Characterization of Simian Virus 40 Large T Antigen by Using Different Monoclonal Antibodies: T-p53 Complexes Are Preferentially ATPase Active and Adenylylated

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We used 21 monoclonal antibodies (PABs 100 to 117, 405, 419, and KT3) specific for different determinants in simian virus 40 (SV40) large T antigen (T) and one antibody specific for p53 that coprecipitates T complexed with p53 (T-p53) to analyze T in SV40-infected CV1 cells. We measured the ATPase specific activity, extent of adenylylation, and p53 content of T precipitated by antibodies directed against the N-terminal region I (0.65 to 0.62 map units), the midregion III (0.43 to 0.28 map units) containing both the ATPase- and nucleotide-binding sites, and the C-terminal region IV (0.28 to 0.17 map units) of T. Lytic T appeared to exist in three different forms with respect to p53 binding and ATPase activity. The most ATPase-active form of T was that precipitated by PAb 122. This T-p53 complex contained only 6% of the total T but contributed 35% of the ATPase activity, on average. Free p53 isolated from 3T6, Ann-1, or L929 cells had no apparent ATPase activity. A second form of T precipitated by several antibodies had little associated p53 but appreciable ATPase activity, accounting for 15 to 20% of total T and 60 to 70% of the ATPase activity. The rest of T constituted the third form and was also depleted in p53 but had a decreased ATPase specific activity. Thus, the remaining 75 to 80% of T had 15 to 20% of the ATPase specific activity. Antibodies specific for region III precipitated T with both altered ATPase activity and altered amounts of bound p53. PABs 104 and 114 reacted with ATPase-active T but inhibited ADP hydrolysis, suggesting that they were inactivating antibodies. T that was preferentially adenylylated in vitro corresponded to T that was also preferentially ATPase active. T bound to p53 was adenylylated to a higher specific activity than total T. In addition, p53 itself was significantly adenylylated under these conditions. The results suggest that ATPase activity and p53 binding are structurally and functionally related and that p53 alters biochemical activities of T and plays a role in productive infection.

Simian virus 40 (SV40) large T antigen (T) can transform cells and is required to initiate viral DNA replication (for reviews, see references 9 and 38). T appears also to function during elongation of replicating SV40 DNA molecules by synchronizing replication fork movement (35). T has an intrinsic ATPase activity (12, 37) that appears necessary for SV40 replication (5, 23). Indeed, T contains a sequence centered at residue 432 that is highly homologous with the nucleotide-binding region of the procaryotic recA protein, a promoter of DNA strand exchange and also an ATPase (29, 30). Covalent modification of T after incubation with the affinity label dialdehyde-ATP affects T-dependent ATPase and in vitro SV40 DNA replication activities; up to a 1:1 stoichiometry of binding of dialdehyde-ATP to T antigen was observed (7, 9, 31). The site of adenylylation is located between residues 412 and 528 in T (8). Direct adenylylation of T with unmodified ATP has also been reported; less than 5% of T was modified (2). Here, the peptide-bound moiety was AMP covalently linked to the protein via a phosphodiester bond to a serine residue, putatively Ser-430 (2, 9). Mutations affecting replication map near the putative adenylylation site, suggesting that adenylylation is important for viral DNA synthesis. While the ATPase site has been reported to be distinct from the nucleotide-binding site (8), interaction between the two sites appears necessary for DNA replication (31). A transformation-related cellular pro-

tein, 53 kilodaltons in size, is physically associated with SV40 T in SV40-infected and -transformed cells (13, 14, 18, 21). p53 appears to be required for cellular DNA synthesis and may regulate cell proliferation (24, 25, 27).

Recently, several new hybridoma lines synthesizing papovavirus-specific monoclonal antibodies (PABs 100 to 117) specific for three different antigenic regions of T were described (15). A map of the binding sites in T for these and other antibodies has been compiled (4, 6, 10, 15, 17). We and others have used these T-specific antibodies as probes to identify and characterize structurally and functionally distinct forms of SV40 T and to analyze changes in specific regions as well as interactions between domains in wild-type T proteins. These studies have indicated the presence of different forms of T, including old compared with newly synthesized T (3, 14), forms more active in a DNA-binding assay (28), and those that are free or associated with replicating or with mature SV40 chromosomes (36). Analysis of the relative amounts of T in various subcellular fractions indicated that about 1.3% of total T is associated with SV40 chromosomes in infected cells (36).

In this study, we used PABs 100 to 117, 405, 419, and KT3 (14, 15, 17, 22) to further characterize and quantify both structurally and functionally distinct forms of T prepared from productively infected CV1 cells. In addition, we used the monoclonal antibody PAb 122 (14), specific for p53, to study T complexed with p53 (designated T-p53). A hypotonic fractionation procedure was used since viral nucleoprotein complexes prepared by this procedure are competent

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to replicate and transcribe faithfully in an in vitro system (32, 33). For all assays, specific activities were determined so that differences that were due to antibodies recognizing different subclasses of T could be distinguished from those reacting with T with low affinity. T bound to p53 was both preferentially ATPase active and adenylated in vitro and had several determinants altered in region III of the T-antigen molecule. This identification of enhanced biochemical activities important for SV40 DNA replication that are associated with the T-p53 complex is evidence that p53 may have an important function in SV40 productive infection.

