

NOTES

Complete Interaction of Cellular 56,000- and 32,000- M_r Proteins with Simian Virus 40 Small-t Antigen in Productively Infected Cells

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Received 25 July 1986/Accepted 16 December 1986

Two cellular proteins are found to be complexed with simian virus 40 small-t antigen in cellular extracts. The complex is a relatively unstable but dynamic one which can dissociate and reform in extracts. In extracts of permissive monkey kidney cells, the small-t antigen appeared to be present in excess, whereas the cellular proteins were nearly entirely committed to the complex in permissive monkey kidney cells.

The small-t antigen of simian virus 40 (SV40) interacts with two cellular proteins that have apparent molecular weights of 56,000 and 32,000 (56K and 32K proteins, respectively) (18). The 56K and 32K proteins are present in immunoprecipitates of wild-type (WT)-SV40-infected cells but not in immunoprecipitates of extracts prepared from cells infected with small-t deletion mutants (16). However, the two proteins can be detected in uninfected- or mutant-infected-cell extracts. When unlabeled extracts that contain small t or partially purified fractions of small t (1) are incubated with radiolabeled uninfected-cell extracts, complexes form and the labeled cellular proteins can be coimmunoprecipitated by antitumor serum. The existence of such a complex in tumor-bearing animals has been suggested by the observation that some tumor-bearing animals induce antibodies that react directly with the cellular proteins in addition to the viral large-T and small-t antigens (14).

56K and 32K proteins which form complexes with small-t antigen have been detected in cells of a variety of mammalian species and are present in uninfected cells at levels that are comparable to those detected after infection (11). In addition, early proteins of other papovaviruses interact with the same two cellular proteins (13), and these proteins are likely to be two of the five proteins that are routinely observed by immunoprecipitation with polyomavirus antitumor sera (15).

The complex between small-t antigen and the two cellular proteins is a relatively unstable one which was disrupted by low concentrations of sodium dodecyl sulfate (SDS). When extracts were adjusted to contain 0.1% SDS in addition to the 0.5% Nonidet P-40 already present in the extract (Fig. 1, lane 1), the 56K and 32K proteins were not coimmunoprecipitated with small-t antigen even though the immunoprecipitation of large-T and small-t antigens was unaffected. These concentrations of detergent are equivalent to those in the commonly used RIPA buffer (4). Similar results were obtained with the detergents Empigen BB and octyl glucoside (data not shown). Interestingly, although the complex was disrupted by 0.1% SDS, immune complexes could be washed with RIPA buffer without loss of the cellular proteins, suggesting that antibody binding can stabilize the complex (data not shown). In contrast to the sensitivity of the complex to detergents, the complex was stable in salt concentrations as high as 500 mM (Fig. 1, lane 3).

The complex was also disrupted by monoclonal antibodies that recognize small-t antigen (5). Immunoprecipitates of extracts treated with pAB419 (Fig. 1, lane 4) contained large-T and small-t antigens in addition to the cellular 30K protein recognized by this monoclonal antibody. The 56K and 32K proteins were not present. The possibility that pAB419 recognized only a subset of small-t antigen was ruled out by sequential precipitations. After two rounds of precipitation with pAB419, the supernatant was incubated with hamster antitumor serum. This resulted in precipitation of trace amounts of the viral antigens, but not the cellular 56K and 32K proteins (lane 6), indicating that the complex had been disrupted by interaction of small t with pAB419 antibodies. As a control, similar sequential precipitations were performed with pAB421, a monoclonal antibody to p53 (Fig. 1, lanes 7 through 9). After two rounds of precipitation with this antibody, hamster antitumor serum was able to cause precipitation of small-t antigen and the associated cellular proteins. Thus, the complex was not disrupted simply by the manipulations involved in antibody clearance experiments. It is important to note that individual antitumor sera have varied in their ability to precipitate the complex, probably depending on dominant small-t epitopes they contain. Pooled antitumor sera have often been inefficient in precipitation of the complex, and best results have been obtained by screening individual serum samples.

