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Monoclonal Antibodies Specific for the Carboxy Terminus of Simian Virus 40 Large T Antigen

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Three mouse hybridomas secreting antibodies against the undecapeptide Lys-Pro-Pro-Thr-Pro-Pro-Pro-Glu-Pro-Glu-Thr, corresponding to the carboxy terminus of simian virus 40 large T antigen, were isolated and cloned. A sensitive enzyme-linked immunosorbent assay was used to characterize the properties of the monoclonal antibodies. All three hybridomas, designated KT1, KT3, and KT4, produced antibodies that immunoprecipitated large T. The antibodies differed in their affinities for the peptide and for the native protein. Antibodies from KT3 precipitated large T better than those from KT1 or KT4. KT3 antibodies also had the highest affinity for the free peptide (5.2×10^6 M^{-1}) as determined by radioimmunoassay; KT1 and KT4 antibodies had ca. 5- and 1,000-fold lower affinities, respectively. Inhibition studies with shorter peptides, overlapping the undecapeptide, revealed the approximate regions recognized by the different monoclonal antibodies. KT3 antibodies bound to a region within the carboxy-terminal six amino acids of large T. Antibodies from KT1 and KT4 reacted with sequences located further towards the amino terminus of the undecapeptide. Surprising results were obtained with KT4 antibodies. Their binding to the undecapeptide was completely inhibited by the undecapeptide itself or the carboxy-terminal hexapeptide. The carboxy-terminal pentamer, on the other hand, slightly enhanced binding, and the carboxy-terminal tetramer, Glu-Pro-Glu-Thr, was strongly stimulatory. A model for this effect is proposed. Using the enzyme-linked immunosorbent assay, we confirmed previous studies (W. Deppert and G. Walter, Virology 122:56–70, 1982) which found that antiserum against sodium dodecyl sulfate-denatured large T reacts strongly with the carboxy terminus of large T. By inhibition studies, we identified the approximate region within the undecapeptide recognized by anti-sodium dodecyl sulfate-denatured large T and compared this region with the region identified by antipeptide serum.

Antisera from simian virus 40 (SV40) tumor-bearing animals have played a crucial role in the identification and characterization of the SV40 large and small tumor antigens (16, 23). Recently, monoclonal antibodies against SV40 large T have been described by several laboratories (3, 8, 9, 14). One of the advantages of monoclonal antibodies over conventional tumor sera is their use for studying the antigenic and functional properties of SV40 tumor antigens, in particular large T. Thus, Martinis and Croce produced monoclonal antibodies which recognize large T from both SV40 and the closely related human BK virus, whereas others recognize only SV40 large T (14). Scheller et al. showed that a monoclonal antibody, monoclonal 7, isolated by Gurney et al. (8), only precipitates a specific subfraction of large T containing most of the DNA replication origin binding activity (17). The region on large T to which monoclonal 7 binds has been shown to map between 0.39 and 0.44 map units on the SV40 genome (4). Another example for the use of monoclonal antibodies has been published by Floros et al. and Mercer et al., who microinjected monoclonal antibodies against large T, thereby inhibiting large-T-specific induction of cellular DNA synthesis in resting cells (6, 15).

Another approach for preparing specific antibodies against SV40 T antigens is to synthesize peptides corresponding to specific regions of the proteins and to raise antibodies against these peptides. This method has been used to prepare antibodies against the amino and carboxy termini of large T (26) and against the C terminus of small T (10), as well as against a large number of other proteins (21; for a review, see reference 24). It would be desirable to combine the advantages of monoclonal antibodies (unlimited supply, ease of handling, and homogeneity) with those of antipeptide antibodies. The main advantage of the latter is that they recognize predetermined regions of proteins, whereas the epitopes recognized by monoclonal antibodies are usually not known. Another advantage of antipeptide sera is that the immune reaction, e.g., immunoprecipitation or immunofluorescence, can be specifically inhibited by peptides. Furthermore, peptides can be used in protein purification by affinity chromatography for eluting proteins from the antibodies (25).

In the present study, a synthetic peptide corresponding to 11 amino acids from the C terminus of SV40 large T was used as an antigen in mice. Three hybridoma cell lines were isolated that secreted antibodies with differing activities against the undecapeptide and SV40 large T.

MATERIALS AND METHODS

Peptides and peptide conjugates. The peptides used in this study are given in Table 1. The 11-amino acid peptide designated Lys-Thr, which corresponds to the carboxy terminus of SV40 large T, was coupled with glutaraldehyde to bovine serum albumin (BSA) and to ovalbumin (OVA) through the lysine residue at the amino terminus. The coupling procedure and the use of such conjugates to produce antibodies in

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rabbits have been previously described (26). A Lys-Thr conjugate was also used in this study to raise antipeptide antibodies in mice. A Lys-Thr peptide conjugated to OVA (Lys-Thr–OVA) was used in immunoassays to eliminate detection of anti-BSA antibodies. The molar ratio of peptide to carrier was 4:1 for the Lys-Thr–BSA conjugate and 1:1 for the Lys-Thr–OVA conjugate. The Lys-Thr peptide and the hexamer were previously synthesized by one of us (G.W.) (26); the pentamer, tetramer, trimer, and dimer were purchased from Bachem, Bubendorf, Switzerland; Pro-Glu, Pro-Pro-Pro, and Pro-Pro were purchased from Serva Chemicals, Heidelberg, Federal Republic of Germany.