MATERIALS AND METHODS

Cells and virus. CV1 cells and SV40 strain wt800 viral stocks were grown as previously described (32). Each 150-mm dish of SV40-infected cells was radiolabeled in vivo with 1 mCi of [35 S]methionine (1,250 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) in 10 ml of Dulbecco modified Eagle medium ($0.2\times$ methionine) plus 3% dialyzed fetal calf serum for 12 to 17 h. At 36 to 42 h postinfection, cells were lysed by hypotonic extraction as previously described (32, 34). The hypotonic buffer consisted of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.8), 5 mM KCl, 0.5 mM $MgCl_2$, and 1 mM dithiothreitol. The cell lysate was pelleted by centrifugation at $20,000 \times g$ for 45 min. Under these conditions, the supernatant fraction contained about 80% of the total T present in the cell and was used for further analysis. On average, about 600 μ l of extract was obtained from each 150-mm dish.

Immunosorption. Hybridoma supernatant PABs 100 to 117, 405, 419, and KT3, all specific for large T, and PAB 122, specific for p53, were prepared as previously described (14, 15, 17, 22). Hybridoma cell lines secreting PABs 405 and 419 were obtained from J. Pipas (University of Pittsburgh). KT3 cells were obtained from G. Walter (University of California, San Diego). NS1 medium (parent nonsecretor murine myeloma line) and MOPC-21 medium (nonspecific immunoglobulin G [IgG] secretor murine line), provided by W. R. Allen (Salk Institute), were used as controls. T-reactive ascitic fluid was generated in weanling hamsters injected intraperitoneally with an SV40-transformed hamster ascitic cell line by the procedure of Lanford and Butel (20). This high-titer ascitic tumor polyclonal antiserum was used for comparison with the different monoclonal antibodies; preimmune hamster serum was used as a control. To immunoprecipitate T contained in cell extracts, we incubated either monoclonal medium or tumor serum and protein A-Sepharose (Pharmacia, Inc., Piscataway, N.J.) with cell extract as previously described (36). In general, each reaction consisted of 150 to 300 μ l of extract (containing 2 to 5 μ g of T) and 200 to 400 μ l of hybridoma medium (up to 20 μ g of specific murine IgG and 200 μ g of nonspecific bovine IgG) or 10 μ l of hamster tumor serum incubated for 30 min in ice. The reaction mix was then incubated further for 30 min at 4°C with shaking after the addition of 50 to 80 μ l of a washed 1:1 protein A-Sepharose suspension (10-mg/ml IgG-binding capacity). The immune complexes were washed three times with NET-Nonidet P-40 (NET-NP-40) buffer (100 mM NaCl, 5 mM EDTA, 50 mM Tris hydrochloride [pH 7.8], 0.05% NP-40) and then were either assayed for associated enzymatic activity or analyzed by sodium dodecyl sulfate (SDS)-gel electrophoresis (see below).

Relative reactivity and p53 content. To determine the relative reactivity of each antibody with T, we measured the amount of immune-complexed radiolabeled T using saturat-

ing amounts of antibody and protein A-Sepharose and a fixed amount of cytosol. Since some of the antibodies used are IgG1 in subclass (15), a second antibody (rabbit anti-mouse IgG) highly reactive with protein A was added as previously described (36). The immune complexes were analyzed by SDS-gel electrophoresis (34). T and p53 were quantified by measuring the total amount of [35 S]methionine counts in each labeled band by using dried gels (without fluor) and an AMBIS Beta Scanner and IBM computer. T was also quantified by densitometric analysis with photographic negatives of SDS gels stained with Coomassie blue. For each experiment, the amounts of T and p53 bound by each antibody were determined and compared with that bound by polyclonal tumor serum. Nonspecific controls (usually less than 0.2% of the label) were subtracted. The ratio of labeled T to p53 in each immune complex was then calculated.

ATPase activity. The amount of ATPase activity associated with each immune complex was assayed as follows. Each precipitate was washed twice in ATPase A buffer (4) consisting of 25 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] (pH 7.0), 0.1 M NaCl, 5 mM $MgCl_2$, and 0.01% NP-40 and incubated at 33°C in 40 μ l of a reaction mix consisting of 1 μ Ci of [α - 32 P]ATP (800 Ci/mmol; New England Nuclear Corp., Boston, Mass.) and 1 to 100 μ M ATP in ATPase buffer. Samples (1 μ l) were removed at 15, 30, and 60 min, spotted on polyethyleneimine-cellulose thin-layer chromatography plates (Brinkmann Instruments Co., Westbury, N.Y.), and chromatographed in 0.75 M KH_2PO_4 (pH 3.5) (37). Appropriate nucleotide and inorganic phosphate standards were analyzed in parallel and then identified by the molybdate-perchlorate staining procedure of Hanes and Isherwood (16). Again, beta-scan analysis was used to quantify total ^{32}P counts in each labeled spot on the dried thin-layer chromatography plates. For each antibody, the rate of ATP hydrolysis was determined by measuring the percent conversion of the input [α - 32 P]ATP to [α - 32 P]ADP with time. Background values for ATP hydrolysis (usually about 2% of the input labeled ATP in 30 min) were subtracted. Antibodies were compared with one another when ADP production was proportionate to time, usually at 30 min. For each antibody measured, the relative ATPase specific activity was determined by normalizing the rate of ATP hydrolysis to the amount of T present in that immune complex after SDS-gel electrophoresis and beta-scan analysis of the dried gel. Using PAB 108 and various concentrations of cell extract, we found that the ATPase activity was proportionate to the amount of T bound to the immune complexes; the different antibodies were compared in this range of T concentration. Protein A-Sepharose-purified monoclonal antibodies gave the same results as hybridoma supernatants.

