Simian Virus 40 Large T Antigen Oligomers: Analysis of Electrophoresis in the Absence of Detergent

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Large T antigen of simian virus 40 is found as monomeric and oligomeric species in transformed cells. These can be demonstrated in cell extracts by velocity centrifugation in sucrose gradients. We analyzed them further in a transformed human line cell (SV80) and a transformed mouse line cell (SVT2). Individual fractions from sucrose gradients were subjected to polyacrylamide gel electrophoresis in the absence of detergent. T-antigen species were then detected by protein blotting and antibody overlay with polyclonal anti-D2 T antibody or monoclonal Pab419, Pab101, or Pbl700 antibody. The rapidly sedimenting species (14S and larger) of large T antigen from both cell lines reproducibly showed two major bands with estimated molecular weights of 670,000 and 850,000. A third band of 1,200,000 was more prominent in SVT2 cells than in SV80 cells. In SV80 cells the slowly sedimenting species of large T antigen (5S to 11S) contained two reproducible bands. A band with a molecular weight of 95,000 was the predominant one in all fractions between 5S and 11S. A relatively minor band with a molecular weight of 230,000 was found in fractions between 9S and 11S. The low-molecular-weight forms were seen in SVT2 cells only when a prominent peak at 5S to 7S was present, that is, when extracts were stored before analysis. In fresh extracts, the low-molecular-weight bands and slowly sedimenting forms were absent.

Simian virus 40 (SV40) large T antigen can be detected as a monomer and as several multimers in extracts from transformed and productively infected cells. These species have been identified by immunoprecipitation following sedimentation of extracts through sucrose gradients (4, 11, 27). Rapidly sedimenting species are distributed around peaks of 16S and 23S (7, 11, 14). These are composed of mature (7, 11, 14, 23, 28), highly phosphorylated (11, 14, 21, 28) T-antigen molecules that have acquired specific posttranslational modifications that permit cation-mediated self-assembly (23) and complex formation with the cellular protein p53 (14, 17, 19, 22). More slowly sedimenting species with S values from 5 to 11 have been found in transformed and lytically infected cells (4, 28). These smaller species can be converted to the more rapidly sedimenting species (7, 14).

The rapidly sedimenting complex with p53 represents the major structural form of large T antigen in nonpermissive cells transformed by SV40 (12, 14), and the complex is stable in such cells (25). Oligomerization is also associated with the gene function for transformation (11, 24). In permissive and semipermissive cells however, only a minor proportion of large T antigen is complexed in this way (8, 10, 11, 15, 17, 21, 23), and the complexes tend to be quite unstable (8, 9, 17). As the maintenance of the transformed phenotype depends on the continued expression of a functional large T antigen (30), the formation of stable, rapidly sedimenting complexes in transformed cells suggests that these complexes may play a role in transformation of nonpermissive cells (26), although not all evidence supports this view (3). The degree of oligomerization may also be responsible for different binding affinities of large T antigen for DNA (4, 5, 13).

Studies on the oligomerization of large T antigen would be greatly facilitated if the oligomeric forms of large T antigen could be resolved better. To this end, we applied sucrose gradient fractions containing sedimented large T antigen from SV40-transformed cells to polyacrylamide gradient gels. Electrophoresis was carried out in the absence of detergent to minimize dissociation of the high-molecular-weight species. The forms of large T antigen resolved by this procedure were detected by the method of protein blotting with antibody overlay. We found three major high-molecular-weight forms and two major low-molecular-weight forms of large T antigen in extracts of SV40-transformed mouse and human cells.

MATERIALS AND METHODS

Cell lines. The SV40-transformed cell lines SV80 (human) and SVT2 (mouse) were cultured in Dulbecco modified Eagle medium as described previously (31).

Preparation of whole cell extracts. The cells were grown to confluency in 150-mm tissue culture dishes. After the medium was removed, the cells were scraped from the dishes into 8 to 10 ml of cold phosphate-buffered saline (PBS). The cell pellets were washed three times in cold phosphate-buffered saline and suspended overnight at 4°C in 100 μl per dish of a buffer containing 50 mM Tris hydrochloride (pH 8.0), 120 mM NaCl, and 0.5% Nonidet beta-40 (6). The extracts were centrifuged at 12,800 × g for 20 min, and the supernatants were loaded directly onto sucrose gradients.

Sucrose gradients. The cell extracts (350 to 500 μl) were layered onto 4.4-ml gradients of 5 to 20% sucrose made up in 10 mM Tris hydrochloride (pH 8.5)–10 mM dithiothreitol–120 mM NaCl. The gradients were centrifuged at 23,200 rpm (50,300 × g) at 4°C for 16 h in a Beckman SW50.1 rotor. The gradients were calibrated with 14C-labeled globulin (6.8S) (New England Nuclear Corp., Boston, Mass.) and 3H-labeled SV40 form I DNA (16S). Fractions of 150 to 300 μl were collected from the bottom of the gradients. Samples from these fractions were analyzed by polyacrylamide gel electrophoresis.