Cells and media. The nonsecretor myeloma line SP2/0-Ag14 (SP2/0 was obtained from G. Köhler, Basel Institute for Immunology) was used in all fusion experiments. Cells were maintained in Dulbecco modified Eagle medium with 4.5 g of glucose per liter (DMEM) containing 10% fetal calf serum (FCS), 0.05 mM mercaptoethanol (MeSH), 1 mM sodium pyruvate, 2 mM L-glutamine, 10,000 U of penicillin-streptomycin per 100 ml, and 200 U of mycostatin (DMEM–10% FCS–MeSH medium). Both myeloma and cloned hybridoma cells were routinely grown in bacteriological petri plates in gas-permeable, sealed plastic bags in a dry incubator at 7.5-10% CO_2 and transferred every 3 days.

The TC7 line of African green monkey kidney cells and SV80 cells (SV40-transformed human fibroblasts) were grown in DMEM with 5% FCS and no MeSH.

Antisera. Rabbit anti-Lys-Thr serum was obtained by immunizing rabbits with a Lys-Thr–BSA conjugate as described previously (26). Mouse anti-Lys-Thr serum was obtained as described below. Rabbit anti-sodium dodecyl sulfate (SDS)-T serum, obtained by immunizing rabbits with denatured, purified large T antigen, was the gift of Wolfgang Deppert (Universitaet Ulm).

Immunization of mice. Ten-week-old female BALB/c mice were immunized with 50 μg of Lys-Thr–BSA per mouse in 200 μl of a 1:1 mixture of complete Freund adjuvant and Lys-Thr–BSA in 0.14 M NaCl. Injections (100 μl each) were made subcutaneously and intraperitoneally. Preliminary studies indicated that a higher dose of antigen did not increase the serum titer. The mice received booster injections on days 16 and 53 with the same dose of coupled peptide in incomplete Freund adjuvant. Mice showing the greatest increase in antipeptide titers after the second booster injection, as determined by enzyme-linked immunosorbent assay (ELISA) (see below), were used for fusions 10 to 11 weeks after the priming immunization. On each of 4 days before the fusion, the mice received additional booster injections by the method of Stälhi et al. (20) with Lys-Thr–BSA in 0.15 M NaCl. The mice were injected intraperitoneally on days 1 and 4 and both intraperitoneally and intravenously on days 2 and 3.

Fusion, screening, and cloning. SP2/0 cells were kept in logarithmic growth for a week before fusion. A feeder cell suspension of 5×10^5 BALB/c spleen cells per ml was prepared the day before the fusion, plated at 0.5 ml per well in 24-well Costar plates, and incubated overnight. Cells were fused at a 1:10 myeloma/spleen cell ratio with a 50% solution of polyethylene glycol (PEG) 4000 in DMEM without serum by the method of Köhler and Milstein (7, 11, 12). The cells were suspended after fusion in DMEM–10% FCS–MeSH medium and plated at 0.5 ml per well in 24-well plates. After 24 h, 1 ml of 2× HAT (1× HAT is 1×10^{-4} M hypoxanthine, 4×10^{-1} M aminopterin, 1.6×10^{-5} M thymidine) in DMEM–10% FCS–MeSH medium was added per well. One-half of the medium was changed with 1× HAT in medium 7 days later and subsequently every 2 to 3 days. Growing cells were detected 4 to 5 days after fusion, and cell supernatants were tested by ELISA for antipeptide antibodies 10, 16, and 21 days after fusion. Positive cell supernatants were further tested for peptide specificity by testing for binding to OVA alone and by inhibition of binding to Lys-Thr–OVA by free Lys-Thr peptide. Cells from wells producing peptide-specific immunoglobulin G (IgG) were cloned by limiting dilution with a conditioned medium consisting of 50% medium harvested from 3-day SP2/0 cultures and 50% fresh DMEM–10% FCS–MeSH medium.

IgG preparation. Antibodies from cell supernatants of cloned hybridomas were concentrated 10- to 15-fold by ammonium sulfate precipitation (50% saturation). The precipitates were suspended in 20 mM Tris–40 mM NaCl, pH 7.8, and dialyzed three times against 20 mM Tris–20 mM NaCl, pH 7.8. For routine use in the ELISA and for immunoprecipitation, the ammonium sulfate precipitates were used.

ELISA. The ELISA with alkaline phosphatase conjugates as described by Voller et al. was used to test antisera, hybridoma cell supernatants, and cell supernatant concentrates (27). To determine IgG content, we added 100 μl of a predetermined dilution (coating saturation, 100 ng of protein per 100 μl) of rabbit anti-mouse-IgG IgG (RAM; Cappel Laboratories, Cochranville, Pa.) in 0.015 M Na_2CO_3–0.035 M $NaHCO_3$, pH 9.6 (coating buffer) to each well of a polystyrene microtiter plate and incubated these mixtures overnight in a humidified chamber at 4°C. After three washes with phosphate-buffered saline–0.5% Tween 20, pH 7.4, 100 μl of cell supernatants or IgG source diluted in phosphate-buffered saline–Tween containing 0.1% OVA (OT buffer)

TABLE 1. Peptides used for immunization of mice and in characterization of antibodies

Designation	Amino acid sequences corresponding to the carboxy-terminal region of SV40 large T
Lys-Thr[a]	NH_2-Lys-Pro-Pro-Thr-Pro-Pro-Pro-Glu-Pro-Glu-Thr-COOH
Hexamer[b]	Tyr-Pro-Pro-Glu-Pro-Glu-Thr
Pentamer	Pro-Glu-Pro-Glu-Thr
Tetramer	Glu-Pro-Glu-Thr
Pro-Glu-Thr	Pro-Glu-Thr
Glu-Thr	Glu-Thr
Pro-Glu	Pro-Glu
Pro-Pro-Pro	Pro-Pro-Pro
Pro-Pro	Pro-Pro

[a] The longer peptide, Lys-Thr, coupled to a carrier protein, was used for immunizations.

