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Stoichiometry of Large T Antigen and pp53 in Complexes Isolated from Simian Virus 40-Transformed Rat Cells

MARTIN I. FREED, IRA LUBIN, AND DANIEL T. SIMMONS*

School of Life and Health Sciences, University of Delaware, Newark, Delaware 19711

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Simian virus 40-transformed cells synthesize high-molecular-weight protein complexes (22 to 30S) that consist of the virus-coded large T antigen (81,500 daltons) and the cellular antigen pp53. These complexes were partially purified from lysates of transformed rat cells by sucrose velocity sedimentation. The stoichiometry of the two proteins in the complex was studied by direct enzyme-linked immunosorbent assays, using alkaline phosphatase-conjugated anti-T and anti-pp53 monoclonal antibodies. The results from these experiments indicate that the T antigen-to-pp53 ratio in the complex is 0.87 ± 0.27. No statistically significant differences were found in this ratio for faster- and slower-sedimenting complexes. These results from enzyme-linked immunosorbent assays and previous molecular weight estimates of the complex suggest that this complex is composed, on the average, of four molecules of T antigen and four or five molecules of pp53.

Cells transformed with simian virus 40 (SV40) contain a protein complex made up of the virus-coded T antigen and a cellular phosphoprotein named pp53 (12, 16). This complex sediments at 22 to 30S (7, 16) and appears to be resistant to heat, partial reduction (9, 12), high salt concentrations (9, 15), and incubation with chelating agents and nonionic detergents (9). The cellular pp53 protein has a molecular weight of 50,000 (50K) to 56K (depending upon the species from which it is isolated) and was originally detected in lysates of SV40-transformed cells by immunoprecipitation with hamster anti-T serum (3, 11–13, 17, 24). Cells infected with SV40 (9, 17, 23) and some lines of normal, uninfected cells (5, 13, 22, 24) also accumulate pp53, although the levels of the protein are generally lower than in transformed cells. Substantial amounts of the protein are made in cells transformed with other papovaviruses (12, 22), adenovirus (21), retroviruses (4, 20), or chemicals (4). Interestingly, the adenovirus tumor antigen (E_{1b} product) (21) binds to pp53 in cells transformed by these viruses. In SV40-transformed and -infected cells, nearly all of the pp53 is complexed with T antigen (7–9, 16), and in some transformed cell lines that produce large amounts of pp53, nearly all of the T antigen is present in this complex (7; this report).

The function of the complex in the cell is not known, and little is known about its structure. In previous work, it has not been possible to obtain a reliable estimate of the ratio of T antigen to pp53 molecules in this aggregate (7, 9, 15, 16). In the present study, we examined the stoichiometry of the two proteins in the complex by direct enzyme-linked immunosorbent assays (ELISAs). We chose to use ELISAs because this method is very sensitive and gives accurate estimates of antigen (and antibody) concentrations (25). Furthermore, it circumvents the difficulties inherent in estimating the levels of these two proteins in the cell from labeling experiments (1).

It was first necessary to find a line of SV40-transformed cells in which the majority of the T antigen and pp53 are complexed to one another. Figure 1 shows that this is the case for a line of transformed rat cells (line 14B). Labeled extracts were incubated with monoclonal antibodies to T antigen (PAb101) or to pp53 (PAb122) (8) followed with fixed *Staphylococcus aureus* to bring down the immune complexes (Fig. 1, lanes a and f, respectively). After the reaction with the first antibody, the cleared supernatants were incubated with either nonimmune medium as a control (Fig. 1, lanes b and g), PAb101 (Fig. 1, lanes c and h), PAb122 (Fig. 1, lanes d and i), or hamster anti-tumor serum (Fig. 1, lanes e and j). The precipitated proteins were subjected to sodium dodecyl sulfate-acrylamide gel electrophoresis (23), and the labeled proteins were detected in the gel by fluorography at −80°C. The X-ray film was scanned with a densitomer to obtain a quantitative estimate of the intensity of each band (data not shown). PAb101 (anti-T) and PAb122 (anti-pp53) precipitated both T antigen and pp53 from the cell extracts (Fig. 1, lanes a and f). Greater than 95% of the pp53 in the extract was initially precipitated with PAb101; a

very small fraction remained in solution and came down with PAb122 (compare Fig. 1, lanes a and d). This indicated that nearly all of the pp53 was complexed to T antigen. Similarly, about 85% of the labeled T antigen in the extract was initially precipitated with PAb122, the remainder (15%) being brought down with anti-tumor serum (compare Fig. 1, lanes f and j). This indicated that a large proportion of the T antigen in this cell line was complexed to pp53. An additional point is that about 90% of the T antigen in the extract was recognized by PAb101 (compare Fig. 1, lanes a and e), showing that this monoclonal antibody binds to most (although not all) of the T antigen molecules, in agreement with the results of Gurney et al. (8).

