Use of Monoclonal Antibodies as Probes of Simian Virus 40 T Antigen ATPase Activity*

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We have investigated the ATPase activity of simian virus 40 (SV40) large T antigen by using monoclonal antibodies as specific probes of enzymatic activity. Three hybridoma cell lines secreting anti-T antigen antibodies were derived from mice that were immunized with D2 T antigen, an SV40 T antigen-related protein. Monoclonal antibodies secreted by these hybridomas bind to three distinct T-antigen determinants. In order to bind to T antigen, the three antibodies required amino acid residues coded by the region of the A gene between 0.37 and 0.29 map unit. Two of these antibodies (DL3C3 and DL3C4) strongly inhibited T antigen ATPase activity. The third antibody (DL3C5) only weakly inhibited the ATPase activity possibly by decreasing the affinity of T antigen for ATP. These results demonstrate that the ATPase activity is intrinsic to T antigen and suggest that the ATPase function of T antigen maps on the SV40 A gene between 0.37 and 0.29 map unit. A T antigen-specific ATPase assay capable of detecting low levels of T antigen in crude extracts of SV40 infected cells was developed by using 3C5 to immobilize an active form of the enzyme. These results indicate that monoclonal antibodies can be used as probes of enzyme structure and function.

T antigen is a 96,000 dalton phosphoprotein coded by the A gene of simian virus 40 (1). This multifunctional protein is required for the induction of viral replication in permissive cells (2, 3) and for the initiation and maintenance of neoplastic metabolism (1, 16, 17). Accordingly, we expect that a thorough characterization of T antigen and its interactions within the cell will contribute to our understanding of the mechanisms of gene regulation and DNA replication.

The characterization of T antigen has been hindered by the difficulty of purifying substantial amounts of the protein to homogeneity from SV40 infected cells. To circumvent this difficulty we have used a naturally occurring adenovirus SV40 hybrid virus (Ad2+D2) to overproduce an SV40 T antigen related protein (D2 T antigen) in HeLa cells (18, 19). This protein, which includes about 90% of the SV40 T antigen (see Fig. 2), can be purified in milligram quantities to homogeneity and is functionally equivalent to authentic T antigen by all criteria which have been applied thus far (15, 18, 20). In addition, we have purified the SV80 T antigen from SV80 cells, a line of human fibroblasts transformed by SV40 virus (20). This protein contains several point mutations near its COOH-terminus and does not support viral DNA replication, but apparently retains its cell transformation functions.1 More recently, we have isolated wild type SV40 T antigen (WT T antigen) from HeLa cells infected with a recombinant adenovirus (Ad-SVR6) which expresses the SV40 A gene under adenovirus transcriptional control (21). The T antigen product of Ad-SVR6 is structurally indistinguishable from authentic SV40 T antigen.

Biochemical analysis of these purified T antigen preparations has revealed a site-specific DNA-binding activity and an ATPase activity that is stimulated by the DNA homopolymer poly(dT) (13, 18, 20, 22, 23). This ATPase activity, in contrast to the DNA binding activity, has not been linked to any of the known biological functions of T antigen. Although the ATPase activity present in purified T antigen preparations from each of these sources can be inhibited by multivalent antisera containing anti-T antigen antibodies (20, 23, 24), we did not have proof that this activity is in fact intrinsic to this viral protein. In addition, we wanted to have a specific T antigen ATPase assay that excludes the cellular ATPase activities present in crude cell lysates. Finally, we hoped to map the region of the A gene that is responsible for the ATPase activity. One possible approach to these problems is to probe the ATPase activity by using monoclonal antibodies directed against various antigenic determinants on SV40 T antigen.

Here we report the use of monoclonal antibodies as specific inhibitors of the T antigen ATPase activity and as a rapid means of isolating an active form of this enzyme from crude lysates. The results suggest that monoclonal antibodies provide an important tool for investigating the structure and function of SV40 T antigen.