[b] In the hexamer, the tyrosine residue was not part of the carboxy-terminal region; it was added during peptide synthesis to mediate coupling of peptide to carrier protein.

was added to the wells and incubated for 2 h at 20°C. The plates were then washed as before and incubated for another 2 h at 20°C with a predetermined dilution of a RAM-alkaline phosphatase conjugate in OT buffer. The plates were washed again and incubated with 100 μl of a 1.6 mM *p*-nitrophenyl phosphate in 10% diethanolamine, pH 9.8, for 30 min to 2 h. The reaction was quantified by reading the absorptance at 405 nm on a Titertek Multiscan. For IgG subclass determination, anti-mouse-IgG –subclass antisera were substituted for RAM in the coating step.

Enzyme immunoassays to test antipeptide antibody were performed by coating the wells as described above with 100 μl of a 1-μg/ml solution of Lys-Thr–OVA conjugate in coating buffer. After washing, 100 μl of cell supernatants (or concentrates) or antisera diluted in OT buffer was incubated for 2 h at 20°C or for 18 h at 4°C. The remainder of the assay was performed as described above. Competition assays were performed by incubating cell supernatant or antisera dilutions with peptides for 2 h at 20°C in a total volume of 100 μl and then proceeding as described above. In the fusion supernatant screening assays, all supernatants that were scored as positive against Lys-Thr–OVA were also tested for binding to OVA alone by coating the wells with OVA (1 μg/ml of coating buffer) in place of Lys-Thr–OVA.

Initially, the ELISA was carried out by using a free peptide as the antigen in the coating step. Preliminary results indicated that the sensitivity of such an assay was insufficient for monoclonal antibody screening. There was also a recurring problem with varying and high backgrounds. Assays with a carrier-bound peptide as the antigen in ELISA gave at least a 10-fold increase in detectable serum titer levels with a consistently low background. This was the sensitivity desired for our monoclonal antibody screening assays.

Radioimmunoassay and affinity determinations. Radioimmunoassays to characterize binding of the monoclonal antibodies to the free Lys-Thr peptide were performed by using ^{125}I-labeled Lys-Thr iodinated with the reagent of Bolton and Hunter (2). The peptide was separated from free ^{125}I on a Sephadex G-10 column equilibrated with 0.25% gelatin–0.05 M PO_4, pH 7.5, and diluted 1:10 with OT buffer. The monoclonal antibodies were titrated by incubation of various amounts of IgG in OT buffer with ^{125}I-labeled Lys-Thr (0.8 ng) for 2 h at 4°C and then precipitation by addition of saturated ammonium sulfate to 50% saturation (1) in the presence of a 1:100 dilution of normal hamster serum. Pellets were washed once with 50% saturated ammonium sulfate. To determine the affinity constant, we incubated the amount of IgG giving 50% binding of labeled peptide with increasing amounts of ^{125}I-labeled Lys-Thr for 16 h at 4°C in a total volume of 350 μl of OT buffer and then precipitated and washed this mixture as described above. The affinity constant was determined by using double reciprocal plots and linear regression analysis as previously described (1). A monoclonal antibody recognizing another peptide was used as the control.

Infection and labeling of cells. Confluent African green monkey kidney cells (TC7 line) growing on 9-cm plastic dishes (Nunc, Roskilde, Denmark) were infected with the large plaque strain of SV40 at a multiplicity of 5 to 10 PFU per cell. The cells were labeled 45 h after infection for 3 h with 250 μCi of [^{35}S]methionine (Amersham-Buchler, Braunschweig, Federal Republic of Germany) per dish in 1.5 ml of methionine-free DMEM supplemented with 5% dialyzed FCS. After labeling, the cells were washed and lysed in 1 ml of lysis buffer (10 mM PO_4, pH 8.0, 140 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 U of aprotinin per ml [13]).

Immunoprecipitation and polyacrylamide gel electrophoresis. For immunoprecipitation of large T, 10 μl of serum or 8 to 11 μg of monoclonal antibody in OT buffer was incubated at 4°C with 50 μl of cell extract. After 90 min, the antigen-antibody complexes were precipitated by further incubation at 4°C for 90 min with constant mixing with either fixed *Staphylococcus aureus* (Pansorbin; Calbiochem-Behring, La Jolla, Calif.) or rabbit anti-mouse IgG coupled to Sepharose 4B (RAM-Sepharose) for those samples containing mouse IgG. RAM-Sepharose was made by coupling 10 mg of RAM to 1 g (dry weight) of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) by the procedure of the manufacturer. After centrifugation, the precipitates were processed and analyzed on SDS-polyacrylamide gels as described previously (13). In later experiments, RAM was coupled to protein A–Sepharose CL-4B (Pharmacia) by the dimethyl pimelimidate method of Schneider et al. (18). In preliminary experiments to isolate native large T, the dimethyl pimelimidate coupling was used to couple monoclonal antipeptide IgG to the RAM-protein A-Sepharose, and this complex was incubated with cell extracts.