To carry out the ELISAs, monoclonal antibodies PAb101 and PAb122 were purified from hybridoma medium by protein A-Sepharose (Pharmacia Fine Chemicals) chromatography according to the method described by Ey et al. (6) except that 3 M NaSCN was used to elute the antibodies. PAb101 and PAb122 are of antibody types immunoglobulin G2a and G2b, respectively (Hybridoma Profiles, August 1982, National Cancer Institute). The purified monoclonal antibodies were conjugated to bovine alkaline phosphatase (Sigma Chemical Co.; type VII-T) according to the method of Voller et al. (25). The same amount of each antibody (1 mg) was coupled to 2.5 mg of enzyme (900 U/mg) so that the two conjugated antibodies had similar specific activities. Extracts of SV40-transformed rat cells were subjected to sedimentation through gradients of 5 to 20% sucrose to partially purify the complexes. Gradient fractions were collected, and serial twofold dilutions (starting with 1:4) were made in prechilled disposable, 96-well (flat-bottomed) polystyrene microtiter plates (Linbro) with the coating buffer of Voller et al. (25) as the diluent. The plates were incubated overnight at 4°C and washed three times with buffer A (0.05 M Tris-hydrochloride [pH 7.8], 0.001 M $MgCl_2$, 1% bovine serum albumin, 0.01% NaN_3) (10). After these washes, 200 μl of alkaline phosphatase-conjugated PAb101 or PAb122 immunoglobulin G (1.25 μg of antibody per ml in buffer A) was added to each well. After an overnight incubation at 4°C, the plates were extensively washed with phosphate-buffered saline (0.0027 M KCl, 0.0015 M KH_2PO_4, 0.14 M NaCl, 0.02 M Na_2HPO_4 [pH 7.4]) containing 0.05% Tween 20 (25), and 200 μl of freshly made substrate solution (1 mg of *p*-nitrophenyl phosphate [Sigma] per ml in 9.7% diethanolamine, 0.0049 M $MgCl_2$, 0.02% NaN_3 [pH 9.8]) was added to each well. The plates were incubated in the dark at room temperature, and the absorbance at 405 nm was measured with an MR580 Microelisa Autoreader (Dynatech Instruments). The reactions were allowed to continue until an absorbance of greater than 1.0 was obtained in the most active sample.

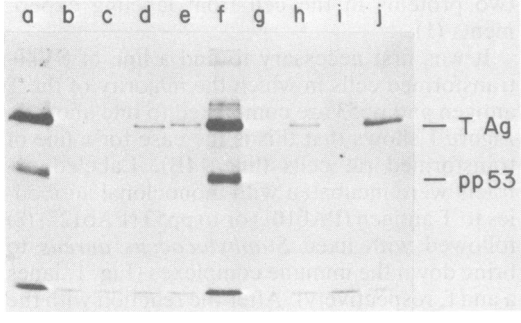

FIG. 1. Immunoprecipitation of T antigen (T Ag) and pp53 from SV40-transformed rat cells. SV40-transformed rat (line 14B) cells were labeled for 3 h with [^{35}S]methionine at a concentration of 20 μCi/ml. The cells were lysed, and immunoprecipitation reactions were carried out in antibody excess essentially as described by Gurney et al. (8). The lysates were first reacted with either PAb101 (lane a) or PAb122 (lane f). The supernatants (lanes b–e for PAb101 and g–j for PAb122) were used in a second immunoprecipitation with nonimmune medium (lanes b and g), PAb101 (lanes c and h), PAb122 (lanes d and i), or hamster anti-tumor serum (lanes e and j). The precipitated proteins were denatured with sodium dodecyl sulfate and applied to a sodium dodecyl sulfate-acrylamide gel (23). The labeled proteins in the gel were detected by fluorography.

Figure 2 shows a plot of enzyme activity versus fraction number for the reactions with anti-T and anti-pp53 antibodies. The two profiles are similar, peaking around fractions 7 or 8. Although the peaks appear to be quite broad, the true antigen distribution is undoubtedly quantitatively different because, under these conditions, a twofold drop in antigen concentration does not result in a twofold drop in activity (see below). The peak fractions correspond to a sedimentation coefficient of 27 to 29S. This value lies within estimates of the sedimentation coefficient of the complex obtained by others (2, 7, 16) and by us (data not shown) as determined by immunoprecipitation reactions of gradient fractions with anti-T and anti-pp53 antibodies. The near coincidence of the anti-T and anti-pp53 reaction profiles in Fig. 2 indicates that the two antigens are similarly distributed in the gradient. Since the majority of the T antigen and pp53 molecules are complexed to one another in this cell line (Fig. 1), it is reasonable to assume that the profiles in Fig. 2 are, for the most part, those for T antigen and pp53 in complexed form.