**MATERIALS AND METHODS**

Cells and Viruses—CV-1 monkey cells were cultured in plastic dishes containing Dulbecco’s modified Eagle’s medium plus 5% fetal

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1 W. Gish and M. Botchan, personal communication.
calf serum, streptomycin (0.1 mg/ml) and penicillin (0.1 mg/ml). SV40 virus, strain 778, was plaque-purified as described previously (25). For the monoclonal antibody-bound ATPase assay, 9-cm plates of nearly confluent CV-1 cells were infected with 20 plaque-forming units/cell of SV40 and incubated for 60–70 h with fetal calf serum reduced to 1.5%.

ATPase Assays—The standard ATPase assay was carried out in a buffer containing 20 μl of 25 mM 1,4-piperazinediethanesulfonic acid (pH 7.0); 0.1 M NaCl; 5 mM MgCl2; 0.01% nonidet P-40; 0–1 mM unlabeled ATP, and 0.5–1 mM of [γ-32P]ATP. For poly(dT)-activated assays, 0.1 μg of poly(dT) was added per 1 μg of T antigen. After incubation with 0.01–1 μg of T antigen for 5–30 min at 20–23 °C, unreacted ATP was precipitated by the addition of 110 μl of acid-washed Norit charcoal (7.5% in 50 mM HCl, 5 mM H3PO4). After pelleting the charcoal by centrifugation, 90 μl of the supernatant was analyzed for free 32P by Cerenkov counting.

Preparation of Nuclear Extracts—All steps were carried out at 20–23 °C unless indicated otherwise. Infected CV-1 cells on 9-cm plates were swelled for 5 min in 5 ml of TD buffer (25 mM Tris pH 8.0; 2 mM MgCl2; 0.5 mM dithiothreitol, and 0.01% phenylmethylsulfonyl fluoride) then lysed for 2 min in TD plus 0.05% nonidet P-40. The plates were gently rinsed twice with 5 ml of TD and extracted with 1 ml of Brian Lysis buffer (0.4 M LiCl, 10 mM Tris, pH 8.0, 0.3 mM dithiothreitol, and 0.01% phenylmethylsulfonyl fluoride) for 5 min. This nuclear extract was used to extract a second plate and then centrifuged for 5 min in an Eppendorf centrifuge at 4 °C to remove insoluble cell debris. The nuclear extract was then assayed directly (standard assays) or diluted 2-fold with 0.2% nonidet P-40 in water the MBA assay.

MBA Assay—The diluted nuclear extract was incubated for 30 min with 1.3 μg of 3C5 followed by incubation for 30 min with 33 μl (settled volume) of protein A Sepharose CL-4B beads with sufficient agitation to keep the beads suspended. The beads were then washed twice with 600 μl of buffer B (10 mM Tris, pH 8.0, 100 mM NaCl, 0.5 mM nonidet P-40) plus 1 mM LiCl and once with 600 μl of buffer B. The beads were assayed for ATPase activity as above except in 10 μl of buffer B with 1 μl of beads.

Chemicals—[γ-32P]ATP and [γ-32P]dATP were synthesized from ADP, GdATP, and H3PO4 (ICN, carrier free) as described previously (26) and purified by elution from DEAE-cellulose with triethylammonium bicarbonate. Poly(dT)7.5S was obtained from P-L Biochemicals. Protein A-Sepharose CL-4B (2 mg of protein A/ml) was purchased from Pharmacia.