RESULTS

Isolation of monoclonal antibodies against the undecapeptide Lys-Thr. The goal of this study was to obtain and characterize monoclonal antibodies against the carboxy-terminal peptide of SV40 large T, Lys-Pro-Pro-Thr-Pro-Pro-Pro-Glu-Pro-Glu-Thr (Lys-Thr), which would also recognize native large T antigen. Mice were immunized with the peptide-BSA conjugate, and their spleen cells were fused with the myeloma SP2/0 as described above. In the fusion experiment from which these monoclonal antibody-producing cells were isolated, 119 of 120 wells had growing colonies. The results for supernatants of five wells were positive against Lys-Thr–OVA in the ELISA. Despite the fact that no cross-reaction between anti-Lys-Thr–BSA serum and OVA had been detected (data not shown), one of the five supernatants bound to OVA and was discarded. Of the remaining four, one was lost before cloning; the remaining three were cloned and designated KT1, KT3, and KT4 by using abbreviations for the first and last amino acids of the immunizing peptide.

ELISA and immunofluorescence testing of antisera and hybridoma cell supernatants. Testing antisera from mice immunized with Lys-Thr–BSA by both immunofluorescence and ELISA indicated that the latter was at least 10 times more sensitive than immunofluorescence. The detectable end titer for one pool of mouse anti-Lys-Thr–BSA serum was 1:2,500 by immunofluorescence with fixed SV80 cells and 1:50,000 by ELISA against Lys-Thr–OVA (data not shown). Because the determined detection range for the ELISA was 1 to 10 ng of IgG per 100 μl, well within the sensitivity required to detect specific IgG in fusion cell supernatants, we decided to use the ELISA as a supernatant-screening assay. The antisera of mice used for cell fusions had titers of 1:10,000 after one booster injection, and all had increased titers ranging from 1:20,000 to 1:100,000 after the second booster injection.

When the cell supernatants from anti-Lys-Thr–producing cloned hybridoma cultures were tested by immunofluorescence (data not shown), one was strongly positive (KT3), one was weakly positive (KT1), and one was negative (KT4). This indicated that one of the Lys-Thr–positive clones would

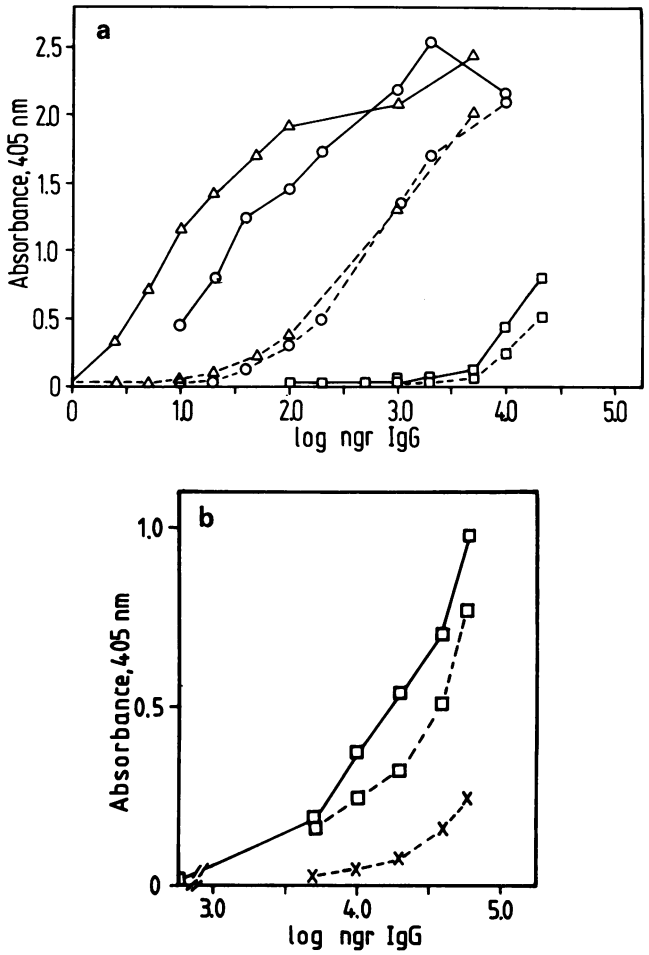

FIG. 1. Titration by ELISA of antipeptide monoclonal antibodies. (a) Titration of antipeptide monoclonal antibodies against Lys-Thr–OVA in the absence and presence of free Lys-Thr. Symbols: KT1 IgG without peptide, O——O; KT1 IgG with the peptide, O----O; KT3 IgG without peptide, Δ——Δ; KT3 IgG with peptide, Δ----Δ; KT4 IgG without peptide, □——□; KT4 IgG with peptide, □----□. The final peptide concentration was 1 μg/100 μl (7.7×10^{-6} M). (b) Titration of KT4 IgG against Lys-Thr–OVA with various amounts of free peptide. Symbols: KT4 IgG without peptide, □——□; with 1 μg of Lys-Thr per 100 μl, □----□; with 10 μg of Lys-Thr per 100 μl, ×----×. Final volumes for all titrations were 100 μl each.

have been lost had immunofluorescence been used as a screening assay.