The ATPase activity of T fractions prepared by sequential and mixed immunoprecipitations was also measured. Sequential immunosorption involved first reacting cell extracts with saturating amounts (two rounds) of a given antibody and protein A followed by protein A beads alone to completely remove all of the first antibody. The supernatant was then reacted with a second antibody and protein A. The washed pair of immune complexes (precipitate PAB 1 and supernatant PAB 1 precipitated with PAB 2) were then assayed in parallel to measure the relative amount of ATPase activity normalized to the amount of labeled T present in each precipitate. Mixed immunosorptions consisted of first reacting extracts with a specific antibody (usually PAB 108 or 122) and washing. Then, saturating amounts of a second antibody were added to the complex, incubated, and

FIG. 1. Regions of SV40 large T antigen used to map monoclonal antibody-binding sites (10, 15, 17, 22). The map positions are shown with the corresponding antibodies that bind to these regions. DNA-binding, ATPase, recA-homologous, ATP-binding, and putative adenylation (Ad) sites are shown.

washed, and the immunoprecipitate was assayed for ATPase activity as before. Again, the activity was normalized to the amount of T present. The amount of specific IgG added was quantified as before (36) to ensure that equal amounts were added.

Adenylation. The extent of *in vitro* adenylation of T and T-p53 complexes recognized by each antibody was measured as follows. Unlabeled cell extracts were incubated with each antibody and the immunosorbent, and the immune complexes were washed twice each with NET-NP-40 and ATPase A buffers as described above. Each immune complex was incubated for 60 min at 33°C in 40 μ l of a reaction mix containing 20 μ Ci of [α - 32 P]ATP and 10 to 100 μ M ATP in ATPase A buffer. The immune complexes were then washed five times in NET-NP-40 buffer and analyzed by SDS-gel electrophoresis. The amount of [α - 32 P]nucleotide-labeled protein was determined with dried gels by beta-scan quantification of the 32 P-labeled T and p53 bands. For each antibody tested, labeled T was then normalized to the amount of Coomassie blue-stained T. The labeled nucleotide-T adduct was further characterized with snake venom phosphodiesterase (Sigma Chemical Co., St. Louis, Mo.) and acid hydrolysis as described by Bradley et al. (2).

RESULTS

Relative reactivity of each monoclonal antibody with T. The locations of the recognition sites in T for PAb 100 to 117, 405, 419, and KT3 are shown in Fig. 1. All precipitate T from extracts of SV40-infected monkey cells (15, 17, 22). The locations of sequences in T known to contain the specific DNA-binding (residues 135 to 230), nucleotide-binding (residues 412 to 528), recA-homologous (centered at residue 432), ATPase (residues 371 to 628), and putative adenylation (residue 504) sites are also shown (2, 4, 5, 8, 9, 30; J. Pipas, personal communication). PAb 122 specifically recognizes the C terminus of p53 and coprecipitates T complexed with p53 (13, 14, 18, 21).

The fraction of total T reactive with these antibodies was measured by protein A-dependent immunoprecipitation as described in Materials and Methods. In general, hybridoma

supernatants were used since affinity-purified antibodies gave the same result but were unstable on storage (36). When necessary, a second antibody was added to the reactions. The immune complexes were fractionated by SDS-gel electrophoresis; representative gels are shown in Fig. 2. Figure 2A shows extracts of T immunoprecipitated with PAb 110 to 117, 122, and KT3 and analyzed by staining with Coomassie blue. Figure 2B shows extracts labeled with [35 S]methionine and reacted with selected PAb 111 to 117 and 419; PAb 101 and 108 are also shown. The relative amount of T (averaged for at least 10 separate determinations) maximally precipitated by each antibody is shown in Table 1.

The results were reproducible and consistent with those previously obtained for PAb 100 to 109 under these conditions (36), that is, antibodies specific for determinants located in region I of T precipitated at least 60% of the T normally reactive with polyclonal tumor serum. By this criterion, PAb 108 and 109 consistently precipitated essentially 100% of the T. All region III antibodies precipitated significantly less T, from 4 to 43%. Four of the five region IV antibodies precipitated 60 to 80% of the T; one (PAb 115) reacted with only 35% of the T. Thus, all but one determinant in the N- and C-terminal regions of T were highly reactive with their corresponding antibodies, while all determinants in region III appeared significantly less reactive in free T. We have previously shown that the PAb 102, 103, 104, and 106 region III are highly reactive with the form of T bound to replicating SV40 chromosomes (36). Therefore, the low extent of reactivity of these antibodies with free T is likely due to inaccessibility of the epitopes rather than poor binding by the antibodies.

Relative T/p53 ratio for each antibody. The amount of [35 S]methionine-labeled p53 coprecipitated with T for each antibody was determined after SDS-gel analysis (see Materials and Methods). A typical result is shown in Fig. 2B. For each experiment, the relative amounts of radiolabeled p53 and T were measured and expressed as a ratio. The results are shown in Table 1 and are averages of from three to six separate determinations. Total T labeled for 16 h and reacted with tumor serum had an average T/p53 ratio of 12 based on

FIG. 2. Relative reactivity of monoclonal antibodies with hypotonic extracts of SV40-infected CV1 cells. Extract (250 μ l) was incubated with hybridoma supernatant (200 μ l) for 30 min in ice, followed by the addition of protein A-Sepharose beads (80 μ l of 1:1 [vol/vol] suspension) in NET-NP-40 plus 1% sucrose buffer. The washed immune complexes were analyzed by SDS-gel electrophoresis. (A) Coomassie blue-stained SDS-gel analysis of extracts reacted with PABs 110 to 117, 122, and KT3. Numbers on right show size in kilodaltons. (B) Autoradiogram of 35 S-labeled extracts reacted with PABs 111 to 117, 101, 108, and 419. Infected cells were labeled in vivo for 16 h with [35 S]methionine before extraction. The relative amounts of 35 S-labeled T and p53 in each lane were measured with an AMBIS Beta Scanner and IBM computer system.

relative radiolabel content. Since both T and p53 contain a similar number of methionines per 100 amino acids, this roughly corresponds to a molar T/p53 ratio of 6.