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Protein transfer (blotting) to nitrocellulose sheets. The proteins were transferred electrophoretically from polyacrylamide gels to nitrocellulose sheets (6) with an apparatus purchased from Bio-Rad Laboratories, Richmond, Calif.

**FIG. 1.** Electrophoresis of SV40 T antigen (T Ag) from SV80 cells in the presence and absence of detergent. An extract from SV80 cells was centrifuged through a sucrose gradient as described in the text. Fourteen fractions (lanes 1 through 14, respectively) were collected from the gradient. In a parallel gradient, a 16S marker sedimented to fraction 5 and a 6.8S marker sedimented to fraction 11. Samples of 10 μl were subjected to electrophoresis on a 7% polyacrylamide gel in the presence of SDS (A) or on a 3 to 25% polyacrylamide gel in the absence of SDS (B). The proteins were transferred to nitrocellulose paper, overlayed with rabbit anti-D2 T antiserum, and made visible by staining. The bottom of the gradient is to the left. In the lanes marked E, uncentrifuged extract was subjected to electrophoresis. The MW estimates (in thousands) on the left of panel B were derived from marker proteins run on the same gel.

**FIG. 2.** Electrophoresis of SV40 T antigen (T Ag) from SVT2 cells in the presence and absence of detergent. Extract from SVT2 cells was centrifuged through a sucrose gradient as described in the text. Fourteen fractions were collected from the gradient. In a parallel gradient, a 16S marker sedimented to fraction 5 and a 6.8S marker sedimented to fraction 11. Samples of 10 μl from the odd-numbered fractions (lanes 3, 5, 7, 9, 11, and 13, respectively) were subjected to electrophoresis on a 7% polyacrylamide gel in the presence of SDS (A) or on a 3 to 25% polyacrylamide gel in the absence of SDS (B). The proteins were transferred to nitrocellulose paper, overlayed with rabbit anti-D2 T antiserum, and made visible by staining. The bottom of the gradient is to the left. The MW estimates (in thousands) on the left of panel B were derived from marker proteins run on the same gel.

**SDS-polyacrylamide gel electrophoresis.** Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed on slab gels (0.3 by 12.5 by 17 cm) containing 7% acrylamide, by the method of Laemmli (18). Samples were diluted in 62.5 mM Tris hydrochloride (pH 6.8)–2% SDS–10% glycerol–2% mercaptoethanol–0.002% bromophenol blue, heated to 100°C for 3 min, and loaded onto the gels. Electrophoresis was at 50 mA for 3 to 4 h.

**Polyacrylamide gel electrophoresis in the absence of detergent.** Slab gels (0.3 by 12.5 by 17 cm) containing linear gradients of 3 to 25% acrylamide in 125 mM Tris hydrochloride (pH 9.6)–20% glycerol were used. The acrylamide to bisacrylamide ratio was 30 to 0.8. The gels were overlaid with a stacking gel of 3% acrylamide which was also made up in this buffer. Samples were diluted in a buffer of 100 mM Tris hydrochloride (pH 6.8)–10 mM KCl–40% glycerol. They were loaded onto the gels and subjected to electrophoresis at 325 V (15 to 10 mA) for 24 h at 4°C in a buffer of 50 mM Tris hydrochloride–120 mM glycine (pH 8.6)–17% glycerol (5 mM thioglycolic acid was added to the cathode chamber). The marker proteins used to calibrate the gels were thyroglobulin (molecular weight [MW], 669,000 [669K]), ferritin (MW, 440K), catalase (MW, 232K), lactate dehydrogenase (MW, 140K), and albumin (MW, 67K) (Pharmacia Fine Chemicals, Piscataway, N.J.).
Electrophoresis was at 40 V (120 to 160 mA) for 16 to 20 h at room temperature.

Detection of blotted proteins. The marker proteins were detected by staining the nitrocellulose blots with 0.1% amido black in a mixture of 45% methanol–10% acetic acid followed by destaining with a mixture of 90% methanol–2% acetic acid. Indirect immunoperoxidase staining was used to identify bands containing large T antigen. The nitrocellulose sheets were soaked in a Tris saline buffer of 10 mM Tris hydrochloride (pH 7.5)–0.145 M NaCl containing 5% bovine serum albumin for 30 min at 37°C to saturate free protein binding sites. Throughout this incubation step and all subsequent ones, the sheets were gently agitated on a shaker platform. The sheets were then incubated with rabbit antiserum against SDS gel-purified D2 T antigen (2) or with monoclonal antibodies Pab101 (15), Pab419 (16), or Pab1700 (31) diluted 1:200 in 1:500 in Tris saline for 30 min at room temperature. The sheets were washed in Tris saline for 30 min with three changes of buffer, the second containing 0.05% Nonidet P-40. The sheets were then treated for 30 min