Titration of KT1, KT3, and KT4 antibodies. A crude IgG fraction from cell supernatants of clones KT1, KT3, and KT4 was prepared by ammonium sulfate precipitation, and the content of mouse IgG was determined by ELISA (see above). All cell supernatants contained between 60 and 100 μg of IgG per ml before concentration, and no anti-Lys-Thr activity was lost during concentration. The IgG preparations were tested in ELISA against Lys-Thr–OVA. Addition of increasing amounts of mouse IgG led to increased binding to Lys-Thr–OVA as indicated by the optical density at 405 nm (Fig. 1). About four times as much KT1 IgG as KT3 IgG was required to get equivalent IgG binding as indicated by the optical density at 405 nm (Fig. 1). To obtain a similar absorbance with KT4, it was necessary to use 1,000-fold more IgG. These data indicate that IgGs from KT3 and KT1 have a much higher affinity for the peptide-OVA conjugate than does that of KT4. The specificity of the immune reaction was demonstrated by the addition of free Lys-Thr peptides (1 μg/100 μl per well). Peptide completely inhibited binding of IgG from KT1 and KT3 as long as the IgG concentration was below 40 ng/100 μl for KT1 and 20 ng/100 μl for KT3 (Fig. 1a). At higher IgG concentrations there was only partial inhibition by the peptide. Peptide at a concentration of 1 μg/100 μl inhibited binding of KT4 IgG only slightly; significant inhibition was obtained with 10 μg/100 μl (Fig. 1b).

Immunoprecipitation of SV40 large T antigen. An important question was whether the monoclonal antibodies reacting with Lys-Thr–OVA would also recognize native large T and be able to immunoprecipitate it. All three monoclonal antibodies precipitated large T, although to differing degrees, KT3 IgG being the most efficient and KT4 IgG being the least efficient (Fig. 2). Precipitates with mouse anti-Lys-Thr serum and with hamster antitumor serum are also shown in Fig. 2. The amount of large T precipitated by the monoclonal antibodies compared well with that precipitated by

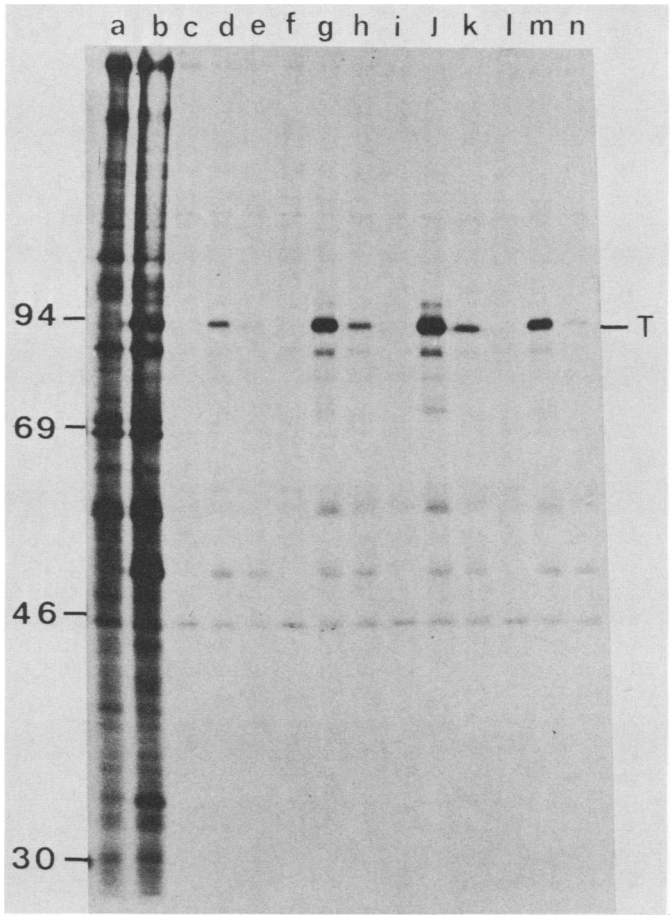

FIG. 2. Immunoprecipitation of SV40 large T by anti-Lys-Thr monoclonal antibodies. Lanes: a and b, 10 μl of hamster antitumor serum; c through e, 10 μl of mouse anti-Lys-Thr–BSA serum; f through h, KT1 IgG; i though k, KT3 IgG; l though n, KT4 IgG. Antibodies were incubated with [^{35}S]methionine-labeled extracts of mock-infected TC7 cells (lanes a, c, f, i, l) and of SV40-infected TC7 cells (lanes b, d, e, g, h, j, k, m, n). In lanes e, h, k, and n, the antibodies were preincubated for 15 min with 10 μg of free Lys-Thr before incubation with the cell extract.

