DETERGENT SOLUBILIZED AND MOLECULAR WEIGHT ESTIMATION OF TUMOR SPECIFIC SURFACE ANTIGEN FROM SV40 VIRUS TRANSFORMED CELLS

Samuel W. Luborsky, Chungming Chang, Samuel J. Pancake and Peter T. Mora

Macromolecular Biology Section, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014

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SUMMARY- Mild detergent treatment of SV40 transformed mouse embryo fibroblasts, followed by $(NH_4)_2SO_4$ precipitation of solubilized proteins and rate zonal centrifugation in a sucrose gradient, provided direct and rapid determination of the sedimentation coefficient (4S) of tumor specific surface antigen(s) (TSSA), as detected by an antibody dependent, cell lysis assay. A molecular weight range of 50,000-60,000 was estimated for the TSSA. These results agree with those also obtained from Sephadex G-150 exclusion chromatography.

INTRODUCTION

Recently, in this laboratory (1) and elsewhere (2,3) interest has centered around solubilization and characterization of various antigens from SV40 virus (SV40) transformed cells, as a model to isolate tumor antigens. These antigens are especially labile. Solubilization has been attempted by partial proteolytic digestion, such as by treatment with papain (2,10), but this resulted in almost complete loss of antigenic activity (10; Pancake, unpublished observations; Hendrickson, personal communication). While detergents have been used to solubilize other membrane bound materials, they were not employed for the tumor associated surface antigens. In view of the timeliness and the wide-spread interest in the study of tumor antigens, we show here the utility of detergent extraction of an SV40 induced antigen.

This report shows that solubilization by a non-ionic detergent can be used to obtain rapidly highly active SV40 TSSA, and to estimate directly the size of the intact TSSA molecule by use of rate zonal centrifugation.

METHODS

The SV40-transformed AL/N mouse embryo cell line, designated SV-AL/N,

and the "spontaneously" transformed AL/N mouse embryo cell line, designated T-AL/N, were used (4,5,6). The SV-AL/N cells were obtained by SV40 infection during an early tissue culture transfer of a mouse embryo cell line from which the T-AL/N cells also originated (7). Cells were cultivated in humidified CO_2 incubators, as previously described (6).

To obtain radioactive labelled cells, a ^{14}C amino acid mixture (New England Nuclear Co.) was added to the tissue culture growth medium (6), to a final concentration of 5 μCi/ml. Exponentially growing cells were then incubated for 16 hrs. The preconfluent cells were collected by scraping and were washed with TBS (0.02M Tris buffered saline, pH 7.4) at 4°C. All further fractionation work was at 4°C.

Antisera which possessed high titers against SV-AL/N cells were prepared by sequential injection, 4 to 6 days apart, of SV-AL/N cells into syngeneic AL/N mice, as described (8).

A modified inhibition assay of complement dependent, antibody mediated, lysis of cells, which is specific to SV40 transformed cell lines (5), was used to measure TSSA activity of solubilized fractions. Residual cytolytic activity (measured by ^{51}Cr release) against SV-AL/N target cells was determined, and inhibition of cell lysis was calculated as follows:

Inhibition % = (1- % lysis by (Ab+Ag)/% lysis by Ab) X 100.

The detergent solubilization procedure employed was a modification of the method used to extract H2 histocompatibility antigens (9). Packed cells were washed 3 times with TBS, then solubilized with 0.5% Triton X-100 solution in TBS, in the presence of 10^{-3}M phenylmethylsulfonylfluoride (PMSF). After incubation, 30 minutes, 4°C, with occasional mixing, and centrifugation at 27,000g for 20 min., the supernatant was passed through a 0.22μ Millipore membrane filter. This cell filtrate was fractionated by ammonium sulfate precipitation. Saturated ammonium sulfate was added dropwise with constant mixing, and the 0-30% and 30-50% ammonium sulfate pre-

cipitates were collected, dissolved in, and dialyzed against, TBS (Pancake et al., in preparation).