In infected monkey kidney (CV-1) cells, only a portion of the total small-t antigen was involved in the complex, whereas nearly all of the cellular 56K and 32K proteins were present in complexed form. Small t was shown to be at least partially free by DEAE-cellulose chromatography (Fig. 2). When extracts of infected cells were applied to DEAE-cellulose, part of the small-t antigen did not bind to the column. This unbound portion varied from 30 to 50% in individual experiments. The remaining small-t antigen bound to the column, as did the cellular 56K and 32K proteins, but they were eluted with 300 mM NaCl and were immunoprecipitated together from the high-salt buffer used. The three proteins coeluted when linear gradients of NaCl were used (data not shown).

The behavior of the small-t antigen on DEAE-cellulose suggests that small-t antigen may bind DEAE as a consequence of its interaction with the cellular proteins. This is consistent with other observations. For example, during

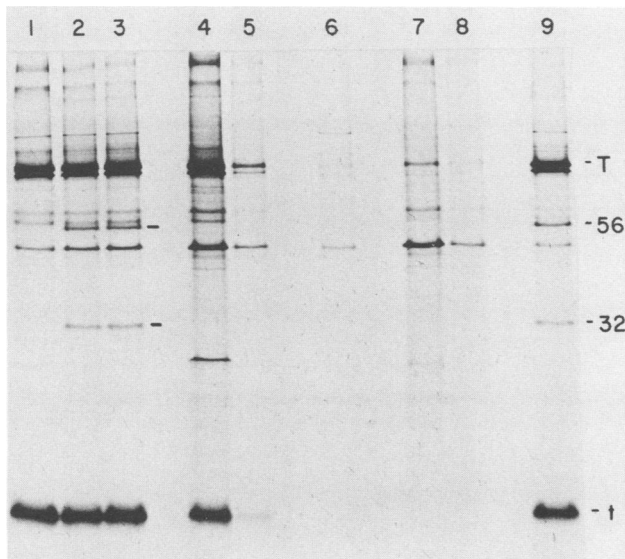


FIG. 1. Disruption of the complex by various agents. Confluent CV-1 cells were infected with WT SV40 at a multiplicity of 10 PFU per cell. Infected cultures were incubated for 24 h and then pulsed overnight with [35 S]methionine. The cells were extracted in Trizma-buffered saline (pH 8) containing 0.5% Nonidet P-40, as described previously (18). Portions of the extract were adjusted to contain either 0.1% SDS or 500 mM NaCl by the addition of 10% SDS or 5 M NaCl from stocks; the extracts were then immunoprecipitated with antitumor serum (anti-t serum). Shown are SDS gels of the proteins immunoprecipitated from extracts in the presence of 0.1% SDS (lane 1) or 500 mM NaCl (lane 2). The precipitate from the unaltered extract is shown in lane 3. Portions of the same extract were used for sequential precipitation with monoclonal antibody pAB419 (anti-T,t; lanes 4 through 6) or pAB421 (anti-p53; lanes 7 through 9). The initial precipitate is shown in lanes 4 and 7. Supernatants from the first precipitation were reprecipitated with the same monoclonal antibodies (lanes 5 and 8). Supernatants from the second precipitation were then precipitated with anti-t serum (lanes 6 and 9).

purification of small t from bacterial clones that express this protein, it was observed that small t was highly aggregated to many bacterial proteins. The aggregated protein could be removed by DEAE-cellulose chromatography, and small-t antigen that did not bind DEAE was shown to be monomeric by gel filtration (1). The monomeric fraction of small t was active in interaction with cellular 56K and 32K proteins. Similarly, monomeric small-t antigen isolated from G200 columns was completely unable to bind DEAE under the conditions used (data not shown).

After DEAE-cellulose chromatography, both bound and unbound fractions of small-t antigen remained active in their ability to interact with additional cellular 56K and 32K proteins. Unlabeled extracts of infected cells were applied to DEAE-cellulose to obtain unbound and salt-eluted fractions (Fig. 2, lanes 5 through 7). After incubation of each small t-containing fraction with radiolabeled extracts of uninfected cells, 56K and 32K proteins were coimmunoprecipitated with antitumor serum that recognized small t. Thus, the small-t antigen present in the unbound fraction represented an active subset of small t which was free of the cellular proteins.