RESULTS

Isolation and Characterization of Monoclonal Antibodies—A purified adenovirus-SV40 hybrid protein (D2 T antigen) containing 90% of the COOH-terminal portion of SV40 T antigen (Fig. 2) was used to immunize mice for the production of monoclonal antibodies against SV40 T antigen. Hybridoma cell lines were obtained from immunized mouse splenocytes as described (27–29). Monoclonal antibodies produced by these hybridomas were purified from ascites fluid or culture medium by affinity chromatography on protein A-Sepharose. Three of these, designated DL3C3, DL3C4, and DL3C5 were shown to have T antigen activity by a nuclear fluorescence assay. (A detailed description of the methods will be presented elsewhere). As shown in Fig. 1, these monoclonal antibodies specifically immunoprecipitate D2 T antigen in the presence of formalin-fixed Staphylococcus aureus cells. The ability of the monoclonal antibody to bind to S. aureus cells indicates that they are of the IgG class (30). The migration pattern of the denatured IgG peptides (Fig. 1) is different for each monoclonal antibody, suggesting that they are derived from distinct clonal cell lines. D2 T antigen is also specifically and quantitatively immunoprecipitated by all three IgGs in the presence of Sepharose beads that have been covalently cross-linked to anti-mouse IgG (data not shown).

Mapping of Monoclonal Binding Sites on T Antigen—The three monoclonal antibodies were tested for their ability to immunoprecipitate a series of truncated T antigens that were derived from adenovirus-SV40 hybrids containing well defined A gene deletions (1). This study localized regions of the T antigen peptide chain that are required for binding of the three monoclonal antibodies (Fig. 2). The 3C3, 3C4, and 3C5 antibodies are similar in that all require the amino acid residues coded between 0.37 and 0.29 map unit. The 3C4 antibody requires the amino acid residues coded between 0.47 and 0.37 map unit. The 3C3 antibody requires the amino acid residues coded between 0.37 and 0.29 map unit. The 3C4 antibody requires the amino acid residues coded between 0.47 and 0.37 map unit.

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Inhibition of ATPase by Monoclonal Antibodies—We have determined that the hydrolysis of ATP and dATP by D2 T antigen follows linear Michaelis-Menten kinetics with or without added poly(dT). The D2 protein has a higher affinity for dATP (Ka = 0.5 μM) than for ATP (Ka = 4 μM) but hydrolyzes both substrates at about the same maximal rate (14 and 18 nmol/min/mg), respectively. The Ka and Vmax for both substrates are increased about 5-fold in the presence of poly(dT). Similar results were obtained when either SV80 or WT T antigen was used instead of D2 T. The substrate dATP was chosen in preference to ATP in most of the experiments presented here since it allows for more sensitive ATPase assays.

Preincubation of purified D2 antigen with the three anti-

Fig. 1. Specific binding of DL3C3, DL3C4, and DL3C5 to D2 T antigen. Purified D2 T antigen (0.5 μg) was incubated with 1 μg of DL3C3 (B), 1 μg of DL3C4 (C), 1 μg of DL3C5 (D), 2 μg of nonimmune mouse IgG (E), and without IgG (F) in 20 μl of buffer B for 10 min at 10 °C. Immune complexes were absorbed by the addition of 8 μl of a 10% suspension of formalin-fixed Staphylococcus aureus Cowan I cells and incubated for 30 min at 0 °C (30). The bacteria were collected by centrifugation, washed three times with 200 μl of buffer B and eluted by boiling for 2 min in 20 μl of sodium dodecyl sulfate-gel sample buffer.

The abbreviation used is: MBA; monoclonal antibody-bound ATPase.

1 D. Lane, manuscript in preparation.
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SV40 A gene coding for amino acid residues that are required for IgG hydrolysis measured in the absence of IgG. Nonimmune mouse standard assay buffer (see "Materials and Methods"). Reactions were initiated by adding 5 µl plasma from single plates of transformed or infected cells. 3C5 is added to the extract and the resulting immune complexes are immobilized on Sepharose beads that have been chemically cross-linked to S. aureus protein A or anti-mouse IgG. Contaminating cellular ATPases are efficiently removed from the immobilized T antigen by washing the beads in a suitable buffer and ATPase activity is retained on the beads is assayed directly. The specificity of the assay is monitored by determining the proportion of immobilized ATPase activity that is inhibited by an anti-T antigen monoclonal IgG such as 3C4. Thus, the protein responsible for the measured activity must be recognized by both monoclonal antibodies.