The labeled T/p53 ratio for antibodies specific for region I and region IV was similar to this. However, several region III antibodies behaved differently, with PABs 103, 104, 106, and 114 recognizing T that was two- to threefold deficient in p53. In contrast, PAB 100 in region III appeared to recognize T that was about two- to threefold enriched in associated p53. PAB 122 (anti-p53) precipitated 6% of the 16-h-labeled T and 4% of 4-h-labeled T, on average. Essentially 90% of labeled monkey p53 is associated with T under these conditions (precipitable with PAB 108 which reacts with total T). With PAB 122, there was an approximately equal ratio of labeled T and p53. From this, we estimate that the molar ratio of T/p53 in the complex reactive with PAB 122 is about 0.5. The p53-specific antibody PAB 246 (39) was also tested, but it precipitated less than 10% of the total monkey p53 compared with that recognized by PAB 122.

These results suggest that the fraction of T precipitated by most antibodies has the same relative amount of bound p53 as total T. Thus, region I- and IV-specific antibodies appear to see similar forms of T as tumor serum. However, several

region III-specific antibodies bound T fractions with altered amounts of bound p53. Four antibodies preferred T that was depleted in bound p53; possible explanations for this include blocking of these epitopes by bound p53 or the presence of distinct subclasses of T with respect to bound p53.

Relative ATPase activity of immune-complexed T. Since many antibodies reacted with less than total T, some precipitated fractions may represent subclasses of T. Also, many antibodies were specific for regions III or IV containing the ATPase domain; some might directly react with elements of the ATPase site. Accordingly, we measured the ATPase activity of T precipitated with the antibodies as described in Materials and Methods. Typical chromatograms of radiolabeled ATP and ADP (the primary hydrolysis product) are shown in Fig. 3. The rate of conversion (nanomoles per minute) for each immune-complexed T fraction was measured. The specific activity was determined by normalizing the ATPase activity to the amount of [35 S]methionine-labeled T found in the same immunoprecipitation fraction. The results shown in Table 1 are averages of at least 10 separate ATPase determinations for each antibody. Since PAB 108 immunoprecipitates nearly all the T reactive with polyclonal tumor anti-T serum, its activity (units of ATP hydrolyzed) and relative amount of T present were both made equal to 1.0. The hydrolysis rate for PAB 108-complexed total T was approximately 12 nmol/min per mg of protein, similar to that previously reported for purified free D2 T antigen (4).

The most surprising result was obtained with PAB 122, which precipitated a small (6%) fraction of T bound to P53 but accounted for up to 40% of the total ATPase activity (T reactive with PAB 108). At 2 μ M ATP, the specific activity of T-p53 complexes was 7.5 times, on average, that of total T. This enhanced ATPase activity was also observed at ATP concentrations of up to 100 μ M. To determine whether this increased ATPase activity was not due to the presence of an ATPase activity associated with P53 itself, we assayed hypotonic extracts of Ann-1, L929, and 3T6 cells, all containing free p53 (13; L. Tack, personal observation). Analysis of the p53 present in 3T6 extracts and immunoprecipitable with PAB 122 is shown in Fig. 4A. No detectable ATPase activity was associated with immune-complexed p53 protein in the absence of T (Fig. 4B); equal amounts of free p53 and p53 complexed to T were assayed in parallel.

While the absolute ATPase activities were different for T precipitated with some antibodies (Fig. 3), the specific activities for many were similar. In general, region I-specific antibodies precipitated most of T and had ATPase specific activities similar to that of PAB 108. However, PABs 111 and 116 appeared to precipitate T that was slightly enriched in ATPase activity. Several region III-specific antibodies (PABs 100, 103, and 106) precipitated T that was up to threefold enhanced in ATPase specific activity. PAB 106 precipitated between 40 and 60% of total T; at the lower concentration of T, the ATPase specific activity was higher than when more T was present. PABs 104 and 114 precipitated T with a highly reduced ATPase specific activity (about three- to fourfold, on average). However, PAB 102 precipitated T with the same ATPase specific activity as total T. Most of the region IV-specific antibodies were like those specific for region I: T was precipitated with an ATPase activity similar to that of total T. PABs 107 and 115 precipitated T that was slightly ATPase active, reacting with 35 to 67% of total T.

These results indicate that four antibodies specific for region III reacted with forms of T that showed the greatest change in ATPase activity. Two bound T that was enhanced

TABLE 1. Characterization of forms of lytic T reactive with different monoclonal antibodies

Specificity (region) ^a	PAb	SDS ^b	% T precipitated ^c	T/p53 ratio ^d	ATPase sp act ^e	Adenylylation of T ^f
T (I)	105	+	82	14	1.1	0.8
	108 ^g	+	98	13	1.0	1.0
	109	+	102	10	0.9	0.8
	110	+	72	11	1.3	0.8
	111	+	64	10	1.6 (+)	1.7 (+)
	112	+	90	9	1.1	0.8
	113	+	90	8	1.0	0.9
	116	+	62	8	1.5 (+)	0.9
	117	+	93	9	1.2	0.9
	419	+	90	12	0.9	1.0
T (III)	100	—	4	5 (+)	1.7 (+)	1.6 (+)
	102	—	43	11	1.1	1.0
	103	—	25	22 (—)	2.7 (++)	2.1 (+)
	104	—	17	25 (—)	0.3 (—)	1.4 (+)
	106 ^g	—	35	21 (—)	2.0 (+)	1.7 (+)
	114	—	19	23 (—)	0.3 (—)	0.7
T (IV)	101	+	86	11	1.0	0.9
	107	+	67	10	1.6 (+)	1.7 (+)
	115 ^g	+	35	8	1.6 (+)	1.6 (+)
	KT3	+	81	10	1.3	0.7
	405	+	66	11	1.4	0.9
T total	Tumor	+	100	12	1.0	1.0
p53	122	+	6	0.8 (++++)	7.5 (++++)	2.8 (++) p53: 1.7 (+)

^a Monoclonal antibodies specific for either T or p53 and polyclonal hamster tumor serum were used. Regions (in map units) of T are: I, 0.65 to 0.62; III, 0.43 to 0.28; and IV, 0.28 to 0.17.