Both newly synthesized and preexisting molecules of small-t antigen showed the same behavior on DEAE-cellulose columns (data not shown). In pulse-chase experiments with chase periods as long as 6 h, at least half the total

small-t antigen bound DEAE. This suggests that the cellular proteins are not simply involved in translocation of newly made small-t antigen in the cell, a situation described for the *src* protein and associated cellular proteins (3). It has not been possible to address this question by using sucrose gradient sedimentation because most of the small t shows more rapid sedimentation than do the cellular 56K and 32K proteins. Similarly, gel filtration chromatography (G200) has not been useful for examining the complex. The large dilutions involved in these columns appear to result in dissociation of the complex, and small-t antigen included in these columns behaved as a monomeric protein, whereas the cellular proteins fractionated together in the 90K to 100K region of the column.

The DEAE-cellulose patterns suggest that small t is present in excess over the 56K and 32K proteins in infected CV-1 cells. However, this interpretation requires that there is not a pool of free 56K and 32K proteins present in the cells. To address this question, advantage was taken of the observation that, after incubation of cell extracts with [3 Hmethyl]S-adenosylmethionine (SAM), the 32K protein was a prominent labeled product (Fig. 3, lane 1). Although a WT-infected-cell extract is shown, labeled 32K protein was also present in extracts of uninfected or mutant-infected

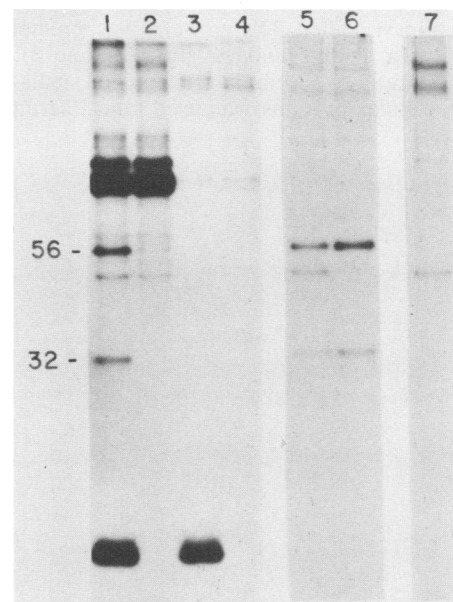


FIG. 2. DEAE-cellulose chromatography of small t and associated cellular proteins. Confluent CV-1 cells were infected with either WT SV40 or the mutant DL-888, incubated for 24 h, and then pulsed overnight with [35 S]methionine. The cells were extracted in 20 mM Tris hydrochloride (pH 8)–80 mM NaCl–2 mM dithiothreitol–0.5% Nonidet P-40. The extracts were applied to DEAE-cellulose columns (1 by 3 cm) and washed with the same buffer. Bound proteins were eluted with buffer containing 300 mM NaCl. Unbound and bound protein fractions were immunoprecipitated with anti-t serum. Shown are SDS gels of the immunoprecipitates of WT DEAE-bound proteins (lane 1), DL-888 DEAE-bound proteins (lane 2), WT unbound proteins (lane 3), and DL-888 unbound proteins (lane 4). A mixing experiment is shown in lanes 5 through 7. Unlabeled extracts of WT- or DL-888-infected cells were fractionated on DEAE-cellulose as described above, added to [35 S]methionine-labeled extracts of uninfected cells, and incubated at 35°C for 15 min before immunoprecipitation. The patterns shown are for proteins that were coimmunoprecipitated with WT DEAE-bound proteins (lane 5), WT unbound proteins (lane 6), and DL-888 unbound proteins (lane 7).

