative cells. The ability of large T antigen to form complexes with p185 in vitro was measured in an in vitro mixing experiment as detailed in Materials and Methods (lanes 5 to 9). "A16 preclearing Ab" denotes the antibody used to preclear unlabelled A16 lysates. "IP Ab" denotes the antibody(ies) used to precipitate the in vitro mixes. Lanes 1 to 4 are standard immunoprecipitates of labelled B2-1 and A16 lysates. C indicates control antibody C6.

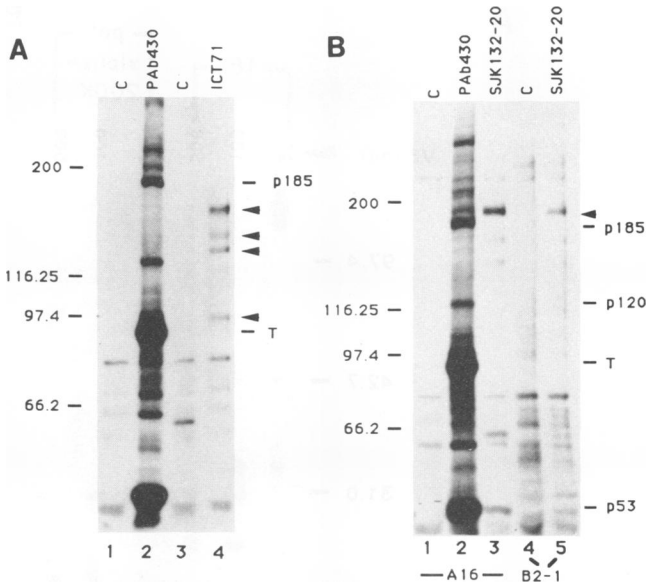


FIG. 4. Comparison of p185 with pol alpha. (A) Metabolically labelled A16 lysates were treated with control monoclonal antibodies (lane 1, C6; lane 3, anti-pp60$^{v\text{-}src}$), PAb430, or the anti-pol alpha monoclonal antibody ICT71, and the immunoprecipitates were analyzed by SDS-PAGE. Arrowheads on the right denote the positions of the four proteins reactive with ICT71. (B) Metabolically labelled A16 (lanes 1 to 3) and B2-1 (lanes 4 and 5) lysates were analyzed as for panel A. SJK 132-20 is an anti-pol alpha monoclonal antibody. Two control antibodies were used: C6 (lane 1) and 180.2E3 (lane 4). The arrowhead on the right denotes the position of the 200-kDa pol alpha protein.

PAb416 is selective: the T antigen-p120 and T antigen-p53 interactions are not significantly affected.

**p185 is present in normal cells.** If T antigen and p185 are in an authentic complex, then p185 should be precipitated by anti-T monoclonal antibodies only when T antigen is present. To test this, we prepared metabolically labelled lysates from B2-1 cells, which do not express T antigen. These lysates were treated with PAb430, and the immunoprecipitates were analyzed by SDS-PAGE; no p185 was precipitated (Fig. 3, lane 1). To determine whether p185 is actually present in these lysates, we performed an in vitro complex experiment. Unlabelled A16 lysates, which contain T antigen, were mixed with labelled B2-1 lysates, and potential T antigen-p185 complexes were allowed to form during an incubation at 0°C. The in vitro mixes were then immunoprecipitated with PAb430 and analyzed by SDS-PAGE. Figure 3 shows that a protein comigrating with p185 is precipitated from the in vitro mix (compare lanes 3 and 6). This protein is not present if a control antibody is used for the immunoprecipitation (lane 5), nor is it present if the disrupting antibody PAb416 is used along with PAb430 in the immunoprecipitation (lane 7), strongly suggesting that the comigrating protein is indeed p185 and therefore that p185 is present in cells not expressing large T antigen. The ability of p185 to be precipitated from the in vitro mix depends on the presence of T antigen, as pretreatment of the unlabelled A16 lysate with an anti-T monoclonal antibody to remove T antigen prevents complex formation (lane 9). These results indicate that p185 is a host protein and that p185 expression is independent of viral protein functions. p185 is also present in normal BALB/c 3T3 cells (data not shown). Note that detectable p53 is not coprecipitated from normal cells in these mixing experiments, presumably because the steady-state p53 level in such cells is low as a result of the absence of the stabilizing effect of T antigen (see reference 41 for a review).