^b Resistant (+) or sensitive (—) to SDS denaturation (15, 17).

^c Relative amount of T precipitated with each antibody with a fixed volume of radiolabeled cell extract plus saturating amounts of antibody and protein A-Sepharose. Control values were subtracted.

^d Relative ratio of labeled T complexed with labeled p53. + denotes an increase and — a decrease in p53 bound to T compared with that bound with tumor serum. Total T immunoprecipitated with tumor serum had a T/p53 ratio of 12.

^e Rate of ATP hydrolysis for each immune complex, corrected for the amount of T and normalized to the results obtained with PAb 108. + denotes an increase and — a decrease in the relative ATPase specific activity.

^f Amount of labeled nucleotide bound to T corrected for the amount of T in the immune complex and normalized to T bound by PAb 108. For PAb 122, the amount of labeled nucleotide bound to p53 was calculated and normalized to the result obtained for T in that sample.

^g Cross-reactive with BK virus large T antigen by immunofluorescence (15).

and two bound T that was inhibited for ATP hydrolysis. Another five antibodies specific for different regions of T showed moderate changes in ATPase activity. Thus, several antibodies bind fractions of T with altered ATPase activity.

Further analysis of ATPase activity and T/p53 ratios of different fractions of T by sequential and mixed immunoprecipitation. Sequential and mixed immunoprecipitations (see

Materials and Methods) were used to define further those fractions of T with altered ATPase activity. In particular, T reactive with region III-specific antibodies was analyzed; all precipitated a fraction of total T (Table 1). Both hybridoma supernatants and purified IgG fractions were used. We wished to determine whether some antibodies directly affected ATP hydrolysis or recognized preferentially active

FIG. 3. ATPase activity of immune-complexed T reacted with different monoclonal antibodies. Extracts were immunoprecipitated with each antibody and washed with NET-NP-40 buffer as described in Materials and Methods. The immune complexes were washed twice with ATPase A buffer and incubated in a reaction mix containing 2 μ M [α -³²P]ATP in ATPase buffer at 33°C. Samples were removed at 30 and 60 min, spotted on thin-layer chromatography plates, and chromatographed as described in the text. The positions of nucleotide and P_i standards are shown. The relative amounts of labeled ADP generated from the input labeled ATP with time were determined with the AMBIS Beta Scanner. NS1, Nonspecific control medium.

FIG. 4. ATPase activity associated with free p53. (A) ^{35}S -labeled extracts of 3T6 cells and SV40-infected CV1 cells immunoprecipitated with anti-T (PAb 108), anti-p53 (PAb 122), and nonspecific control medium (NS1). (B) ATPase activity associated with p53 in 3T6 extracts compared with total T and T-p53 complexes in SV40-infected CV1 extracts. Washed immune complexes were incubated for 30 min at 33°C, and samples were chromatographed as before.

and inactive forms of T. For example, it was not clear whether the lack of activity for T bound to either PAb 104 or 114 was due to inhibition via antibody binding to elements constituting part of the ATPase site or to recognition of an inactive form of T.

Mixed immunoprecipitation allows analysis of the effect of adding a second antibody to either total T or a subset of T after precipitation with a first antibody. The results of measuring the ATPase activity of such mixed immunoprecipitates are shown in Table 2. When PAb 108 was used to precipitate total T and then PAb 108 or another region I antibody (PAb 110 or 111 are shown) was added to the washed immune complex and analyzed, the ATPase specific activity was unchanged, as expected. Similarly, no significant change was observed when any of PAb 100, 103, and 106 (region III) or 101 and 115 (region IV) was added to total T. Thus, PAb 100, 103, 106, and 115 may recognize an ATPase-active form of T as the increased ATPase specific activity observed for them in Table 1 does not seem to be due to binding and activation by the antibody. However, it is possible in these mixing experiments that prior binding of PAb 108 changes the affinity of T for a second antibody or that binding of PAb 108 induces a conformational change in T that prevents ATPase activation with a second antibody. Addition of PAb 122 to total T or T without p53 had no effect, indicating that this antibody does not enhance ATPase activity on binding to total T nor does it contain an associated ATPase activity of its own.

In contrast, when PAb 104 or 114 was used in a mixed immunoprecipitation reaction, the ATPase activity of total T was decreased three- to fivefold. This result indicates that PAb 104 and 114 recognize an ATPase-active form of T but that binding of the antibody inhibits this activity. Therefore, these two antibodies do not appear to recognize an ATPase-inactive subclass of T. PAb 104 and 114 bind less than 20% of the total T and inhibit 65 to 70% of the total T-associated ATPase activity. Since T-p53 represents about 30% of the total ATPase activity and PAb 104 and 114 both prefer T lacking p53 (Table 1), these two PAb could have been inhibiting only T without p53. The results (Table 2) comparing the addition of PAb 104 and 114 to T-p53 and T depleted of p53 show that both forms of T are inhibited compared with adding other antibodies such as PAb 108 or 101; however,

the ATPase activity was consistently twofold more inhibited for T depleted in p53 than for T-p53. Thus, PAb 104 and 114 appear to preferentially inhibit T depleted in p53; they also appear to inhibit, but less strongly, the ATPase activity of

