Interaction of Simian Virus 40 Small-T Antigen Produced in Bacteria with 56K and 32K Proteins of Animal Cells

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Small-t antigen produced in bacteria interacted with two animal cell proteins with molecular weights of 56,000 and 32,000, as did the viral antigen from infected cells. Demonstration of this specific interaction required the enrichment of native, monomeric small-t antigen from extracts in which much of the small-t antigen was highly aggregated.

The small-t antigen of simian virus 40 appears to have a function associated with cellular growth control in both permissive and nonpermissive infections. It plays a role in the efficient transformation of some growth-arrested cell lines (2, 5, 7). In primary baby mouse kidney cells, small-t antigen has been reported to promote continued cell cycling induced after infection (4). In a related system, small-t antigen has been shown to allow CV-1 monkey kidney cells to escape from a growth-arresting block imposed by theophylline and other methyl xanthine derivatives (6).

The mechanism through which small-t antigen affects arrested cells is unknown but may be related to its ability to cause actin cable disorganization (3) or to interact specifically with two animal cell proteins, 56K and 32K (10). We monitored the interaction of small-t antigen with these proteins as an indication of the functionality of the viral antigen in partial purifications and in studies of small-t antigen expressed by bacterial clones. We report here that small-t antigen produced by bacterial clones can interact specifically with 56K and 32K proteins of monkey kidney CV1 cells and that this is therefore an intrinsic property of small-t antigen. Partial purification of small-t antigen in native form was crucial for the detection of this property.

We used bacterial clone HP1 (9) for studies of non-denatured small-t antigen produced in bacteria. As originally described by workers in Tjian's laboratory, HP1 produces small-t antigen as approximately 0.2% of the bacterial protein under the control of a *lac* promoter. Although the protein can be extracted from bacterial cultures in soluble form, much of the protein is in a highly aggregated, high-molecular-weight form. Monomeric small-t antigen can be isolated by gel filtration, but a rapid partial separation of aggregated and monomeric small-t antigen can be achieved by DEAE-cellulose chromatography. In the experiment shown in Fig. 1A, extracts of HP1 prepared by Nonidet P-40 lysis of lysozyme-EDTA-treated bacteria were applied to DEAE-cellulose in the presence of 80 mM NaCl at pH 8. Approximately 20% of the bacterial protein did not bind DEAE, and about 10% of the small-t antigen was identified in this fraction by immunoprecipitation. The percentage of small-t antigen which did not bind DEAE varied somewhat (10 to 30%) in a series of experiments and may reflect the total level of small-t antigen expressed within a culture.

Immunoprecipitation of small-t antigen from DEAE unbound fractions showed only a low level of coprecipitation of bacterial proteins, which was one indication that most of the small-t antigen was unaggregated. Further indication of this came from Sephadex G200 chromatography, in which most of the total small-t antigen migrated as monomeric protein.

The remaining small-t antigen could be eluted from DEAE with 250 mM NaCl (Fig. 1A, lane 2), and this contained small-t antigen which was highly aggregated. Immunoprecipitation of this fraction resulted in the coprecipitation of large numbers of bacterial proteins. After G200 chromatography of bound proteins, nearly all the small-t antigen was found in the void fractions (data not shown). The heterogeneous behavior of small-t antigen on DEAE-cellulose is not unique to protein produced in bacteria but is characteristic of small-t antigen produced in infected animal cells as well (8). A portion (25 to 30%) of the small-t antigen from infected cells does not bind DEAE, and this fraction is monomeric in nature and is free of the associated 56K and 32K proteins which bind DEAE along with the remaining small-t antigen (unpublished observations).

Another useful rapid chromatography step involves Thiol-Separose (Fig. 1B). Over 80% of the small-t antigen from DEAE unbound fractions bound Thiol-Sepharose and could be eluted with 20 mM dithiothreitol. Only 10% of the total bacterial protein bound this column. Thiol-Sepharose chromatography of unfractionated extracts of HP1 has not been useful because the bacterial proteins to which small-t antigen aggregates bind Thiol-Sepharose and are eluted along with small-t antigen.