Table I shows that the MBA assay is specific for both purified D2 T antigen and SV40 T-antigen in nuclear extracts from SV40-infected cells, as most of the 3C5 bound ATPase activity was detected in mock-infected cells. In contrast, no T antigen-specific ATPase activity was detected in mock-infected cells. To further substantiate the specificity of this assay, the proteins bound to the beads were analyzed by SDS-gel electrophoresis. The only protein band visualized from infected cells were those corresponding in mobility to IgG heavy chain, IgG light chain, and SV40 T antigen. The T antigen band was not detected in beads prepared from mock-infected cell extracts (data not shown).

This assay is sensitive enough to detect as little as 2 ng of T antigen or about 0.4% of the T antigen present in a single 9-cm plate of SV40-infected CV-1 cells (~2×10^6 cells). The ATP concentrations are given in the standard assay buffer (see "Materials and Methods"). Reactions were initiated by adding 5 µl of 80 µM [γ-^32P]ATP (0.5 µCi) and assayed as described. Activity is expressed as the percentage of the rate of hydrolysis measured in the absence of IgG, O, nonimmune mouse IgG, □, DL3C3, ●, DL3C4, ▲, DL3C5.

The activation of the ATPase by poly(dT), no significant reduction in the activation of dATPase activity by poly(dT) was observed, which indicates that the 3C5 binding site is not required for the interaction of T antigen with poly(dT). The ability of the three monoclonal antibodies to inhibit T antigen ATPase activity is not restricted to the D2 T antigen. The effect of these antibodies on the ATPase activities of SV80 and WT T antigens did not differ from that of the D2 protein (data not shown).

**Monoclonal Antibody-Bound ATPase Assay**—The availability of an anti-T monoclonal antibody (3C5) that does not strongly inhibit T antigen ATPase has allowed us to study the ATPase activity of T antigen in nuclear extracts by using a monoclonal antibody bound ATPase (MBA) assay. In this assay, T antigen in crude cellular extracts is selectively immobilized on 3C5 IgG bound to Sepharose beads. The extracts are rapidly prepared from single plates of transformed or infected cells. 3C5 is added to the extract and the resulting immune complexes are immobilized on Sepharose beads that have been chemically cross-linked to S. aureus protein A or anti-mouse IgG. Contaminating cellular ATPases are efficiently removed from the immobilized T antigen by washing the beads in a suitable buffer and ATPase activity is retained on the beads is assayed directly. The specificity of the assay is monitored by determining the proportion of immobilized ATPase activity that is inhibited by an anti-T antigen monoclonal IgG such as 3C4. Thus, the protein responsible for the measured activity must be recognized by both monoclonal antibodies.

**Table I Monoclonal antibody bound ATPase assay**

<table>
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<tr>
<th>T Antigen source</th>
<th>Standard assay</th>
<th>MBA assay</th>
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<td>-3C4 +3C4</td>
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<tr>
<td>Mock-infected CV-1</td>
<td>2.1 2.1</td>
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**Fig. 3. Inhibition of D2 T antigen ATPase by anti-T antigen monoclonal antibodies.** D2 T antigen (0.2 µg) was incubated for 10 min at 0 °C with the indicated amount of IgG in 15 µl of 1.33 × standard assay buffer (see "Materials and Methods"). Reactions were initiated by adding 5 µl of 80 µM [γ-^32P]ATP (0.5 µCi) and assayed as described. Activity is expressed as the percentage of the rate of hydrolysis measured in the absence of IgG, O, nonimmune mouse IgG, □, DL3C3, ●, DL3C4, ▲, DL3C5.
We have used monoclonal antibodies directed against T antigen as probes to examine the ATPase activity of SV40 large T antigen. Anti-T antibodies that were secreted by hybridoma cell lines were identified by nuclear immunofluorescence and immunoprecipitation assays. Their probable binding sites on T antigen were localized by testing their ability to immunoprecipitate a series of truncated T antigens. These anti-T antibodies were used to develop a rapid, sensitive, and highly specific assay for T antigen ATPase activity in crude extracts of SV40-infected cells.