TABLE 2. ATPase activity of different forms of T in mixed immunoprecipitates^a

T fraction ^b	PAb addition (specificity) ^c	ATPase sp act ^d
Total T	108 (I)	1.0
	110 (I)	0.9
	111 (I)	0.8
	100 (III)	0.7
	103 (III)	0.8
	104 (III)	0.2 (--)
	114 (III)	0.3 (--)
	106 (III)	0.8
	101 (IV)	0.8
	115 (IV)	0.8
	122 (p53)	1.1
T-p53	108 (I)	3.4 (++)
	104 (III)	1.0
	114 (III)	1.2
	101 (IV)	3.3 (++)
	122 (p53)	4.3 (++)
T without p53	108 (I)	0.9
	104 (III)	0.1 (--)
	114 (III)	0.3 (--)
	122 (p53)	0.7
PAb 114-reactive T	101 (IV)	0.3 (--)
	103 (III)	0.2 (--)
	106 (III)	0.2 (--)
	122 (p53)	0.3 (--)

^a Data for one typical experiment are shown.

^b Extracts were precipitated with the first PAb (108 [total T], 114, 122 [T-p53]) or sequentially with PAb 122 followed by PAb 108 (T without p53) and washed.

^c PAb 2 was added to the PAb 1 immune complex, washed, and assayed for ATPase activity.

^d ATPase activity was corrected for the amount of T in each complex and normalized to total T precipitated with PAb 108. Shown is the relative increase (+) or decrease (-) in ATPase specific activity.

TABLE 3. ATPase activity of sequentially immunoprecipitated T fractions^a

First round			Second round		
PAb 1	Specificity	ATPase 1	PAb 2	Specificity	ATPase 2
108	I	1.0	108	I	1.0
104	III	0.4 (--)	108	I	0.3 (--)
114	III	0.3 (--)	108	I	0.4 (--)
100	III	2.2 (+)	108	I	1.0
103	III	2.8 (++)	108	I	0.6 (-)
106	III	2.6 (++)	108	I	0.8
115	IV	2.2 (+)	108	I	0.6 (-)
122	p53	4.2 (+++)	122	p53	4.6 (+++)
100	III	1.4 (+)	122	p53	3.7 (++)
104	III	0.2 (--)	122	p53	3.0 (++)
114	III	0.4 (--)	122	p53	2.5 (+)
122	p53	11.8 (+++)	108	I	0.8
122	p53	5.6 (+++)	104	III	0.4 (--)
122	p53	6.2 (+++)	114	III	0.4 (--)
122	p53	4.3 (+++)	100	III	2.3 (+)

^a Data from one typical experiment are shown. The PAb 1 immunoprecipitate was assayed for ATPase activity in parallel with the supernatant from PAb 1 sequentially immunoprecipitated with PAb 2. Specificity is defined in Table 1. ATPase specific activity is the rate of ATP hydrolyzed per microgram of T normalized to PAb 108 and is shown as a relative increase (+) or decrease (-).

T-p53 complexes. When PABs 103, 106, and 122 were added to PAB 114-precipitated T, no change in ATPase activity was observed, consistent with the presence of active forms of T rather than activating antibodies.

Sequential immunoprecipitation allows separation of total T into different fractions which can be assayed in parallel for ATPase activity. The data for these analyses are shown in Table 3. For PAB 108, about 90% of the T was precipitated in the first round with a specific activity of 1.0; when PAB 108 was used in the second round, the remaining T also had a specific activity of 1.0. In contrast, precipitation first with either PAB 104 or 114 followed by precipitation of supernatant T with PAB 108 resulted in a depletion of ATPase specific activity for the remaining T. This is consistent with recognition and inactivation of an ATPase-active subclass of T subsequent to antibody binding. When PAB 104 or 114 was followed by PAB 122, about half of the ATPase activity usually associated with T-p53 remained; thus, PABs 104 and 114 bind to some but not all of the ATPase-active T. When T was sequentially reacted with PAB 103, 106, or 115 and then with PAB 108, the remaining T was depleted in ATPase activity. This is consistent with these PABs preferring an ATPase-active form of T. PABs 103, 106, and 115 each precipitate about 30 to 40% of the T, on average. Sequential precipitation with PAB 100 followed by PAB 122 resulted in a decrease in activity for the PAB 122 precipitate. This is consistent with PAB 100 recognizing some of the ATPase-active T-p53 normally precipitated by PAB 122 (Table 1). There was no detectable change in ATPase activity for the PAB 108 precipitate after reacting with PAB 100; however, since PAB 100 binds such a small amount of total T (less than 5%), a significant difference was not expected. When PAB 122 was followed by PAB 100, the specific activity was still high, indicating that the form of T lacking p53 present in the PAB 100 fraction of T also has enhanced ATPase activity. Thus, the T-p53 recognized by PAB 100 does not account for all the elevated ATPase activity of this fraction. The reverse experiment with PAB 100 followed by PAB 122 showed that some but not all of the ATPase-active T-p53 is recognized.