**p185 is not pol alpha.** Several studies have provided evidence that T antigen forms a complex with pol alpha (12, 18, 52). This interaction is thought to be critical for directing pol alpha to the viral origin of replication and the subsequent initiation of DNA synthesis (52, 62). The ability of pol alpha from permissive and semipermissive species, but not that from nonpermissive species, to direct in vitro SV40 origin-specific DNA synthesis suggests that the SV40 host range is due, at least in part, to species-specific differences in pol alpha (40, 63). Indeed, recent studies of the T antigen-pol alpha interaction in vitro indicate that T antigen has an approximately 10-fold-higher affinity for pol alpha from a semipermissive species than for pol alpha from a nonpermissive species (12). However, other evidence supports the notion that T antigen retains at least some ability to complex with rodent pol alpha (19, 20). This evidence, along with the recent demonstration that an N-terminal, 83-amino-acid fragment of T antigen appears to bind to the purified 180-kDa large subunit of pol alpha (12), led us to test whether the p185 identified in our studies is the large subunit of pol alpha.

We obtained two monoclonal antibodies against human pol alpha that cross-react with the mouse protein. These were used to immunoprecipitate pol alpha from mouse cell lysates so that it could be compared with p185 precipitated with PAb430 from the same lysates. Anti-pol alpha mono-

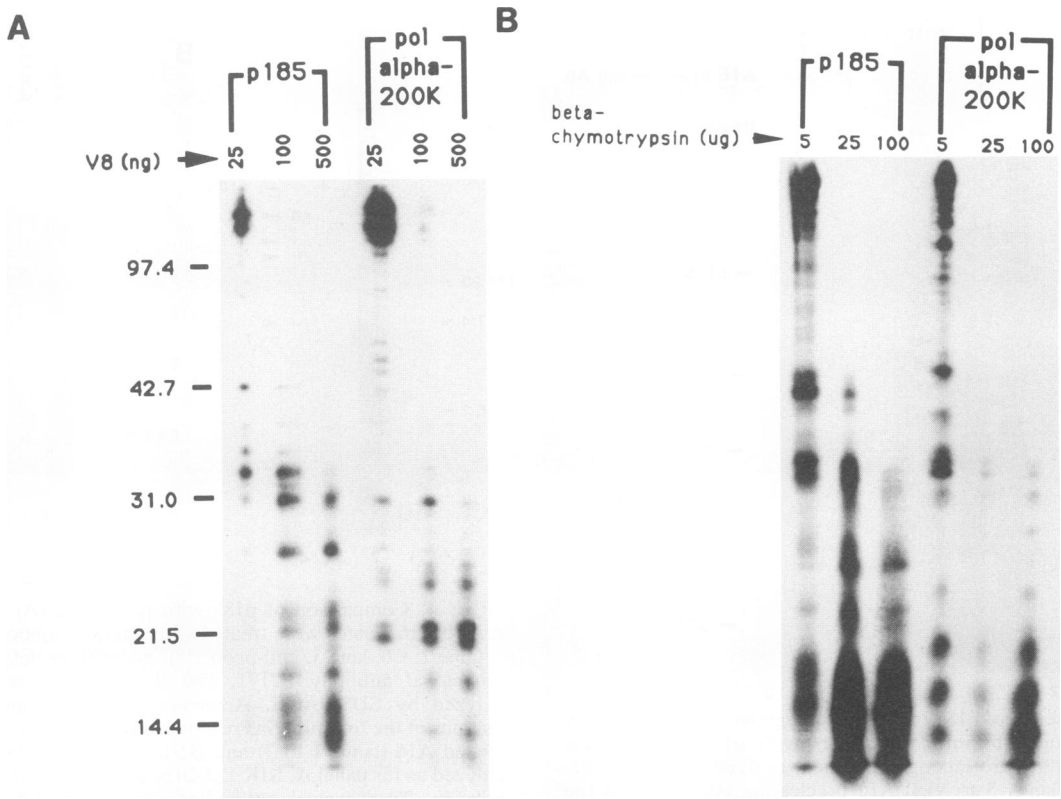


FIG. 5. Partial protease digestion of gel-purified p185 and pol alpha. p185 and the pol alpha 200-kDa protein were gel purified and digested with *S. aureus* V8 protease (A) or with β-chymotrypsin (B) during gel electrophoresis as detailed in Materials and Methods.

