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Abstract

Simian virus 40 (SV40) large T antigen was partially purified from small amounts of SV40-infected and SV40-transformed cells by immunoaffinity chromatography with high recovery. T antigen, in both crude and partially purified states, was detected rapidly by a sensitive and quantitative enzyme-linked immunosorbent assay (ELISA). Stability of the partially purified T antigen was found to increase by addition of 0.01% bovine serum albumin (BSA).

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PARTIAL PURIFICATION OF SIMIAN VIRUS 40 LARGE T ANTIGEN BY IMMUNOAFFINITY CHROMATOGRAPHY AND ITS DETECTION BY ENZYME-LINKED IMMUNOSORBENT ASSAY

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Abstract. Simian virus 40 (SV40) large T antigen was partially purified from small amounts of SV40-infected and SV40-transformed cells by immunoaffinity chromatography with high recovery. T antigen, in both crude and partially purified states, was detected rapidly by a sensitive and quantitative enzyme-linked immunosorbent assay (ELISA). Stability of the partially purified T antigen was found to increase by addition of 0.01 % bovine serum albumin (BSA).

Key words : SV40 T antigen, affinity chromatography, ELISA.

SV40 large T antigen, the product of the A gene of SV40, has a number of functions in both lytic infection and cell transformation (1, 2). To examine the roles of T antigen in *in vitro* systems, T antigen has been purified from cultured cells on a large scale (3, 4). The D2 hybrid protein of the defective adenovirus-SV40 hybrid, $Ad2^{+}D2$, has been used instead of authentic T antigen (5). In these studies, T antigen has mainly been assayed by the complement fixation test, which is a time-consuming and rather tedious procedure. In this report, we describe the partial purification of T antigen from small amounts of SV40-infected and SV40-transformed cells by immunoaffinity chromatography. A rapid and quantitative estimation of relative concentrations of T antigen in those preparations was achieved by the ELISA technique. In addition, heat stability of partially purified T antigen was examined by ELISA.

MATERIALS AND METHODS

Cells and virus. African green monkey kidney cells (CV-1) grown in Eagle's minimum essential medium (MEM) supplemented with 10 % bovine serum (BS) were infected with SV40, strain 777, at 50-100 multiplicity of infection (MOI). After infection the cells were incubated at 37 ℃ for 36-40 h in MEM with 3 % BS. VA-13 (SV40-transformed WI-38) cells were grown in Eagle's MEM with 10 % BS.

Preparation of cellular and nuclear extract. A whole cell extract of SV40 infected CV-1 (2 g) or mock-infected CV-1 (1 g) was prepared as described by Henderson and Livingston (6). A nuclear extract of VA-13 cells (2 g) was prepared according to the method of Kriegler *et al.* (7)

with some modifications.

Immunoaffinity chromatography of T antigen. The immunoadsorbent used was Sepharose 4B (Pharmacia) coupled with hamster anti-T IgG by the CNBr activation procedure. The whole cell extract was fractionated between 30-60 % ammonium sulfate saturation, and the final pellet was dissolved in buffer A (0.05 M Tris-HCl, pH 7.8, 10 % glycerol, 0.15 M NaCl, 1 mM dithiothreitol, 100 μg/ml phenylmethylsulfonylfluoride). After dialysis against buffer A, this fraction was applied to the immunoaffinity column equilibrated with buffer A, and T antigen was eluted with buffer A containing 5 M LiCl. The eluate was concentrated by ultrafiltration (PM-10, Amicon) and dialyzed against buffer A.

ELISA procedure. ELISA was performed using anti-T hamster serum and alkaline phosphatase-conjugated antibody (8) against hamster IgG according to the method of Kilton *et al.* (9), except that 30 μl of test solution diluted with buffer A was added to each well and that buffer containing 1 % gelatin, in place of BSA, was used as the coating buffer. After stopping the enzyme reaction, the product (p-nitrophenol) formed in each solution was determined by measuring the absorbance at 400 nm. Control values from the wells to which only preimmune hamster serum was added were subtracted from each value to obtain antigen-specific absorbance.