Gel filtration of the 30-50% $(NH_4)_2SO_4$ fraction on Sephadex G-150 was carried out as described in the legend of Fig. 1. The combined 0-50% $(NH_4)_2SO_4$ fraction was layered on a preformed 5-20% linear sucrose gradient prepared in TBS, and centrifuged and collected as given in the legend of Fig. 2.

RESULTS

Detergent treatment with Triton X-100 produced rapid solubilization of SV40 induced tumor specific surface antigen (TSSA) activity. This TSSA activity, present in SV-AL/N cell filtrates, was concentrated by $(NH_4)_2SO_4$

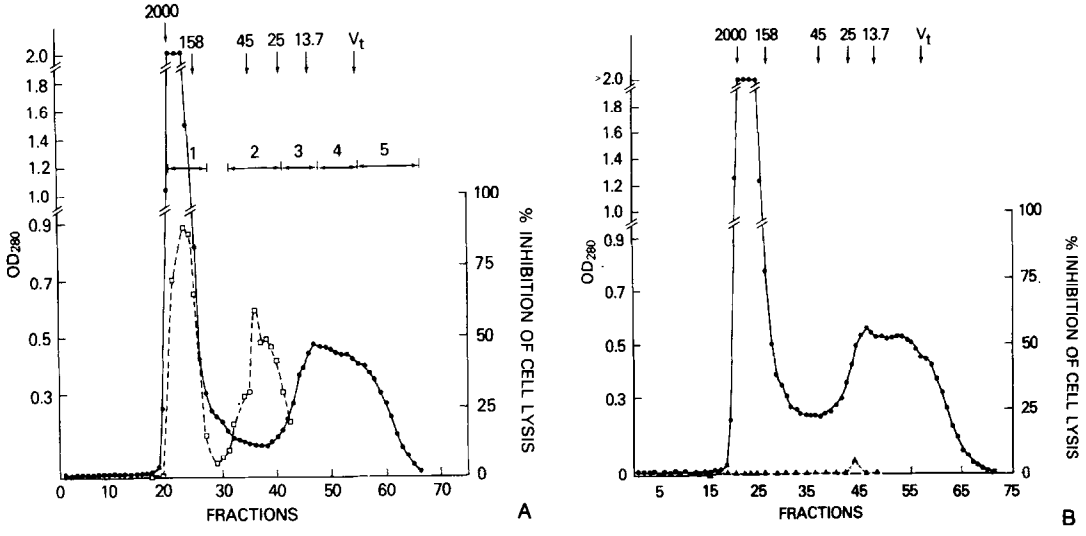

Figure 1. Fractionation of 30-50% $(NH_4)_2SO_4$ fraction from SV-AL/N and T-AL/N cell filtrates, by gel permeation. Sephadex G-150 column (63 x 1.6cm) was prepared in TBS; 30-50% $(NH_4)_2SO_4$ fractions in TBS layered on top. Elution was with TBS (6ml/hr). Arrows show elution positions of molecular weight standards (x 10^{-3}), in decreasing order: Blue Dextran, aldolase, ovalbumin, chymotrypsinogen A, ribonuclease A (Pharmacia); V_t=total bed volume. Bars show fractions pooled. Percent inhibition of cell lysis was measured and calculated as described in Methods (data averaged from duplicate assays).
A: Fraction from SV-AL/N cells; input: 2.0 ml of 34 A_{280}, $9x10^5$ cpm/ml; recovery of input: >46% A_{280}, 85% cpm (data not shown). Fractions after 43 were cytolytic.
B: Fraction from T-AL/N cells; input: 1.8 ml of 55.5 A_{280}; recovery: >53% of input A_{280}. Fractions after 48 were cytolytic.

precipitation. An aliquot of cell filtrate, which contained 30µg protein, for example, inhibited 43% of serum mediated cytolytic activity, while the same amount of protein from the 0-30% $(NH_4)_2SO_4$ fraction inhibited 61%, and from the 30-50% fraction inhibited 77%, of the cytolytic activity. In contrast, in T-AL/N cell filtrates and all of its $(NH_4)_2SO_4$ precipitates there were no detectable SV40 specific TSSA activities (<10% inhibition).