By combining DEAE-cellulose, Thiol-Sepharose, and G200 chromatography, substantial purification of small-t antigen was achieved (Fig. 2). In the experiment shown in Fig. 2, small-t antigen was detected only in regions of a G200 column where a myoglobin (17K) marker eluted, and the profiles shown represent total proteins from these fractions, and not immunoprecipitates. Small-t antigen can be concentrated after G200 chromatography with phenyl-Sepharose chromatography and elution with Nonidet P-40. A combination of these four steps provides a scheme which results in substantial enrichment of monomeric small-t antigen within 24 h. By following radioactivity in the small-t protein, we estimated that over 75% of the monomeric protein present in DEAE unbound fractions can be recovered in nondenatured form.
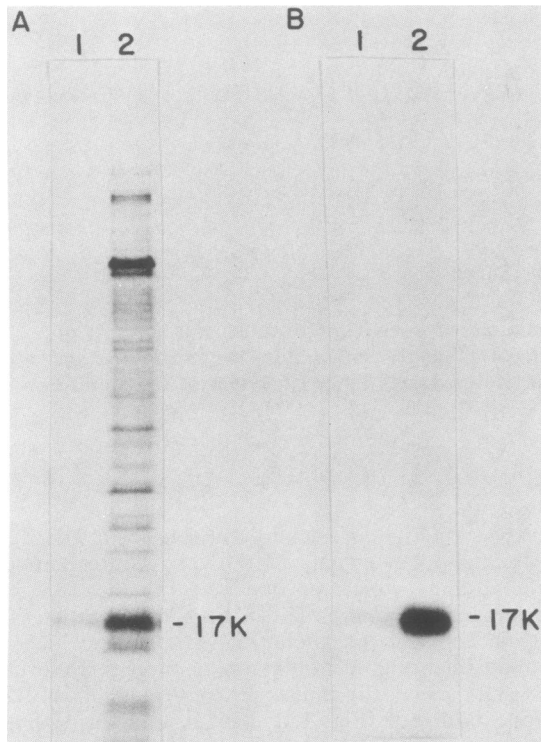
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FIG. 1. DEAE-cellulose and Thiol-Sepharose chromatography. A 5-ml log-phase culture of HP1 was labeled with [^{35}S]methionine and then incubated on ice with lysozyme in the presence of 50 mM EDTA. After 15 min, Nonidet P-40 was added to a final concentration of 0.1% to lyse the bacteria. The extract was adjusted to 80 mM NaCl and then applied to a 2-cm column of DEAE-cellulose (A). After removal of unbound proteins with 20 mM Tris (pH 8) containing 80 mM NaCl, proteins bound to the column were eluted with 250 mM NaCl. Equivalent percentages of the unbound and bound protein fractions were used for immunoprecipitation. Lane 1, Precipitate of proteins that did not bind DEAE; lane 2, precipitate of the eluted proteins. (B) Proteins from fractions that did not bind DEAE were adjusted to 300 mM NaCl and then applied to a 1-cm column of Thiol-Sepharose in the presence of 1 mM EDTA. Unbound proteins were washed from the column with 20 mM Tris (pH 8)–300 mM NaCl–1 mM EDTA. The column was then eluted with 20 mM dithiothreitol in the same buffer. Aliquots of the proteins in the unbound (lane 1) and bound (lane 2) fractions were then immunoprecipitated.

Monomeric small-t antigen from bacterial extracts had the ability to interact with the 56K and 32K proteins from animal cells (Fig. 3). In the experiment shown in Fig. 3, HP1 extracts were applied directly to G200 columns, and pooled fractions were then concentrated with phenyl-Sepharose chromatography. The concentrated fractions were incubated with radioactive extracts of CV-1 cells for 15 min at 35°C and then immunoprecipitated with either antitumor serum that recognized small-t antigen (serum 349) or antitumor serum that recognized only the large-t antigen, but not the small-t antigen (serum 245). The 56K and 32K proteins from CV-1 cells extracts were coimmunoprecipitated only when antiserum 349 was used. Similar results were obtained with small-t antigen after DEAE-cellulose chromatography to remove most of the aggregated small-t antigen. The interaction with the cellular 56K and 32K proteins, however, was more difficult to demonstrate, because aggregates were not

completely removed in this step and lysozyme used to disrupt bacteria was present in the fractions. Both of these factors tended to increase the background precipitation of bacterial proteins and to obscure detection of the 56K and 32K proteins, even when bacterial proteins were not radiolabeled. The use of lysozyme was preferred, however, because other procedures, such as sonication, reduced the solubility of the small-t antigen from bacteria (unpublished observations). Interaction of small-t antigen with the 56K and 32K proteins was also detected with small-t antigen eluted from Thiol-Sepharose and with small-t antigen obtained from a combination of all of these chromatographic steps.

The interaction of small-t antigen with specific proteins is advantageous as a simple assay that can be easily used for a series of column fractions or for testing the function of small-t antigen after a variety of treatments. For example, other bacterial clones which express small-t antigen at a much higher level than HP1 have been used to purify small-t antigen in denatured form (1). It has been reported that, after renaturation by dialysis, these preparations promote actin
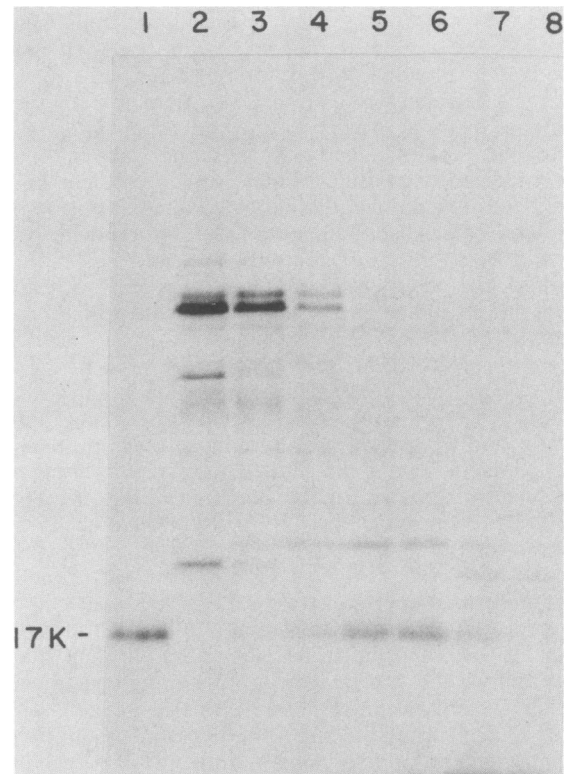