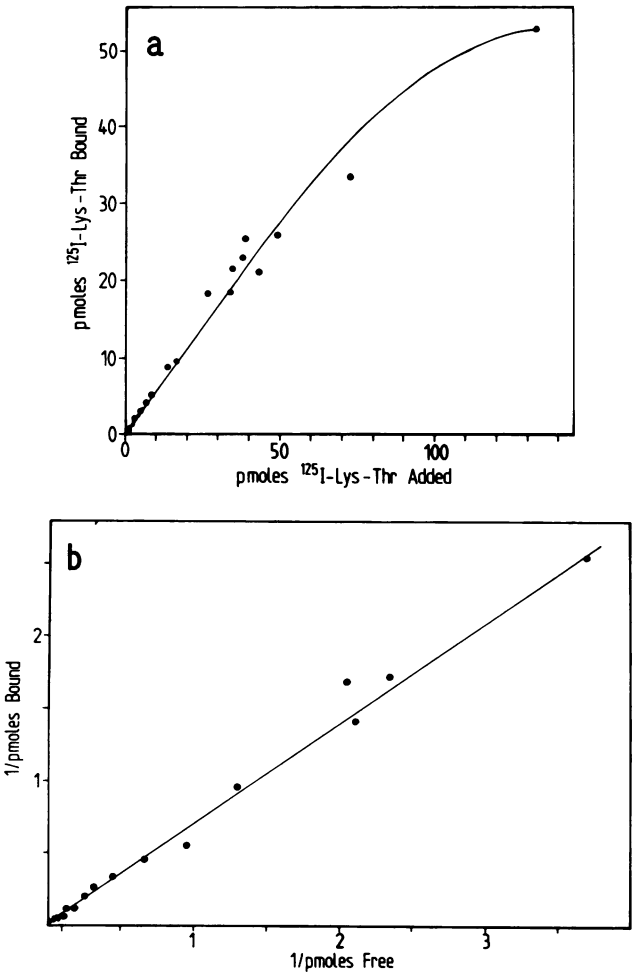

FIG. 3. (a) Binding of KT3 IgG to ^{125}I-labeled Lys-Thr. KT3 IgG (25 μg) was incubated with increasing amounts of iodinated peptide. Data are cumulative results of two experiments and are corrected for background. (b) Double reciprocal plot of data presented in (a).

hamster antitumor serum. Preincubation of the antibodies with free Lys-Thr peptide inhibited immunoprecipitation of large T by the monoclonal antibodies. Although incomplete inhibition of large T precipitation was obtained in this experiment, subsequent experiments with increased amounts of free peptide resulted in complete inhibition (data not shown).

Affinity of KT3 IgG for Lys-Thr. The data (Fig. 1 and 2) indicate that the relative binding capacities of the three monoclonal antibodies both to coupled Lys-Thr peptide and to native large T were similar; KT3 IgG was the most efficient, and KT4 IgG was the least efficient. Since KT3 IgG bound best in both cases, the affinity constant of this IgG for free peptide was determined by using ^{125}I-labeled Lys-Thr labeled with the reagent of Bolton and Hunter (2) (Fig. 3). The linearity of the plot in Fig. 3b confirmed the homogeneity of KT3 IgG expected of monoclonal antibodies. The affinity constant for free Lys-Thr calculated from these data was 5.2×10^6 M^{-1} (coefficient of correlation, 0.99). Preliminary data (not shown) for KT1 and KT4 IgGs indicate that their affinity constants were less than that of KT3 IgG by factors of 6 and 1,000, respectively. These relative differences in affinity are similar to the result obtained in the ELISA.

Characterization of epitopes recognized by monoclonal antibodies. Since an 11-amino acid peptide was used for immunization, it was possible that independently isolated monoclonal antibodies would recognize different regions of the peptide. To determine which region within the original immunizing peptide was recognized by the three monoclonal antibodies, we conducted ELISA inhibition studies with short peptides, overlapping the sequence of the Lys-Thr peptide (Table 1), to test for inhibition of binding to Lys-Thr–OVA. Results obtained with KT3 IgG are shown in Fig. 4. The carboxy-terminal hexamer inhibited as well as the Lys-Thr peptide, whereas the pentamer and the tetramer inhibited to a lesser degree. The tripeptide Pro-Glu-Thr was about 50- to 100-fold less efficient than the longer peptides, whereas Pro-Glu had no detectable effect. Since the hexamer inhibited as well as the undecapeptide, we inferred that the region recognized by KT3 IgG is contained completely within the carboxy-terminal six amino acids. Furthermore, the inhibition obtained with the pentamer, tetramer, and trimer suggests that the center of the epitope is Pro-Pro-Glu-Pro. We cannot exclude the possibility that the actual carboxy terminus is also recognized, although there was no significant inhibition by the dipeptide at the concentrations tested.

Inhibition studies with KT1 are shown in Fig. 5. The hexamer inhibited about a third as well as did the Lys-Thr peptide. All other peptides were completely inactive. We surmised that the amino terminus of the hexamer contains part of the recognized region and that the region to the left of Pro-704 is also recognized. The finding that Pro-Pro-Pro and Pro-Glu did not inhibit KT1 binding could be due to their small size and low affinity for the antibody.

Surprising results were obtained with antibodies from KT4 (Fig. 6). The hexamer inhibited binding to the Lys-Thr–OVA almost as well as did the undecapeptide. The pentamer slightly but reproducibly enhanced binding, whereas the tetramer was strongly (factor of 2 to 3) stimu-

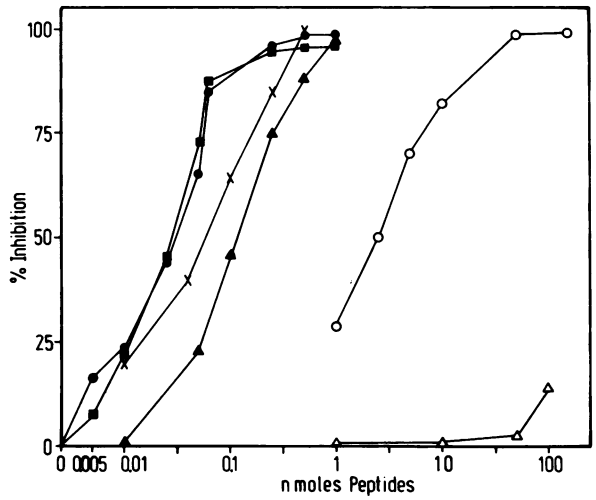

FIG. 4. Characterization of the antigenic region recognized by KT3 IgG by competitive ELISA with free peptides. KT3 IgG (10 ng) was incubated with increasing amounts of Lys-Thr (●), hexamer (■), pentamer (×) tetramer (▲), Pro-Glu-Thr (○), and Glu-Thr (△). Percent inhibition was calculated by comparing the decrease in optical density at 405 nm with that of a control without any peptide. All peptides listed in Table 1 were tested; peptides not shown did not inhibit binding.