The inhibition of T antigen ATPase activity by monoclonal antibodies provides strong evidence that this activity is intrinsic to T antigen. Although we and others have previously shown that multivalent antisera inhibits this activity (20, 23, 24), it was possible that the antisera contained one or more antibodies that cross-reacted with cellular proteins or that the binding of several antibodies to each T antigen polypeptide could alter the conformation of the protein and, thus, indirectly inhibit a cellular ATPase that was tightly bound to T antigen. The use of monoclonal antibodies for these experiments virtually eliminates these possibilities because it is extremely unlikely that these objections could apply to all three of the monoclonal antibodies tested. Furthermore, inhibition was observed when using T antigen preparations from several different sources.

The fact that both the D2 T antigen (which lacks the normal NH2-terminal peptide) and SV40 T antigen (which contains several COOH-terminal mutations) exhibit wild type levels of ATPase activity (20) suggested that the terminal portions of the polypeptide are not required for the ATPase function. Furthermore, monoclonal antibodies known to be directed against the NH2 terminus (0.65 -0.53 map unit) and COOH terminus (0.29 -0.50 map unit) of T antigen (31) do not affect the ATPase activity. This study suggests that a centrally located segment of T antigen is involved in the hydrolysis of ATP. The two monoclonal antibodies that strongly inhibited the ATPase activity of T antigen (3C3 and 3C4) required amino acid residues coded between 0.37 and 0.29 map unit for binding. The most plausible explanation for these observations is that these residues are involved in both antibody binding and ATP hydrolysis. The 3C5 binding site, however, is likely to be located at some distance from the active site as 3C5 is a weak inhibitor of the ATPase activity. Similarly, the 3C5 site is also located at some distance from the putative poly(dt) binding site. These findings, although incomplete, provide a first step towards the construction of a structure-function model of the multifunctional T antigen protein. Further studies utilizing a more diverse collection of monoclonal antibodies in conjunction with additional functional assays (e.g. binding to the SV40 origin of replication) should allow us to form a more detailed physical map of T antigen functional sites.

The most important question that remains to be answered about the T antigen ATPase activity is what role, if any, does it play in the biological functions of this viral protein. One attractive possibility is that the ATPase activity is directly coupled to the T antigen-dependent initiation of DNA replication. This could involve ATP-dependent local unwinding of DNA by T antigen bound to the origin of replication. Recent studies have shown that an ATP-dependent DNA unwinding activity cannot be attributed to T antigen alone (32); however, it cannot be excluded that T antigen may express this activity when in association with cellular replication proteins. It is also possible that the uncoupled hydrolysis of nuclear ATP may facilitate T antigen-dependent DNA replication by altering DNA structure in vivo. This hypothesis is consistent with the recent observations that DNA replication is inhibited by ATP in vitro and that the nuclear concentration of ATP is lowered as cells enter S phase (33, 34).

Monoclonal antibodies directed against T antigen are expected to be valuable tools for determining the function of T antigen ATPase activity. In addition to their use as specific ATPase inhibitors in vitro they can be microinjected into cells to specifically block T antigen ATPase in vivo (35, 36). Furthermore, monoclonal antibodies attached to a solid matrix may be useful in isolating native complexes of T antigen and cellular proteins that are involved in viral replication and/or cellular transformation. The MBA assay should allow a rapid survey of mutant T antigens containing lesions that disrupt the ATPase active site. In vitro and in vivo analysis of such mutant T antigens should help clarify the role of the ATPase activity in the biological functions of this viral protein.

In conclusion, the results presented here indicate that monoclonal antibodies are potentially useful reagents for the study of any enzyme, and particularly those whose functions are not clearly established. Their most important potential uses include 1) the identification of an enzymatic activity as being intrinsic to a particular protein; 2) their use as highly specific enzyme inhibitors for both in vitro and in vivo studies; 3) the construction of structure-function maps of proteins; and 4) the rapid isolation of active enzymes or complexes from crude extracts.

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REFERENCES
The ATPase Activity of Simian Virus 40 T Antigen