cells. However, the 32K protein was present in immunoprecipitates of only the WT-infected-cell extracts (lanes 3 through 5). Detection of the 32K protein in whole extracts was quite surprising because the 32K protein is a minor component of the cell extract and has never been detected clearly by one-dimensional gel electrophoresis of total cell extracts. The nature of the modification of the 32K protein by SAM was not determined. After acid hydrolysis of the gel-purified labeled protein, most of the radioactivity was lost as volatile material, inconsistent with the presence of one of the commonly methylated amino acids (lysine, histidine, or arginine) which are acid stable (8). These amino acids have not been detected by two-dimensional thin-layer chromatography or with the amino acid analyzer. In addition, the modification does not appear to be a carboxymethylation of aspartic or glutamic acid. The modification was stable at room temperature at pH 11 even in the absence of any detergents (data not shown), although similar incubations of carboxymethylated proteins result in complete removal of the methyl group with the formation of methanol (16). Although the nature of the modification remains unknown, labeling with SAM provided a useful tool for monitoring the 32K protein in this study as well as in procedures such as column chromatography.

Extracts labeled with SAM were used to monitor the 32K protein in WT-infected-cell extracts before and after immunoprecipitation. Labeled 32K protein was precipitated in association with small-t antigen (Fig. 3, lane 3). In this experiment, antitumor serum that recognized small-t antigen but not the cellular proteins was used (anti-t serum). After

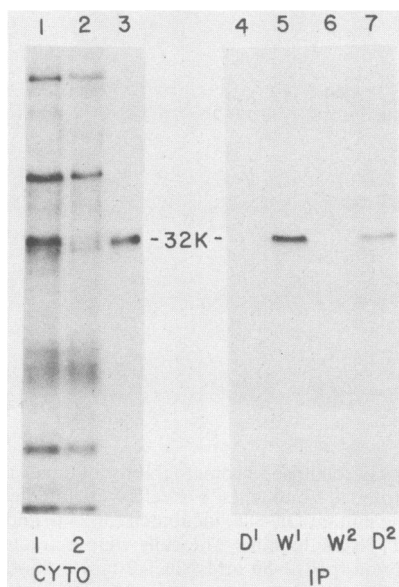


FIG. 3. Labeling of the 32K protein with SAM. Extracts were prepared from unlabeled WT- or DL-888-infected CV-1 cells at 40 h postinfection and then incubated for 20 min at 35°C with [³H]methyl-SAM (70 Ci/mmol, 5 μM). A portion of the labeled WT-infected-cell extract was saved for direct analysis, and then the extracts were immunoprecipitated with anti-t serum. The patterns shown are for the labeled proteins in the WT extract (CYTO) (lane 1), the proteins remaining in the supernatant after precipitation (lane 2), the initial WT precipitate (IP) (lanes 3 and 5), and the initial DL-888 precipitate (lane 4). After the initial precipitation with anti-t serum, the supernatants were reprecipitated with anti-56K,32K serum. The proteins remaining in the WT supernatant are shown in lane 6, and those remaining in the DL-888 supernatant are shown in lane 7.

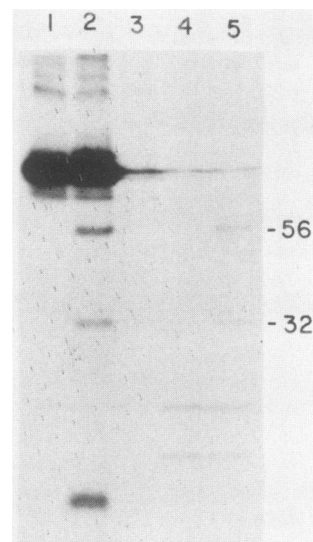


FIG. 4. Clearance of 56K and 32K proteins by coimmunoprecipitation with small-t antigen. CV-1 cells were infected with either WT SV40 or DL-888 for 24 h and labeled overnight with ³H-amino acids. Extracts were then precipitated with anti-t serum. Supernatants from the initial precipitation were divided for secondary precipitation with either anti-t serum or anti-56K,32K serum. The patterns shown are for the initial DL-888 immunoprecipitate (lane 1), the initial WT immunoprecipitate (lane 2), the secondary precipitation of the WT supernatant with anti-t serum (lane 3), the secondary precipitation of the WT supernatant with anti-56K,32K serum (lane 4); and the secondary precipitation of the DL-888 supernatant with anti-56K,32K serum (lane 5).