clonal antibody ICT71 precipitated four species, ranging in size from 97 to 165 kDa (Fig. 4A, lane 4). These are presumably proteolytic breakdown products of the intact large subunit of pol alpha that have been noted in many previous studies (see, for example, reference 16). None of the proteins precipitated by ICT71 comigrated with p185. The other anti-pol alpha antibody, SJK 132-20, precipitated a single major protein of 200 kDa (Fig. 4B, lane 3). Again, no 185-kDa protein was present. When B2-1 lysates (which do not contain T antigen) were treated with SJK 132-20, a 200-kDa protein once more was the major specific protein found (Fig. 4B, lane 5). This result argues against the possibility that SJK 132-20 is prevented from binding to a putative 185-kDa pol alpha protein in A16 lysates as a result of T antigen blocking the relevant epitope. These data suggest that p185 and pol alpha are not the same protein. p185 and pol alpha were compared directly by examining the partial digest products generated by treating each protein with V8 protease (Fig. 5A) or β-chymotrypsin (Fig. 5B). The results show that p185 and pol alpha are not related.

**Mapping of the p185 binding domain on T antigen.** Complex formation of T antigen with p53, pRB, and p120 is believed to play an integral role in T antigen's ability to deregulate cellular proliferation, since mutations in T antigen which abrogate binding to one or more of these proteins also diminish at least some aspects of T antigen's transforming capacity (9, 14, 44, 60). We were therefore interested in defining the domains of T antigen which are required for the interaction with p185 and determining whether these sequences are also important for T antigen's transforming function. To address this question, we expressed a panel of mutant T antigens in B2-1 cells and assayed each mutant for its ability to complex with p185 and to transform.

Immunoprecipitation analyses indicated that five of the six mutants assayed form a complex with p185. Complexing phenotypes were the same whether assayed by transient or stable expression of T antigen; we have presented the results from assays in which the highest levels of T antigen were expressed. Mutant *dl*1137, which encodes only the N-terminal 121 residues of T antigen, complexes with p185 (Fig. 6, lane 4). The truncated T antigen encoded by this mutant migrated off the gel shown, but an identical immunoprecipitate electrophoresed for a shorter length of time indicated that a T antigen of the expected size is expressed at a level comparable to that of the wild-type protein (data not shown). The ability of *dl*1137 to complex with p185 demonstrates two points: (i) the 121 N-terminal residues of T antigen are sufficient for complex formation, and (ii) since *dl*1137 does not bind p53, T antigen-p185 complex formation is independent of the T antigen-p53 interaction. Mutant 5002Δt encodes a T antigen containing two N-terminal amino acid substitutions and is also deleted for intron sequences that renders it unable to express a full-length small-t-antigen protein (43, 44a). The ability of this mutant to form a complex with p185 indicates that the interaction is independent of small-t-antigen expression (Fig. 6, lane 6). Mutant 3213 encodes a large T antigen containing two amino acid substitutions within the domain required for binding pRB and p120, while 3214 contains one substitution at the boundary of this same domain and another, more C-terminal, substitution (47). Both mutants complex with p185 (Fig. 7, lanes 4 and 6), indicating that complex formation can occur

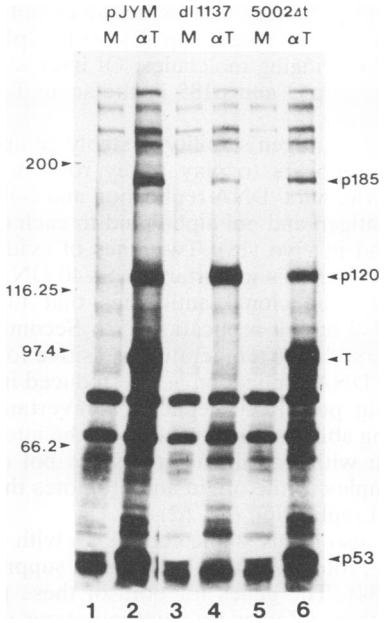


FIG. 6. p185-binding abilities of T-antigen mutants *dl*1137 and 5002Δt. B2-1 cells transiently transfected with three T-antigen constructs were metabolically labelled and treated with monoclonal antibodies; immunoprecipitates were analyzed by SDS-PAGE. Antibody M is control antibody M73; anti-T (αT) is PAb430.