FIG. 3. Comparison of T antigen from SVT2 and SV80 cells after electrophoresis in the absence of detergent. Extracts from SV80 and SVT2 cells were sedimented on sucrose gradients. Seven fractions were collected from each. In a parallel gradient, a 16S marker sedimented to fraction 2 and a 6.8S marker sedimented to fraction 6. Samples of 25 µl were subjected to electrophoresis on a 3 to 25% polyacrylamide gel in the absence of SDS. The proteins were transferred to nitrocellulose paper, overlayed with rabbit anti-D2 T antiserum, and made visible by staining. The bottom of the gradients is to the left. The MW estimates (in thousands) on the right were derived from marker proteins run on the same gel.

FIG. 4. Overlay of T antigen with monoclonal antibody Pab101 after electrophoresis in the absence of detergent. Extracts from SV80 and SVT2 cells were sedimented on sucrose gradients. Fourteen fractions were collected from each. In a parallel gradient, a 16S marker sedimented to fraction 3 and a 6.8S marker sedimented to fractions 10 and 11. Duplicate samples of 25 µl from fractions 3, 6, 9, and 12 were subjected to electrophoresis on a 3 to 25% polyacrylamide gel in the absence of SDS. The proteins were transferred to nitrocellulose paper, overlayed with monoclonal antibody Pab101 (A) or rabbit anti-D2 T antiserum (B), and made visible by staining. The bottom of the gradients is to the left. The MW estimates (in thousands) on the left were derived from marker proteins run on the same gel.
at room temperature with horseradish peroxidase conjugated to goat anti-rabbit antibody (for reactions with anti-D2 T antibody) or goat anti-mouse antibody (for reactions with monoclonal antibody) diluted to 1:2,000 to 1:4,000 in Tris saline and bovine serum albumin and washed again. For the color reaction, the sheets were soaked in 25 ml of freshly prepared Tris saline containing 0.1% bovine serum albumin, 1.5 mg of 3,4,3',4'-tetra-amino-biphenyl hydrochloride per ml, and 1 mg of imidazol per ml to which 5 ml of 30% H$_2$O$_2$ was added immediately before use. The color reaction was terminated by rinsing in water. The sheets were dried between filter papers and stored to protect them from light.

**RESULTS**

Large T antigen from SV80 cells could be identified as a single species with an MW of 86K in SDS-polyacrylamide gels when blotted and reacted with anti-D2 T serum. It sedimented broadly through the sucrose gradients from about 5S to about 23S (Fig. 1A). The blotted proteins did not react with normal rabbit serum (data not shown). When the same fractions were analyzed by electrophoresis in the absence of detergent, we observed the pattern seen in Fig. 1B. The slowest sedimenting species of T antigen migrated as a single band at about 95K. Those sedimenting between 7S and 11S showed, in addition to the 95K band, a minor band with an apparent MW of 230K. The more rapidly sedimenting forms were resolved into two bands at 670K and 850K.

Large T antigen from SVT2 cells could be identified as two immunoreactive bands of 92K and 86K in SDS-polyacrylamide gels when blotted and reacted with rabbit anti-D2 T serum. It sedimented almost entirely as a high-molecular-weight species of greater than 11S if the extracts were analyzed without being frozen first for storage (Fig. 2A). If extracts were frozen and thawed before gradient analysis, we found more material that sedimented at 5S to 7S. Normal rabbit serum did not react with any protein bands in these gels (data not shown). When the same fractions from these gradients were analyzed on polyacrylamide gels in the absence of detergent and then blotted and reacted with anti-D2 T serum, we observed the pattern seen in Fig. 2B. The T-antigen species sedimenting in the middle of the gradient were resolved into two bands with estimated MWs of 670K and 850K. Those sedimenting towards the bottom of the gradient migrated as a single band estimated to be 1,200K.

A direct comparison on the same gel of the electrophoresis patterns of extracts run in parallel gradients is shown in Fig. 3. This shows the reproducibility of the patterns and demonstrates that SV80 and SVT2 have some bands in common, specifically those that migrate at 670K and 850K.