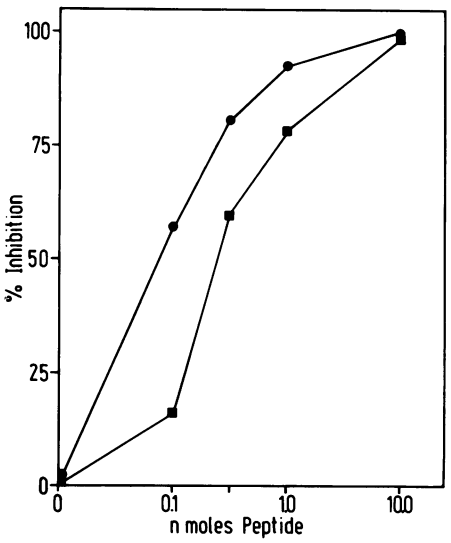

FIG. 5. Characterization of the antigenic region recognized by KT1 IgG by competitive ELISA with various free peptides. KT1 IgG (40 ng) was incubated with peptides as designated in the legend to Fig. 4; no other peptides inhibited binding of antibody.

latory. Pro-Pro-Pro and Pro-Glu inhibited IgG binding (Fig. 6). These data suggest that the sequence around Pro-Pro-Pro-Glu is recognized by the antibodies from KT4. The stimulation phenomenon will be discussed later.

Inhibition by free and carrier-bound Lys-Thr of monoclonal antibody KT3. The affinity of an antibody to a peptide might vary depending on whether the peptide is presented free or bound to a large-molecular-weight carrier. Binding of KT3 IgG to Lys-Thr–OVA was about 10 times more inhibited by Lys-Thr–BSA than by free peptide (Fig. 7). Lys-Thr–OVA had an intermediate effect. The inhibition curves are based on the total amount of peptide added, taking into account the peptide/carrier molar ratios (see above). We conclude from these results that the affinity of monoclonal antibody KT3 is higher for carrier-bound than for free peptide.

Reaction of anti-SDS-denatured large T and hamster anti-tumor sera with Lys-Thr. In a previous publication, we demonstrated, using immunofluorescence, that rabbit or mouse antibodies against purified SDS-denatured large T recognize the carboxy terminus of native and denatured large T (5). We did not detect a reaction of hamster anti-SV40 tumor serum, presumably directed against native large T, with the carboxy terminus. The best interpretation of these findings was that because the carboxy terminus can be recognized by antipeptide serum, it is exposed on native large T, but it is not immunogenic. It becomes strongly immunogenic, however, upon denaturation with SDS. Using the ELISA, we quantitatively measured the titer of rabbit anti-SDS-T serum against the carboxy terminus. Rabbit anti-SDS-T serum had a titer of 1:10,000 to 1:40,000, almost as high as that of rabbit or mouse anti-Lys-Thr serum (Fig. 8a). This indicates that the antigenicity of the carboxy-terminal peptide is similar whether it is presented as part of a denatured protein or coupled to a carrier protein.

When tested in the ELISA, hamster anti-SV40 tumor serum also showed a weak positive reaction against Lys-Thr–OVA (Fig. 8b); however, the titer was 1,000 to 10,000 times lower than that of the anti-SDS-T serum. Controls showed that the reaction was inhibitable with peptide and negative when OVA was used as coat instead of Lys-Thr–OVA and was therefore specific for Lys-Thr. In view of this large difference in titer, it is not surprising that by immunofluorescence we were previously unable to detect this activity in hamster SV40 tumor sera.

A preliminary characterization, by using ELISA with free peptides, of the regions within the Lys-Thr peptide recognized by rabbit anti-SDS-T and anti-Lys-Thr–BSA sera was performed. Although the hexamer completely inhibited the binding of anti-SDS-T serum with Lys-Thr–OVA, a 100- to 1,000-fold-higher concentration of the hexamer was needed than of Lys-Thr (data not shown). This result suggests that anti-SDS-T serum recognizes sequences within the hexamer but that sequences to the amino-terminal side of the hexamer are also necessary for binding. Rabbit anti-Lys-Thr–BSA serum was inhibited nearly to the same extent by both Lys-Thr and the hexamer (data not shown), indicating that the sequences recognized are located predominantly within the hexamer.

DISCUSSION

We have isolated and characterized monoclonal antibodies against an 11-amino acid peptide, corresponding to the C terminus of SV40 large T antigen, which are able to immunoprecipitate native large T. We also prepared hybridomas secreting monoclonal antibodies against polyomavirus medium T (Grussenmeyer, MacArthur, and Walter, unpublished data). Using similar methods, Tamura and Bauer prepared monoclonal antibodies against the C terminus of $pp60^{src}$ of Rous sarcoma virus (22).