in the absence of binding to pRB and p120. Mutant *dl*1135 is missing residues 17 to 27, a region of T antigen that is necessary for the protein's ability to complement an adenovirus E1A N-terminal deletion mutant in focus formation assays (64). This deletion also destroys the epitope recognized by PAb430 (44a; unpublished observation), necessitating the use of anti-T monoclonal antibodies that recognize

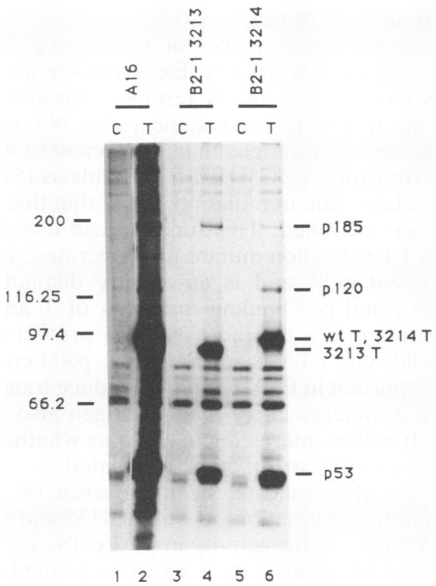


FIG. 7. p185-binding abilities of T-antigen mutants 3213 and 3214. G418$^r$ pools of B2-1 cells cotransfected with two T-antigen constructs plus pSV2neo were analyzed as for Fig. 6. Antibody C is control antibody C6; T is PAb430.

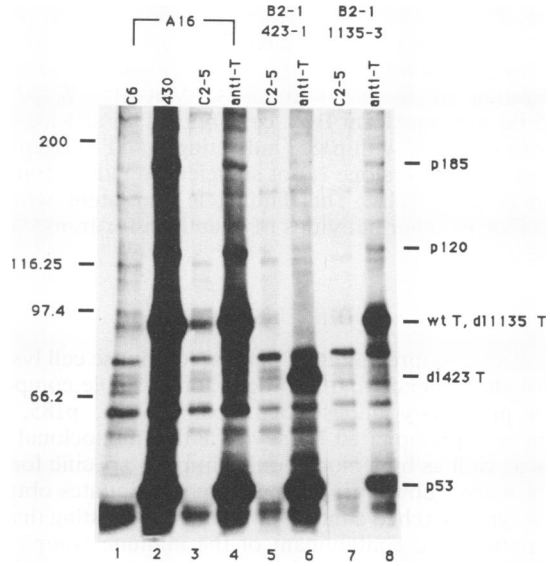


FIG. 8. p185-binding abilities of T-antigen mutants *dl*423 and *dl*1135. B2-1 423-1 and B2-1 1135-3 are subcloned lines expressing T-antigen mutants *dl*423 and *dl*1135, respectively. They were analyzed for the presence of T antigen-p185 complexes as for Fig. 6. C6 and C2-5 are control antibodies; anti-T represents a combination of two anti-T monoclonal antibodies, PAb423 and PAb101.

other epitopes in T antigen. T antigen-p185 complexes are detected with a combination of two such antibodies, PAb423 and PAb101 (Fig. 8, lane 8). Mutant *dl*423, missing residues 2 to 108, complexes with little, if any, p185 (Fig. 8, lane 6). This finding demonstrates that sequences contained within this deleted region are necessary for the T antigen-p185 interaction, in agreement with the results from mutant *dl*1137, which also indicate the importance of the N terminus of T antigen.

Table 1 summarizes the ability of each mutant to complex with p185 and to induce focus formation of B2-1 cells. Three of the six mutants (3213, 3214, and 5002Δt), like wild-type T antigen, transform B2-1 fibroblasts and complex with p185.

TABLE 1. p185-binding and focus-forming abilities of T-antigen mutants

| T antigen | Amino acid change | p185-binding ability[a] | Relative focus-forming ability[b] |
|---|---|---|---|
| pJYM | | + | 1.0 |
| 3213 | Glu-107→Lys<br>Glu-108→Lys | + | 0.9 |
| 3214 | Ala-116→Thr<br>Glu-141→Lys | + | 1.2 |
| 5002Δt | Leu-19→Phe<br>Pro-28→Ser | + | 0.3[c] |
| *dl*1137 | 122–708 deleted | + | <0.005[c] |
| *dl*1135 | 17–27 deleted | + | <0.003 |
| *dl*423 | 2–108 deleted | − | 0.04 |

[a] Determined by immunoprecipitation analysis as shown in Fig. 6 through 8.