this precipitation, little 32K protein could be detected in the supernatant (lane 2), suggesting that nearly all of this cellular protein was associated with small-t antigen in extracts. In a parallel approach, extracts were precipitated with anti-t serum and proteins remaining in solution after this initial precipitation were reprecipitated with anti-56K,32K serum (14). Essentially all the SAM-labeled 32K protein was removed from WT-infected-cell extracts by initial precipitation with anti-t serum (Fig. 3, lane 5). Supernatants from this precipitation contained nearly undetectable quantities of the 32K protein, as determined by precipitation with anti-56K,32K serum (Fig. 3, lane 6). In contrast, no labeled 32K protein was observed after initial immunoprecipitations of extracts from mutant-infected cells (lane 4), but the 32K protein was detected when supernatants from the initial precipitation were precipitated with anti-56K,32K serum (lane 7).

A similar experiment was performed with cells labeled metabolically with ³H-amino acids (Fig. 4). Again, immunoprecipitation of WT-infected-cell extracts with anti-t serum removed all the 56K and 32K proteins from the extract (lane 2), although the same antiserum did not cause the precipitation of these proteins from mutant-infected-cell extracts (lane 1). Supernatants from the first round of immunoprecipitation were then used for precipitation with additional anti-t serum (lane 3) or with anti-56K,32K serum (lanes 4 and 5). The 56K and 32K proteins were detected in supernatants of the mutant-infected-cell extract but not in those of the WT-infected-cell extract.

The gels shown in Fig. 4 were used to quantitate the viral and cellular proteins by excision of the bands for counting in a scintillation counter. The total amounts of each protein

were estimated by comparing the radioactivity in each protein to the specific activity of the total extract protein (counts per minute per microgram of protein as determined by the method of Lowry et al. [6]). Large-T antigen, the 56K protein, the 32K protein, and small-t antigen accounted for 0.49, 0.017, 0.011, and 0.037% of the total cell protein, respectively. In addition, when appropriate adjustments were made for the molecular weights of the proteins, it was apparent that the 56K and 32K proteins were present in approximately equimolar amounts in the immunoprecipitates, whereas small-t antigen was present in excess (three- to fourfold).

The two cellular proteins appeared consistently in the same relative ratios both in infected- and uninfected-cell extracts. This was observed both by coprecipitation with added small-t antigen and by direct precipitation with anti-56K,32K serum. It is of interest in this regard that the two cellular proteins cofractionate in gel filtration columns, even in extracts from uninfected cells, with an apparent molecular weight of 100K. Thus, the two proteins may normally interact with each other in cells.

In summary, in permissive infections of CV-1 cells, small-t antigen was present in excess over the 56K and 32K proteins, which were nearly completely committed to the complex. Interaction of the cellular proteins with small t could either activate or inhibit an activity of these proteins. If small t functions to block the activity of these proteins, sufficient amounts of small-t antigen are present, at least in CV-1 cells, to effect this. The multiplicities of infection used in these experiments (5 to 10 PFU per cell) are the same as those required for small-t antigen to overcome the growth arrest of CV-1 cells by theophylline and other methylxanthines (9, 12). Therefore, it is possible that the interaction of cellular proteins with small t may play a role in this cellular alteration.

The relative amounts of small-t antigen and the two cellular proteins are not known for nonpermissive infections, although it is clear that less small-t antigen is produced. Thus, it might be of interest to determine whether the amounts of small t needed to promote anchorage-independent growth (2, 7, 17) or microcolony formation (10) correlate with the amounts needed to interact with the cellular 56K and 32K proteins. The high multiplicities of virus needed to demonstrate these effects of small-t antigen suggest that complete interaction of the cellular proteins with small-t antigen may be required. However, determination of the role these cellular proteins play in the uninfected cell will be needed for final interpretation of the significance of this complex to SV40 infection.

This investigation was supported by Public Health Service grant CA21327 from the National Cancer Institute.

The technical assistance of Karen Mayer and Bansi Joshi is gratefully acknowledged.

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