To demonstrate that the rabbit anti-D2 T serum that we used in these blotting procedures was specifically identifying SV40 T antigen, we compared its overlay pattern with that produced by reaction with monoclonal antibodies against large T antigen. We chose Pab101 (15) which reacts with a site toward the C-terminal end of large T antigen, Pab419 (16) which reacts with a site toward the N-terminal region of large T antigen, and Pab1700 (31) which reacts with an internal site on native large T antigen. Figure 4 demonstrates that for extracts from both SV80 and SVT2 cells, Pab101 gave the same pattern of reactivity as rabbit anti-D2 T antiserum. In this experiment, the extract from SVT2 cells had been stored frozen before analysis. A band of 95K is present in fractions toward the top of the gradient. Pab419 gave the same pattern of reactivity as rabbit anti-D2 T for T antigen from SV80 cells (Fig. 5). Interestingly, Pab1700 identified only high-molecular-weight species in an extract in which anti-D2 T antiserum was able to identify all species (Fig. 6). This antibody reacts with native but not denatured T antigen (31). These results with monoclonal antibodies indicate that each of the protein bands identified in the gels run in the absence of detergent contains T antigen.

**DISCUSSION**

We analyzed SV40 large T antigen by polyacrylamide gel electrophoresis in the absence of detergent. We found that each of the major species of T antigen, as defined by velocity sedimentation, corresponded to one or two distinct protein bands on the gel. From the rough estimates of molecular weights from standards run on these gels, we found a correlation in the size of these protein bands with the S values from sucrose gradients. The 23S species was associated with a T-antigen band with an apparent molecular weight of 1,200K, the 14S to 16S species was associated with bands of 670K and 850K, and the 5S to 7S species was associated with a major band of 95K and a minor band of 230K.

Just as there is variation in the velocity sedimentation patterns of T antigen derived from different cell lines, we found some variation in the electrophoresis pattern between
the transformed human cell line SV80 and the transformed mouse line SVT2. The 23S species from SVT2 gave a distinct band on electrophoresis that was not as evident in analyses of SV80 cells. The smaller species associated with sedimentation values of 5S to 7S in SV80 cells were not as apparent in fresh extracts from SVT2 cells.

We think it unlikely that the bands we observed on these non-denaturing gels are the result of aggregation of T-antigen molecules induced by electrophoresis conditions, since migration on the gels was appropriate for the sedimentation value. The bands on the gels undoubtedly contain T-antigen since they were detected by protein blotting with antibody overlay. The antibodies we used were highly specific for large T antigen: the polyclonal antibodies in anti-D2 T antiserum have reliably detected T-antigen species in a number of studies (1, 2, 31), and the monoclonal antibodies Pab101, Pab419, and Pab1700 have also been shown to react with high affinity and specificity with T antigen (15, 16, 31). The T antigen-p53 complex and the other oligomers of T antigen preferentially react with Pab101 (6, 15, 29). Results of our studies confirm this reactivity of Pab101 with oligomers and show further that there are at least three to four species of such oligomers. The native forms of T antigen in the nucleus and on the cell surface react with Pab1700, while the denatured forms do not. This holds for SDS-denatured nuclear T antigen and formaldehyde-fixed membrane T antigen (31). Since this antibody did not react with the 95K species, we suggest that the native form of T antigen in transformed cells is predominantly oligomeric both in the nucleus and in the plasma membrane. But, since we examined total extractable T antigen, our results do not preclude a metabolic role for small amounts of the monomeric species in these cells.

The estimates of MW provided by the migration patterns on the nondenaturing gels should not be regarded as accurate. Since the gels contained a gradient of polyacrylamide concentration, migration was probably to some extent controlled by molecular size. However, since detergent was not present, the distribution of charge was not even over the proteins, and this would result in deviation from the migration expected on the basis of size alone. It would be reasonable to guess that the 95K band represents the monomer species of T antigen, but we cannot speculate as to what combinations of dimers, tetramers, or other oligomers make up the other bands. Also, other proteins, such as the p53 family, may be associated with these more slowly migrating bands. Other work has shown that the T-antigen-p53 complex has four T-antigen molecules and that its precursor is the 16S form, which also has four T-antigen molecules (29). The 16S form also reacts with a monoclonal antibody against p53 (20). We do not yet know which of the individual bands that we have found contain p53 or are related to it immunologically.

This method of analysis by electrophoresis in the absence of detergent should allow for more precise definition of the species associated with the post-translational modifications of T-antigen oligomers and, eventually, their role in the biology of lytic infection and transformation.

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LITERATURE CITED


FIG. 6. Overlay of T antigen with monoclonal antibody Pab1700 after electrophoresis in the absence of detergent. Extract from SV80 cells were sedimented on sucrose gradients. Seven fractions were collected. In a parallel gradient, a 16S marker sedimented to fraction 2 and a 6.8S marker sedimented to fraction 6. Duplicate samples of 25 µl from fractions 2, 4, and 6 were subjected to electrophoresis on a 3 to 25% polyacrylamide gel in the absence of SDS. The proteins were transferred to nitrocellulose paper, overlayed with monoclonal antibody Pab1700 (A) or rabbit anti-D2 (RaD2) T antiserum (B), and made visible by staining. The bottom of the gradients is to the left. The MW estimates (in thousands) on the left were derived from marker proteins run on the same gel.