[b] Determined by transfecting DNA constructs encoding the various T antigens (5 to 9 μg each) into B2-1 cells and then plating the transfected cells into focus assays as detailed in Materials and Methods. The results are presented as the number of foci induced by each mutant relative to the number induced by wild-type T antigen.

[c] Results from one experiment. Results for all other mutants are averages of two to four experiments.

Mutant *dl*423 shows a significant reduction in both phenotypes. These data suggest that T antigen-p185 complex formation plays a role in the ability of T antigen to deregulate proliferation of mouse fibroblasts. Mutants *dl*1137 and *dl*1135 do not transform B2-1 cells but retain at least some ability to complex with p185, indicating that T antigen-p185 complex formation alone is not sufficient to induce transformation of B2-1 cells. This finding is consistent with the importance of other activities of T antigen for transforming ability.

## DISCUSSION

Using immunoprecipitation analysis of mouse cell lysates, we provide evidence that T antigen forms stable complexes with a previously undescribed host protein, p185. This protein is coprecipitated by seven anti-T monoclonal antibodies as well as by a monoclonal antibody specific for p53. p185 is absent, however, from immunoprecipitates obtained with isotype-matched control antibodies, suggesting that it is not a nonspecific contaminant of the immune complex. In addition, treatment of B2-1 lysates containing E1A with an E1A-specific monoclonal antibody immunoprecipitates a number of previously described E1A-binding proteins, including p120, but not p185 (data not shown). p185 precipitation by anti-T antibodies is not the result of direct recognition of p185 through epitopes shared with T antigen, given that the antibodies capable of precipitating p185 are directed against several different epitopes on T antigen and against p53. One anti-T antibody, PAb416, is able to disrupt preformed T antigen-p185 complexes and to block the formation of these complexes in vitro. This finding is notable for two reasons. First, it is further evidence against p185 being a nonspecific contaminant of anti-T immunoprecipitates, since little or no p185 is found in PAb416 immunoprecipitates despite the presence of T antigen. Second, the location of the epitope recognized by PAb416 (amino acids 82 to 130 [1]) suggests that the N terminus of T antigen is important for complex formation. Thus, the PAb416 epitope overlaps the region of T antigen that retains sufficient structural information to complex with p185 (residues 1 to 121), as defined by mutant *dl*1137. PAb416 may directly compete with p185 for T-antigen sequences, or it may perturb some higher-order structure of T antigen that is required for complex formation. Finally, the ability of PAb430 to coprecipitate p185 depends on the presence of T antigen. No p185 is precipitated by this antibody from lysates which do not contain T antigen, even though p185 is present in these lysates, as shown by in vitro mixing experiments.

Although p185 and pol alpha are approximately the same size and appear to require a similar region of T antigen for complex formation, we believe that they are unrelated proteins, as suggested by the following evidence. First, no protein that comigrates with p185 is recognized by either of two monoclonal antibodies directed against pol alpha. Second, partial peptide maps of p185 and pol alpha generated by two different proteases are dissimilar. We have also found that an anti-T monoclonal antibody previously shown to block the in vitro association of T antigen and pol alpha (52) does not block T antigen-p185 complex formation (data not shown).

Do T antigen and p185 interact through direct protein-protein contacts or indirectly, through another protein which acts as a molecular bridge? As only unfractionated whole cell lysates were used in this study, either alternative is possible. The protein-binding properties of *dl*1137, which does not bind p53, and 3213, which does not bind pRB or p120 (Fig. 7) (6a), indicate that p53 as well as pRB or p120 do not act as the bridging molecules. Of interest, however, is the presence of p53 and p185 in the same T-antigen complexes (Fig. 2B).

SV40 large T antigen's ability to stably complex with host cell proteins appears to play a key role in its biological functions during viral DNA replication and cellular transformation. T antigen and pol alpha bind to each other, both in vitro (52) and in vivo (18). Two lines of evidence indicate that this interaction is important in SV40 DNA replication. First, anti-T monoclonal antibodies that block complex formation also inhibit replication (52). Second, a T-antigen mutant (*tsA*58) that is temperature sensitive for its ability to induce viral DNA replication is also reduced in its ability to complex with pol alpha; replication revertants regain pol alpha-binding ability (19). Presumably, the interaction of the viral protein with pol alpha directs the pol alpha-primase enzyme complex to the origin and promotes the initiation of bidirectional replication (52, 62).