These results suggest that there are different forms of T with altered ATPase activity. T-p53 accounted for 5% of the

total T and 30 to 40% of the total activity and thus was preferentially ATPase active. There appeared to be two forms of T without p53. One consisted of about 15 to 20% of the T and about 60% of the ATPase activity, suggesting another enhanced ATPase-active subclass. This form was recognized best by PABs 104 and 114. PABs 100, 103, 106, and 115 also appeared to react with this form of T. The other form of T lacking p53 is inferred to be significantly less ATPase active and appears to consist of the remaining T (75% of the total) with about 10 to 15% of the ATPase activity. Two PABs (104 and 114) appeared to recognize an ATPase-active form of T but were inactivating upon binding; these antibodies preferred to bind T without p53 than to bind T-p53. Kinetic (Lineweaver-Burk) analysis indicated that the V_{max} for ATP hydrolysis for total T of 12 nmol/min per mg of T decreased to 1.5 to 2.0 nmol/min per mg of T for total T with added PAB 104 or 114; the K_m was relatively unaffected (data not shown). No activating antibodies were observed for ATPase activity. PAB 100 appears to bind an ATPase-active form of T, some of which is associated with p53.

In vitro adenylation. Bradley and co-workers (2) have observed that a small fraction of purified SV40 T is adenylylated in vitro when incubated with labeled ATP. Accordingly, we incubated immune-complexed T with [α -³²P]ATP as described in Materials and Methods. Figure 5 shows the results when T and T-p53 complexes were immunoprecipitated with different antibodies (PABs 105 to 110 and 122) and incubated with labeled ATP. The labeled immune complexes were fractionated on SDS gels and analyzed by autoradiography. After extraction of the α -³²P-labeled T band from the dried gel, digestion with snake venom phosphodiesterase generated [³²P]AMP. Acid hydrolysis of the extracted labeled T band generated [³²P]phosphoserine. Alkaline phosphatase had no effect on the amount of specific ³²P incorporation into T. These results are all consistent with in vitro adenylation of immune-complexed T. We observed that labeled ATP incorporation into T was linear with increasing ATP concentration, up to 1 mM, as reported previously (2); however, the degree of nonspecific adenylation of T increased at greater than 50 μ M concentrations of ATP, when

FIG. 5. Adenylation of T and T-p53 complexes immunoprecipitated with different antibodies. Immune complexes (Fig. 3) were washed with ATPase A buffer and incubated with 20 μ M [α - 32 P]ATP for 60 min at 33°C in 40 μ l of ATPase A buffer. The complexes were washed five times with NET-NP-40 and analyzed by SDS-gel electrophoresis and autoradiography. (Left) Results for extracts reacted with PAb 105 to 110. (Right) Total T (PAb 108) and T-p53 complexes (PAb 122).

other proteins such as IgG were modified. We estimate that about 1% of the T is specifically adenylylated at 50 μ M ATP. The addition of an ATP regeneration system (creatine kinase and creatine phosphate) increased the extent of specific adenylylation of T consistent with donation of the nucleotidyl moiety by ATP rather than the ADP generated by the ATPase reaction.

For each antibody analyzed, the relative specific activities (amount of 32 P incorporated per microgram of T) were calculated and normalized to PAb 108 (see Materials and Methods). The results are shown in Table 1. Several of the region III antibodies (PAb 100, 103, and 106), as well as one region I (PAb 111) and two region IV (PAb 107 and 115) antibodies, recognized preferentially adenylylated forms of T. T bound to p53 (coprecipitated with PAb 122) was the most adenylylated, with a two- to threefold-greater specific activity than T without p53 (sequential precipitation with PAb 122 followed by PAb 108) or total T (PAb 108). These same antibodies also recognize forms of T with enhanced ATPase activity. Thus, there is a correspondence between enhanced ATPase-active and adenylylated T. When the p53 content of the adenylylated forms of T was analyzed, there was no correlation. PAb 122 and 100 both reacted with T-p53, while PAb 103 and 106 preferred T depleted in p53 and PAb 107, 111, and 115 all bound T with the same T/p53 ratio as total T. Therefore, we conclude that essentially all enhanced ATPase-active forms of T are preferentially adenylylated in vitro, regardless of the amount of bound p53.

Besides adenylylation of T, we observed that p53 was also covalently labeled in the presence of [α - 32 P]ATP (Fig. 5; Table 1). Quantification of the amount of labeled nucleotide indicated that p53 was adenylylated to about the same degree as the T with which it was complexed. Sequential immunosorption of a PAb 122 supernatant with PAb 108 showed that the labeled p53 band had been removed (data not shown).

DISCUSSION

We used 22 monoclonal antibodies specific for T and T-p53 complexes to analyze extracts of SV40-infected CV1

cells. The antibodies specific for T each recognized one of three separate regions in T: the N-terminal region I at 0.65 to 0.62 map units, the midregion III at 0.43 to 0.28 map units, and the C-terminal region IV at 0.28 to 0.17 map units. The relative reactivity of these antibodies with forms of T which differ in their ATPase activity, extent of adenylylation, and p53 content was characterized. Our major conclusions are as follows.

Relationship between ATPase activity and p53 binding. Using PAb 122, about 6% of the total long-term 35 S-labeled lytic T was complexed to p53. This T-p53 fraction was the most ATPase active form of T and accounted for 30 to 40% of the ATPase activity. T reacted with PAb 100, 103, 104, 106, and 114 had altered ATPase activity and altered amounts of complexed p53 compared to total T (Table 1). Thus, several region III antibodies bound forms of T altered for both p53 binding and ATPase activity. That the same structural determinants in T are affected suggests a correspondence between these two activities. That the most ATPase-active fraction of T is bound to p53 is also consistent with a relationship between p53 binding and ATPase activity. Other region III antibodies (PAb 414 and 205) appear to inhibit the in vitro association of p53 with SV40 T, while another (PAb 250, which recognizes the upstream end of the ATPase site) binds poorly to T-p53 complexes (19; S. Mole, J. Gannon, M. Ford, and D. P. Lane, *Structure and Function of SV40 Large T Antigen*, in press). It is possible that complexed p53 inhibits the reactivity of some of these PAb with their corresponding epitopes, but there may be other explanations. For example, some of these antibodies may prefer to bind newly synthesized T, which is known to be depleted in bound p53; p53 preferentially binds to mature forms of T (3). Studies with deletion and point mutants of SV40 T are also consistent with a relationship between the ATPase domain and p53 binding (5, 26; J. Pipas, personal communication). Together, these results indicate that sequences within and flanking the ATPase domain are important for interaction with p53.