RESULTS

Partial purification of T antigen. The whole cell extract from SV40-infected CV-1 cells was first fractionated by ammonium sulfate precipitation. The fraction containing T antigen was then applied to the column of hamster anti-T IgG immobilized on Sepharose 4B. After washing the column with a sufficient amount of buffer A, T antigen was successfully eluted with 5 M LiCl in neutral buffer (Fig. 1). When 5 M LiCl was replaced by 1.5 M KSCN, the same elution pattern

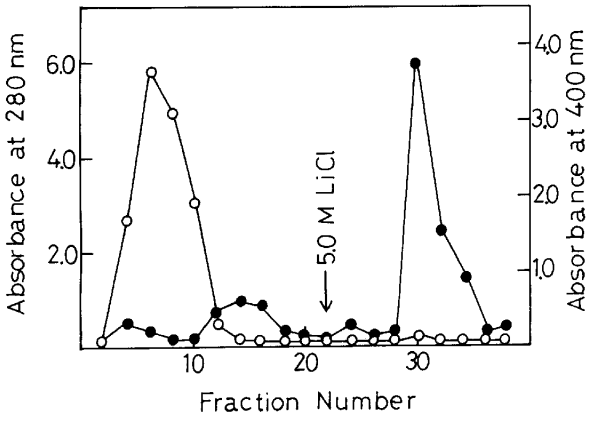

Fig. 1. Immunoaffinity chromatogram of T antigen extracted from SV40-infected CV-1 cells. Crude T antigen was extracted from 2 g of SV40-infected CV-1 cells. The ammonium sulfate fraction dissolved in 4 ml of buffer A was applied to an anti-T IgG Sepharose column (10 ml). Fractions of 2 ml were collected, and aliquots were assayed for T antigen by ELISA. Absorbance at 280 nm (○—○), amount of T antigen represented by absorbance at 400 nm (●—●).

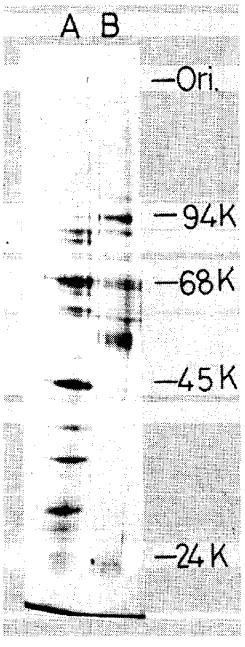

Fig. 2. SDS-PAGE of the partially purified T antigen. The antigen-enriched fraction from the affinity column was electrophoresed in 10 % polyacrylamide gel containing 0.1 % SDS (16). Gels were stained with silver (17). A partially purified T antigen fraction from SV40-infected CV-1 whole cell extract (A), and from VA-13 nuclear extract (B). Molecular weight markers used were phosphorylase a (94K), BSA (68K), ovalbumin (45K), and trypsinogen (24K).

was obtained (data not shown). A 83-fold purification of T antigen was attained with relatively high yield (79 %) by this single step when the relative concentration of T antigen was estimated by ELISA as described below. When VA-13 nuclear extract was fractionated by this column, the increase and recovery of the antigenic activity was 26-fold and 41 %, respectively. The staining patterns after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the partially purified fractions are shown in Fig. 2. Although these fractions were not homogenous with respect to polypeptide composition, protein bands observed around the 84-94 K dalton region appear to correspond to T antigen. In order to prevent the binding of non-specific proteins to anti-T IgG Sepharose, the column was washed with buffer containing 0.5 % Tween 80 after application of the sample (10), or the sample was pretreated by passing it through a column of normal hamster IgG Sepharose. However, the contaminating bands were not eliminated by these treatments (data not shown).

Stability of T antigen. Although the immunoreactivity of T antigen as revealed by ELISA was quite stable in the cellular and nuclear crude extracts, that of the partially purified T antigen was extremely labile to heat and dilution. Addition of 0.01 % BSA to the buffer, however, significantly increased the stability of T antigen incubated at 37℃ (Fig. 3). Similarily, 0.01 % BSA preserved the antigenic activity of T antigen upon dilution (data not shown).

Determination of T antigen by ELISA. To examine whether ELISA can be used for quantitative determination of T antigen throughout its purification, the crude extract of SV40-infected CV-1 and T antigen fractions from the immunoaffinity

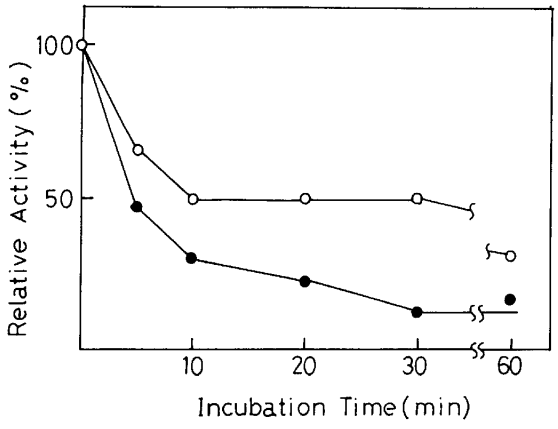

Fig. 3. Heat stability of partially purified T antigen at 37℃ in the presence or absence of BSA. Partially purified T antigen (10 μg/ml) was incubated at 37℃ with (○—○) or without (●—●) 0.01 % BSA in buffer A. At the indicated times the remaining antigenic activity was measured by ELISA.