T antigen also forms stable complexes with p53 and pRB, two cellular proteins that possess tumor suppressor activity (9, 30, 32, 34). The genes for both of these proteins have been found to be affected by point mutations or deletions in several types of murine and human tumors; in addition, exogenous pRB can suppress the proliferation of certain tumors, and wild-type p53 expression inhibits the transforming effects of a number of oncogenes (for reviews, see references 36 and 37), including SV40 T antigen under certain conditions (17). Analysis of T-antigen mutants has uncovered a correlation between the ability of T antigen to deregulate cellular proliferation and its ability to bind one or both of these proteins (9, 14, 44, 60). These data have provided strong support for a transformation model in which complex formation with T antigen serves to inactivate the proliferation suppression functions of p53 and pRB (21). Given the similarities between p120 and pRB in primary structure and T-antigen-binding characteristics, p120 may play a similar role in the negative control of cell proliferation; complex formation with T antigen thus may also result in inactivation of p120 function (15).

The functional consequences of T antigen-p185 complex formation are presently unclear. Extensive studies of mutant T antigens indicate that the N terminus harbors a discrete transforming function; for instance, the N-terminal 121-residue fragment of T antigen that is sufficient to bind p185 is capable of transforming C3H10T1/2 fibroblasts (54). A transformation-related function that resides within this region has recently been identified. This function can complement an adenovirus E1A deletion mutant to cooperatively transform primary rodent cells and is apparently distinct from the pRB-, p120-, and p53-binding functions of T antigen (42). The E1A mutant fails to bind a 300-kDa protein (p300) that binds to wild-type E1A, suggesting that p300 complex formation is important in E1A's ability to induce transformation (55). Thus, it appears likely that T antigen also complexes with p300. It will be interesting to examine whether p185 and p300 are structurally or functionally related.

Our results also indicate the importance of N-terminal sequences in transformation, as both *dl*1135 and *dl*423 show diminished transforming activity in B2-1 cells. The defects in these mutants apparently are not related to binding of pRB or p120, since complex formation with these proteins is not required for transformation of this line (mutant 3213 retains the ability to transform B2-1 cells). We cannot rule out the possibility that the deletions in *dl*423 and *dl*1135 have dis-

turbed a more C-terminal transforming domain; however, both mutants retain the ability to complex with p53 (Fig. 8). The inability of *dl*423 to complex with p185 is consistent with p185 complex formation playing a role in the N-terminal transforming activity of T antigen. The nontransforming mutant *dl*1135, however, retains p185-binding activity, suggesting that the sequences deleted from *dl*1135, while important in transformation, are not required for p185 complex formation. Comparison of relative binding levels, though, is hampered somewhat by the inability of *dl*1135 to be recognized by PAb430, the anti-T antibody that coprecipitates the highest relative amount of p185; *dl*1135 may actually complex with less p185 than does wild-type T antigen. Alternatively, mutant *dl*1135 may define another N-terminal transforming domain of large T antigen, distinct from a putative p185 transforming domain. In this regard, *dl*1135 will not complement the aforementioned E1A mutant that fails to bind p300 (64).

The N terminus of T antigen has also been implicated in the protein's ability to transactivate the SV40 late promoter and other viral promoters (54, 65). Indeed, the N-terminal fragment expressed by *dl*1137 transactivates the adenovirus E2 gene at wild-type efficiency (54), suggesting the possibility that T antigen-p185 complex formation could also play a role in T-antigen-mediated transactivation. Provocative new evidence suggests that pRB functions as a negative regulator of transcription by binding to the cellular transcription factor E2F and thereby preventing E2F from activating various viral and cellular genes (2, 4, 6). E1A transactivation of these genes presumably results from E1A-pRB complex formation inducing the release of E2F, which is then competent to transactivate (2, 4). However, sequences of T antigen that are required for transactivation of certain promoters map upstream of the pRB-binding domain, suggesting that another N-terminal domain is involved (65). Finer genetic mapping of the region(s) of T antigen important for p185 complex formation will be required to determine whether this transactivation domain, or other functional domains, are involved. Molecular cloning of the p185 gene should offer further insight into p185 function. Identification of conserved functional domains, as well as analysis of p185 expression during the cell cycle, may reveal similarities between p185 and the other host cell proteins that appear to play crucial roles in T-antigen function.

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