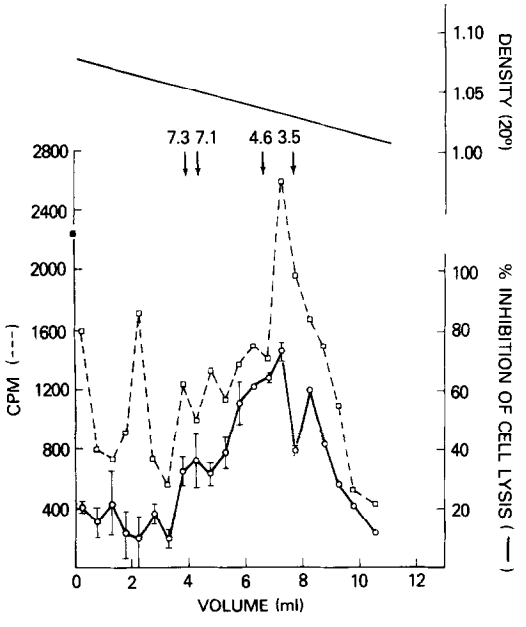

Figure 2. Representative distribution of protein and TSSA activity in SV-AL/N cell extract after rate zonal centrifugation (11). SV-AL/N cells, in log phase growth, labelled with ^{14}C mixed amino acids (1.3 ml packed cell vol.), were solubilized with 0.5% Triton X-100 containing PMSF. After centrifugation and filtration, the cell filtrate was fractionated by $(NH_4)_2SO_4$ precipitation (see Methods). Combined 0-50% precipitate after dialysis (1.2 ml, containing 15 mg, $7.7x10^5$ cpm protein), layered on linear 5-20% preformed sucrose gradient in TBS containing PMSF, in the SW41 centrifuge tube, and centrifuged 20 hrs., 4°C, 40,000 rpm. Fractions collected by bottom puncture. Figure shows distribution of radioactivity in 5µl aliquots: -- ☐-- (■: total counts in pellet), and percent inhibition of serum mediated cytolytic activity, measured and calculated as described in Methods, after dialysis against TBS: -o- (in duplicate- error bars show the two values). Input: 1.2ml of 16 A_{280}, $6.4x10^5$ cpm/ml; recovery: 67% of input cpm. Arrows indicate sedimentation coefficients of marker proteins (Pharmacia) run and measured simultaneously in separate tubes, in order of decreasing S-values (with molecular weight indicated): aldolase (158,000); γ-globulin (153,000); albumin (68,500); ovalbumin (45,000).

Gel exclusion chromatography of the 30-50% $(NH_4)_2SO_4$ fraction on a Sephadex G-150 column resulted in the distributions of biologic activity and protein shown in Fig. 1A. The TSSA activity eluted both with the excluded volume and at a subsequent position, with apparent molecular weights of >200,000 and 25,000-50,000, respectively. When (non-SV40 transformed) T-AL/N cells were extracted and fractionated similarly, no TSSA activity was detected (Fig. 1B).

To estimate the sedimentation coefficient, S, of TSSA, the 0-30% and 30-50% $(NH_4)_2SO_4$ fractions were combined, layered on a sucrose gradient, and centrifuged (Fig. 2). The TSSA activity banded broadly at 2.5-4.5S, with a peak near 4S, the latter corresponding to an estimated molecular weight of 50,000-60,000. There is possibly a lower molecular weight TSSA component of about 30,000-35,000, which has appeared in at least three other independent cell labelling and fractionation experiments similar to that shown in Fig. 2. A small pellet from the bottom of the tube was resuspended in TBS; no significant inhibition of cytolytic activity was detected.