To determine the molar ratio of T antigen to pp53 in the complex, it was necessary to calculate the relative antigen concentration of these

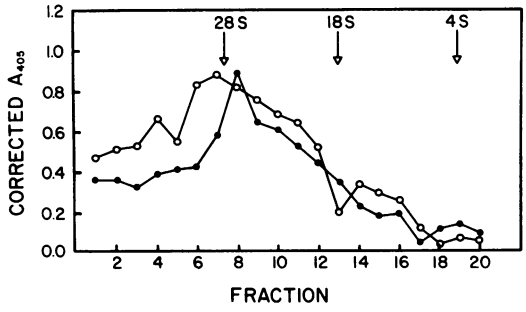

FIG. 2. ELISA of T antigen-pp53 complexes. SV40-transformed rat (line 14B) cells were grown to confluency in roller bottles. The cells were washed three times with ice-cold phosphate-buffered saline and lysed in 4 ml of 0.01 M Tris-hydrochloride (pH 9.0)–0.14 M NaCl–0.001 M dithiothreitol–0.5% Nonidet P-40. The lysate was clarified by centrifugation at 4°C for 10 min at 16,000 × *g* and concentrated to 0.6 ml by dialysis against dry Sephadex beads at 4°C. The concentrate was layered on a 5 to 20% sucrose gradient in 0.01 M Tris-hydrochloride (pH 7.4)–0.14 M NaCl–0.001 M dithiothreitol and centrifuged at 21,200 rpm for 15 h at 4°C in a Beckman SW41 rotor. Fractions (0.6 ml) were collected in the cold and assayed with enzyme-conjugated anti-T (●) and anti-pp53 (○) antibodies as described in the text. The plotted absorbances at 405 nm (A_{405}) were derived from assays of a 1:8 dilution of each fraction and are corrected by the plate reader for extraneous noise due to dirt or occlusions on the plastic. Sedimentation is from right to left, and the position of HeLa cell rRNAs is noted at the top.

two proteins in various fractions of the gradient. These values were estimated by plotting absorbance (enzyme activity) versus antigen dilution for the fractions (Fractions 6 through 11) containing most of the complex (Fig. 3). These curves describe the reactions of T antigen and pp53 with their corresponding monoclonal antibodies and, for most of these fractions, are sigmoidal in shape. To estimate relative antigen concentrations, the point on the curve that is most sensitive to changes in antigen concentration was determined. This point is called the 50% point and can be used as an estimate of antigen concentration. It is defined as that point which is halfway between the reaction plateau values at high and low antigen concentrations. The low plateau values were obtained directly from the figure. The high plateau values were estimated by extrapolation of the curves shown in Fig. 3. These extrapolations were somewhat difficult for fraction 9 and are likely to be slightly different from the true values. However, the anti-T and anti-pp53 reaction curves for this fraction are nearly superimposable, and therefore their plateau values are probably very similar. Table 1 lists the relative antigen concentrations of both T antigen and pp53 at the calculated 50% point for each of fractions 6 through 11 of the complex peak. The ratio of these two values is also shown in Table 1 and represents the deduced T antigen-to-pp53 ratio in each of these six fractions. The average antigen ratio (and hence molar ratio since the antibodies are monoclonal) is 0.87 ± 0.27. Extending these calculations for the fractions beyond 6 and 11 had little effect on the average molar ratio. Since no consistent differences were observed between the calculated molar ratios in different fractions, it appears that sedimentation did not separate complexes with different relative amounts of T antigen and pp53.

In determining the accuracy of the calculated T antigen-to-pp53 ratio, we should consider that some of the anti-T activity (up to 15%) shown in Fig. 2 may be due to a reaction with small amounts of uncomplexed T antigen (Fig. 1) present in fractions 6 through 11. This point alone might indicate that the calculated ratio is too high. However, not all (about 90%) of the T antigen molecules are recognized by PAb101 (see above), and consequently the T antigen concentration measured by ELISA is somewhat lower than it should be. This would have an approximately equal effect in the opposite direction, and therefore we think that a value of 0.87 ± 0.27 is reasonably accurate for this ratio. The simplest interpretation of these data is that T antigen and pp53 are, on the average, present in approximately a one-to-one molar ratio in the complex. This interpretation does assume, however, that the two proteins of the complex are, on the average, equally available for antibody binding. This assumption is not unreasonable because PAb101 and PAb122 precipitate the complex with about equal efficiency (unpublished data).