Inhibition of ATPase activity by two new region III antibodies. PAb 104 and 114 inhibited ATPase activity about three- to fivefold when added to T, apparently by decreasing the V_{max} . These two antibodies appear to recognize a different epitope(s) than PAb 204, another region III-specific monoclonal anti-T previously isolated by Lane and co-workers that inhibits ATP hydrolysis (4). PAb 204 recognizes a determinant located at residues 453–469, very near the recA-homologous nucleotide-binding site at residue 432, and also prefers to bind T lacking p53 (Mole et al., in press). The PAb 204 epitope is SDS resistant, while the PAb 104 and 114 epitopes are both SDS sensitive (4, 15).

Different forms of ATPase-active T. Quantification of the ATPase activity associated with mixed and sequentially immunoprecipitated T fractions indicated different ATPase-active fractions of T. The most likely interpretation of our results is that there are at least two forms of T with enhanced ATPase activity (Fig. 6). The most active is T complexed with p53, recognized by PAb 122. This form may also preferentially react with PAb 100. The other form of T is a small subclass that is depleted in p53 and reactive with PAb 104 and 114. This fraction may also react with PAb 103 and 106. PAb 104 and 114 reacted with ATPase-active T and inhibited activity; thus, they are inactivating antibodies. They presumably interact directly with elements contained within the active site of T or alter a structural determinant in region III that is important for the integrity of the enzymatic site. That a subclass of T without p53 is also preferentially

FIG. 6. Different forms of ATPase-active T recognized by different antibodies. The width of the arrow indicates the relative preference of a specific antibody for a given form of T or T-p53.

ATPase active rules out bound p53 as the only cause of enhanced ATPase activity. A third form of T appears to be significantly less ATPase active but was not specifically recognized by any of the antibodies and was inferred from sequential and mixed immunoprecipitations (see Results). There was no evidence for an inactive T-p53 complex, but it remains a possibility.

Relation of adenylation of T to ATPase activity. Essentially all enhanced ATPase-active forms of T were preferentially adenylylated. T complexed with p53, the most ATPase-active fraction, was the most adenylylated compared with total T. Preferential *in vitro* adenylation of immune-complexed T was also associated with the same ATPase-active forms of T recognized by certain region III-specific antibodies. Not only are the adenylation and ATPase sites, both associated with replicative function, located in the same region of T (Fig. 1), they appear to affect the same antigenic determinants. Some of these subclasses of T precipitated by region III antibodies are depleted in p53; another is enriched. Thus, adenylation corresponds best with preferentially ATPase-active forms of T, regardless of bound p53. p53 itself was adenylylated under these conditions.

Reactivity of midregion III is most altered in functionally active forms of T. All antibodies directed against both the N-terminal (region I) and all but one of the C-terminal determinants (region IV) in free T reacted as, or nearly as, efficiently with T as polyclonal hamster tumor serum. However, all midregion III antibodies (PABs 100, 102, 103, 104, 106, and 114) were significantly less reactive than tumor serum, as seen previously with a subset of these antibodies (36). The predominant form of free T in infected cell extracts has a middle region that appears poorly reactive, while both termini are highly reactive with their specific corresponding antibodies. Based on the results of the functional assays presented in this paper, this decrease in reactivity appears to be due to the ability of these antibodies to recognize structurally and functionally distinct subclasses of T. PABs 104 and 114 were significantly less reactive with 16-h-labeled T than previously observed for 2-h-labeled extracts (15), suggesting they prefer newer forms of T. Previously, we have shown that region III is altered when T is bound to replicating SV40 chromosomes; however, while T bound to mature chromosomes has several unreactive determinants compared with T bound to replicating DNA complexes, PAB 104 reacts with both chromosomal forms (see reference 36). This suggests that T bound to both forms of chromatin has ATPase activity.

Most of region III is sensitive to both SDS and thermal

denaturation (6, 15); thus, specific antigenic sites within this region could affect the overall structure of T rather than directly interacting with the ATPase active site. The oligomerization of T may also affect its reactivity with a given antibody since T from lytically infected cells is predominantly tetrameric (14 to 16S) in structure (1, 11). Monomer units of T within this complex may be arranged such that a particular region is shielded resulting in poor reactivity with a corresponding antibody. It is also possible that all four potential binding sites are not equally reactive with a given antibody; however, as long as one is accessible, the tetrameric protein could bind efficiently to the antibody.

Summary. That T-p53 complexes are preferentially ATPase active and adenylylated, activities that are both associated with SV40 replication, is significant. This observation suggests a role for T-p53 in productive infection, perhaps in regulating ATPase activity. Bound p53 may protect enhanced ATPase-active forms of T with increasing age or oligomerization of the T. The significance of p53 adenylation is unclear. A small fraction of T is adenylylated in cells, but a similarly small fraction of the total T in cells is bound to replicating SV40 chromosomes (36). We are further characterizing the reactivity of these antibodies toward other forms of T and T-p53 complexes of different age and degree of oligomerization. We are also measuring the reactivity of specific mutant T antigens toward different antibodies and comparing the results with that of wild-type T. In this way, we can determine whether a localized alteration or a broad conformational change is responsible for particular defects in T functions and better characterize the structural and functional correlates of the various forms of T present in lytically infected cells.

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