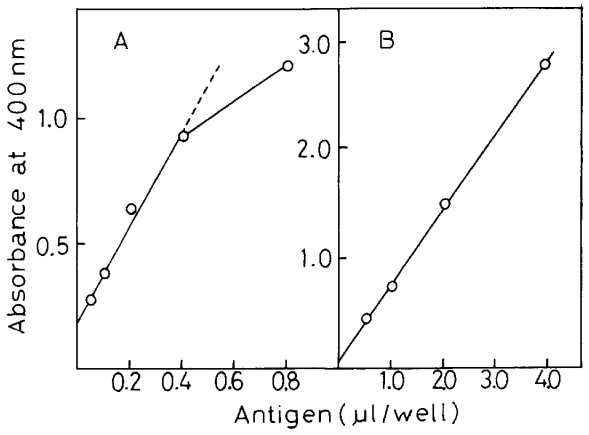

Fig. 4. Quantitation of T antigen by ELISA. ELISA was performed as described in Materials and Methods. Crude extract of SV40-infected CV-1 cells (7.5 mg/ml) (A) and the partially purified T antigen fraction (30 μg/ml) (B) were tested as antigens. Hamster anti-T serum (or preimmune serum) and enzyme-labeled antibody were used at the dilution of 1:200 and 1:150, respectively. Each plot represents the average of three measurements from each of which the absorbance of the corresponding control reaction with preimmune serum was subtracted.

column were used as test antigens. When the test solution contained less than 30 μg/ml of protein, BSA was added so that the total protein concentration was 30 μg/ml in order to protect the antigen. As shown in Fig. 4, the amount of T antigen (volume of antigen added to the system) was proportional to the absorbance at 400 nm over a wide range. With the crude extract, however, the linearity was lost in the high concentration region, probably because of non-linear binding

to the immunoplate surface with other proteins present at high T-antigen concentrations (above 100 μg/ml). Very little reaction was observed when preimmune serum or extract from mock-infected CV-1 cells were used as antibody or antigen, respectively. The titration data fit the line $Y = aX + b$. The intercept, "b", of the line for the crude extract was significantly greater than that for the partially purified fraction. It appears reasonable to assume that the slope, "a", directly correlates with the concentration of T antigen in the sample. This assumption was confirmed by an experiment in which a mixture of the crude extract and partially purified fraction was used as an antigen (data not shown). The result showed that the "a" value had an additive character, indicating that relative concentrations of T antigen in various samples can be assessed by comparing the slopes of the titration curves.

DISCUSSION

When only a small amount of cultured cells are available and conventional multiple chromatographic procedures are used, it is quite difficult to purify T antigen, even partially, with high yield. Therefore, we tried to isolate T antigen in a single step by immunoaffinity chromatography. T antigen bound to immunoadsorbent was eluted by addition of chaotropic ions such as LiCl and KSCN in neutral buffer. The recovery determined by ELISA was relativery high. Immunoaffinity chromatography has been used by Del Villano and Defendi (11) for the purification of T antigen. However, the recovery obtained by their procedure was much less than that described in this report, perhaps due to the difference in buffer composition. Schwyzer *et al.* (12) isolated T antigen by immunoaffinity chromatography to analyze the T antigen with SDS-PAGE. In their procedure the T antigen bound as an immune complex to Protein A-Sepharose was eluted with SDS. Our data indicate that the T antigen eluted by the chaotropic agent retains immunoactivity. The fractions purified by the column were not homogenous in SDS-PAGE gels stained with silver. The contaminating proteins may include degradation products of T antigen (12, 13) or cellular proteins associated with T antigen (14, 15). We attempted further purification by several methods, but did not succeed.

Relative concentration of T antigen was estimated with ELISA as described in Results. In general, the ELISA titer of antigen is estimated by using a standard curve calibrated with highly purified antigen (9). In the present experiments, the immunoactivity of T antigen was represented by the slope of the titration curve such that are titer of T antigen could be defined as an increase of 1.0 O. D. at 400 nm. By using this titer unit, the recovery and specific activity could be calculated. We found that the protein concentration of test antigen considerably affected the linearity of the titration curve. Above 100 μg/ml the protein dose not bind quantitatively to the surface of the immunoplate well. Below 30 μg/ml, immunoactivity of T antigen considerably decreases owing to its lability.

Therefore, the total protein concentration of the test solution should be adjusted to within the range of 30 to 100 μg/ml.

In conclusion, immunoaffinity chromatography and ELISA are useful for the rapid purification and sensitive assay of T antigen when small samples are used.

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