It is possible to obtain an independent estimate of the composition of the complex from published data. Bradley et al. (2) determined the molecular weights of monomers, dimers, and tetramers of T antigen by gel exclusion chromatography. The values they obtained corresponded, within ±10%, to the values estimated

TABLE 1. Calculation of T antigen/pp53 ratios

Fraction no.	Relative antigen concn		
	T antigen	pp53	T antigen/pp53[a]
6	1/28	1/24	0.85
7	1/27	1/30	1.11
8	1/22	1/20.5	0.93
9	1/16	1/12	0.75
10	1/46	1/19.5	0.42
11	1/23.5	1/27.5	1.17

[a] Average = 0.87 ± 0.27.

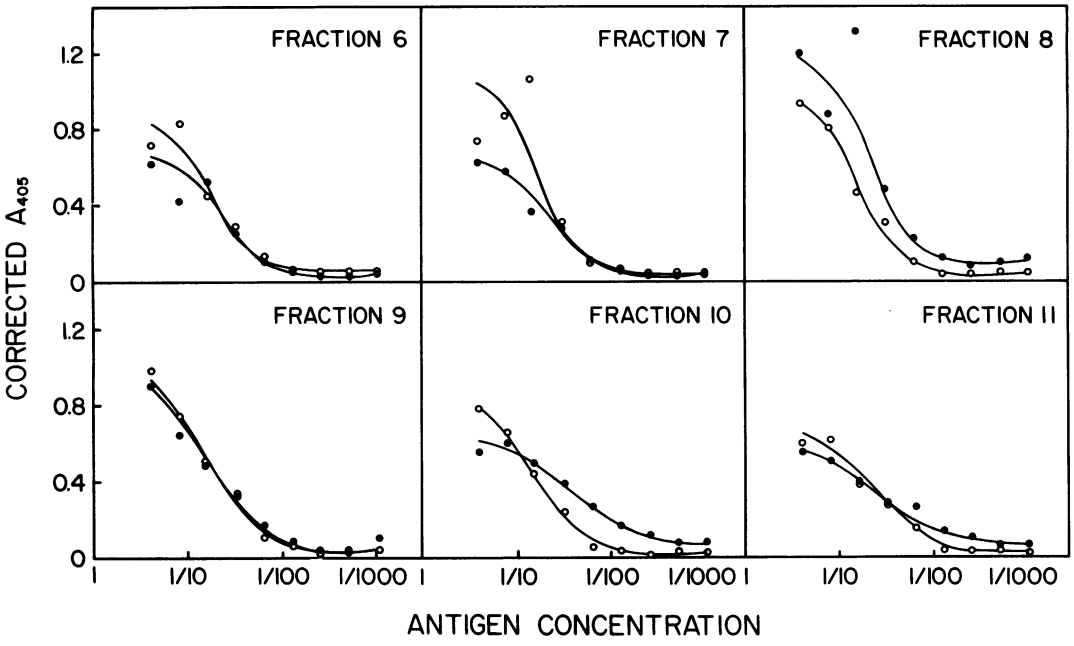

FIG. 3. Antigen dilution curves of ELISAs of T antigen-pp53 complexes. Cell lysates were subjected to sedimentation in sucrose gradients as described in the legend to Fig. 1. Fractions were collected and tested by ELISAs with monoclonal anti-T (●) and anti-pp53 (○) antibodies as described in the text. Corrected absorbances at 405 nm (A_{405}) were plotted versus antigen concentration for fractions 6 through 11 of the gradient.

directly from sedimentation coefficients. With their values for the sedimentation coefficients of the T antigen-pp53 complex and of the T antigen tetramer, the relationship described by Martin and Ames (14) gives a calculated molecular weight of 560K ± 10% for the complex. This calculation makes the assumption that the molecular weight values of the complex as determined by gel exclusion and by sedimentation are within 10% of one another. Second, it seems likely that the complex contains a tetramer of T antigen, because Meyers et al. (18) have reported that the tetrameric form of the T antigen analog, D_2T, preferentially binds, in vitro, to SV40 DNA at the origin of replication, and Reich and Levine (19) recently have shown that the T antigen-pp53 complex displays the same SV40 DNA-binding specificity as uncomplexed (without pp53) T antigen. Consequently, a complex with an approximate molecular weight of 560K and tetrameric T antigen component might be expected to contain four or five molecules of pp53 (based on molecular weights of 81.5K for T antigen and 53K for pp53).

The deductions made above about the composition of the complex and our own estimates derived from ELISA results are entirely consistent with one another. That four molecules of pp53 are associated with the protein complex is supported by observations of McCormick et al. (15) which indicate that the pp53 isolated from F9 embryonal carcinoma cells is in a tetrameric form and binds to purified T antigen in vitro. However, a complex consisting of four molecules of T antigen and five molecules of pp53 cannot be excluded. Although not enough information is available to predict the actual arrangement of the individual protein subunits, additional information on the structure of the complex might provide insights into its function in the cell and its possible role in cellular transformation with SV40 and other papovaviruses.

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