FIG. 2. G200 chromatography of small-t antigen. A 50-ml culture of HP1 was labeled with [^{35}S]methionine and extracted as described in the legend to Fig. 1, and a protein fraction that did not bind DEAE-cellulose was then applied to a Thiol-Sepharose column. The fraction recovered from Thiol-Sepharose by elution with 20 mM dithiothreitol was applied to a G200 column (2.5 by 40 cm) (upward flow). Fractions of 2.5 ml were collected with a flow rate of 15 ml/h. The void volume of the column was 80 ml (fraction 32), and a myoglobin marker (17K) eluted in fractions 68 through 78. Proteins in the fractions shown were precipitated with trichloroacetic acid for analysis on sodium dodecyl sulfate gels. The patterns shown are the total proteins from fractions 61, 65, 69, 73, 75, 81, and 85 (lanes 2–8, respectively). Lane 1, Immunoprecipitate from the Thiol-Sepharose-eluted proteins used as a marker for small-t antigen.
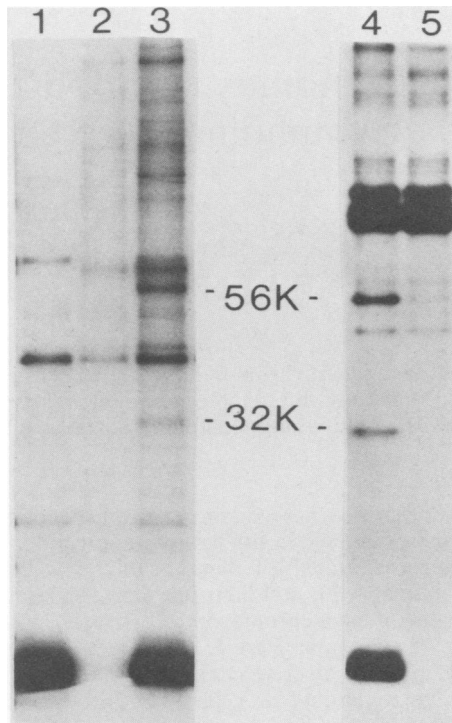
FIG. 3. Coprecipitation of cellular 56K and 32K proteins. A 20-ml culture of HP1 was labeled with [^{35}S]methionine, extracted, and applied to G200 as described in the legend to Fig. 2. Fractions 68 through 80 were pooled, adjusted to 0.8 M NaCl, and applied to a column (1 by 2.5 cm) of phenyl-Sepharose. After a low salt wash, bound proteins were eluted with 1% Nonidet P-40 in 20 mM Tris (pH 8)–50 mM NaCl. Aliquots of the concentrated fractions were either immunoprecipitated directly (lane 1) or mixed with [^{35}S]methionine-labeled extracts of CV1 monkey kidney cells and incubated for 15 min at 37°C before immunoprecipitation (lanes 2 and 3). Antibodies used were antiserum 245 (lane 2) and antiserum 349 (lanes 1 and 3). Antiserum 349 was unable to directly recognize the 56K and 32K proteins, as shown by the absence of these protein in immunoprecipitates of radiolabeled extracts from cells infected with the small-t antigen deletion mutant dl888 (lane 5). Large-T and small-t antigens and the two cellular proteins appear in immunoprecipitates from wild-type infected cells (lane 4).

cable disorganization after microinjection. In contrast, we were unable to retain the ability of small-t antigen to interact with these specific proteins after reversible denaturation, although immunoprecipitable small-t antigen was recovered.

The interaction of small-t antigen with the cellular proteins is an assay that demands that most, if not all, of the small-t antigen participate if the cellular proteins are to be detected. In preparations which contain only a small percentage of active or nondenatured molecules, the native small-t antigen would interact with the cellular proteins, but the decreased amounts of these proteins immunoprecipitated would be very difficult to detect on sodium dodecyl sulfate gels. Such preparations would appear to be active in assays such as microinjection to monitor actin cable disorganization. Therefore, use of coimmunoprecipitation should be an advantage in purifications in which the recovery of large amounts of native or functional small-t antigen is necessary and should allow the direct study of small-t antigen for a variety of enzymatic or binding activities in the future.

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