DISCUSSION

Solubilization by proteolytic enzymes, such as papain, is known to fragment membrane antigen molecules, such as the H2 murine histocompability antigens (12). In the past the use of papain for solubilization of SV40 induced TSSA resulted in poor yield of this activity (10, also Pancake, unpublished observations). We have employed Triton-X 100 detergent to solubilize and characterize apparently intact TSSA.

Fractionation and characterization of similar antigens (1,4,10), including SV40 induced T antigen (3), have generally employed gel filtration, ion exchange chromatography, gel electrophoresis, etc. To estimate molecular weight, these techniques require use of standards with similar molecular properties. However, the molecular nature of TSSA is unknown. Nevertheless, by the use of arbitrarily chosen protein standards, the second peak of TSSA activity (Fig. 1A) is estimated to have a molecular

weight of ∿45,000. The TSSA activity also appeared in the excluded volume (Fig. 1A). The molecular nature and solubility of this component are unknown.

This communication reports simple techniques to recover and separate soluble TSSA activity, and characterize it by sedimentation velocity in sucrose density gradients (Fig. 2). Such experiments permit direct estimation of sedimentation coefficient based solely on physical parameters (geometry of tube and rotor, time and g values, density and viscosity of the solvent), and thus provide a size estimate in relative terms between similar types of molecules (11); no assumptions as to the molecular nature of the TSSA activity are required. In the high molecular weight region, the sucrose gradient yielded a small pellet which contained no measurable TSSA activity. The nature and solubility of the TSSA activity observed in the excluded volume region in the gel filtration experiment is ambiguous (Fig. 1). It may result from use of a different $(NH_4)_2SO_4$ fraction; or perhaps sucrose facilitated solubilization and dissociation. The sedimentation coefficient of the single broad band of TSSA activity was calculated to be 4.0S. A molecular weight of 48,000-60,000 was calculated (11) by assuming a spherical shape for TSSA. These values agree well with S values and molecular weights (4S; 50,000-60,000) estimated from protein marker positions; they also fall into the range of the lower molecular weight peak observed by the independent method of gel filtration (Fig. 1). This method thus provides an unambiguous estimate of size, as well as a fractionation and purification of the sample, and thus permits simultaneous assay for many possible (labile) antigen activities in the same sample.

Work is in progress to characterize and compare other SV40 induced antigens, such as tumor specific transplantation and T antigens from similar SV40 transformed cells.

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REFERENCES

1. Smith, R. W., Morganroth, J. and Mora, P. T. (1970) Nature 227, 141.
2. Law, L. W., Henricksen, O. and Appella, E. (1975) Nature 257, 234.
3. Henderson, I. C. and Livingston, D. M. (1974) Cell 3, 65.
4. Smith, R. W. and Mora, P. T. (1972) Virology 50, 233.
5. Pancake, S. J. and Mora, P. T. (1976) Cancer Research 36, 88.
6. Mora, P. T., Brady, R. O., Bradley, R. M. and McFarland, V. W. (1969) Nat. Acad. Sci. USA 63, 1290.
7. Takemoto, K. K., Ting, R. C. Y., Oser, H. H. and Fabisch, P. (1968) J. Nat. Cancer Inst. 41, 1401.
8. Pancake, S. J. and Mora, P. T. (1974) Virology 59, 323.
9. Schwartz, B. D. and Nathenson, S. G. (1971) J. Immunology 107, 1363.
10. Drapkin, M. S., Appella, E. and Law, L. W. (1974) J. Nat. Cancer Inst. 52, 259.
11. Martin, R. G. and Ames, B. N. (1961) J. Biol. Chem. 236, 1372.
12. Henning, R., Milner, R. J., Reske, K., Cunningham, B. A. and Edelman, G. M. (1976) Proc. Nat. Acad. Sci. USA 73, 118.