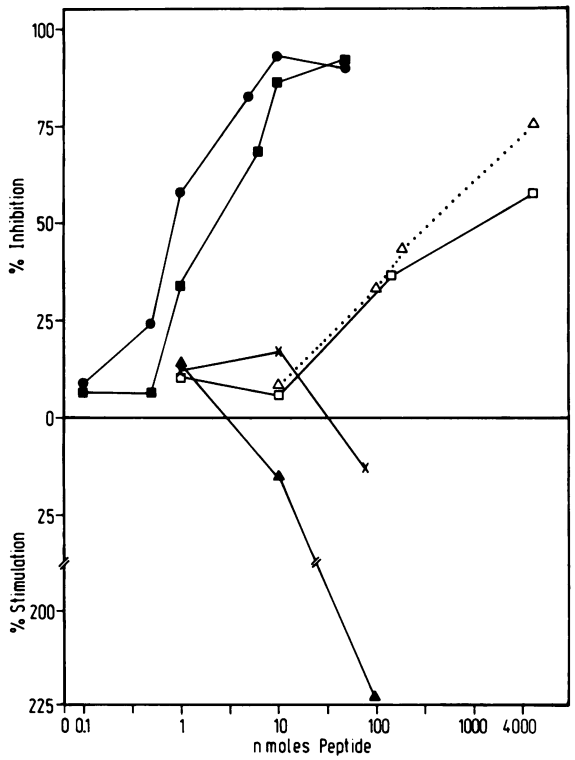

FIG. 6. Characterization of the antigenic region recognized by KT4 IgG by competitive ELISA with various free peptides. KT4 IgG (20 μg) was incubated with peptides, designated as in the legend to Fig. 4 except for Pro-Pro-Pro (□) and Pro-Glu (△). Percent stimulation was calculated by comparing the optical density at 405 nm in the presence of peptide with that of a control without peptide.

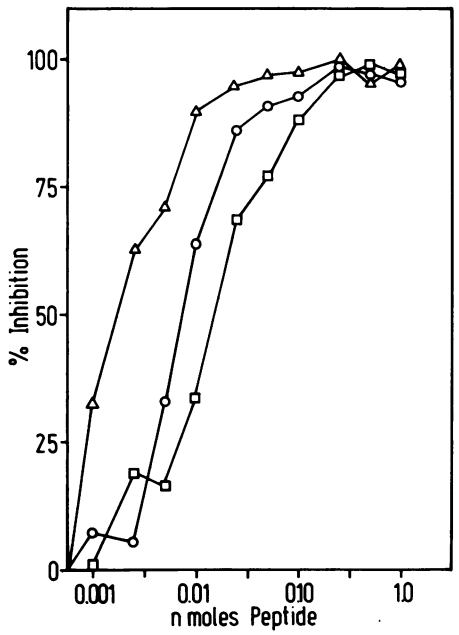

FIG. 7. Comparison of inhibition of binding of KT3 IgG to Lys-Thr–OVA by free and coupled Lys-Thr. KT3 IgG (10 ng) was incubated with increasing amounts of free Lys-Thr (□), Lys-Thr–OVA (○), and Lys-Thr–BSA (△). For incubation with the coupled peptides, the amount of protein added was corrected to give the amount of peptide indicated on the graph.

It is apparent that for many studies monoclonal antipeptide antibodies are preferable over conventional immune sera or antipeptide sera prepared in rabbits, both of which are limited in quantity and must be individually characterized. Furthermore, because the region recognized by the monoclonal antipeptide antibodies is known and because of the various uses of peptides in combination with the antibodies, these antibodies are also preferable to monoclonal antibodies prepared by immunization with the entire protein, native or denatured. In the latter case, the region recognized is usually not known and must be deduced by using mutant analysis (4) or other elaborate procedures.

It should be pointed out that antipeptide sera in general and monoclonal antipeptide antibodies in particular could be useful for detecting very small amounts of an antigen of known amino acid sequence. With a peptide-carrier conjugate as coat and antipeptide antibodies at high dilution, the antigen or protein in question could then be titrated in the ELISA. This assay would be particularly useful if the antigen is present in serum or tissue and therefore cannot be labeled with radioactive amino acids or if the amount of tissue available is very small. A similar assay could be set up for measuring small amounts of antibodies against a particular antigen.

Aside from the practical uses of antipeptide monoclonal antibodies, there are important questions about antibody structure, affinity, the nature of the antigen-combining site, and immunogenicity that can be approached by studying monoclonal antipeptide antibodies. For instance, it is feasible to purify monoclonal antibodies against a defined peptide sequence, to crystallize them, and to determine the structure of the IgG molecule by X-ray crystallography. Crystals could be obtained with or without peptides, and the effect of antigen binding on antibody structure could be investigated in great detail.

It is interesting that all three hybridomas described in the present study secreted antibodies recognizing both the peptide and the native protein. One might have expected that some or even most of the antibodies reacted only with the peptide. However, since the C terminus of native large T might be flexible enough to assume most configurations which can occur in the free or carrier-bound peptide, our finding might not be so surprising. These considerations originally prompted us to choose peptides from the ends of large T when we began making antipeptide antibodies in rabbits (26).

The monoclonal antibodies from KT1, KT3, and KT4 differ in their affinities for the undecapeptide, with KT3 having the highest and KT4 having the lowest. A quantitative determination of the affinity of KT3 IgG for free peptide resulted in a value of $5.2 \times 10^6\ M^{-1}$. KT1 and KT4 antibodies, by estimation, have, respectively, 5- and 1,000-fold-lower affinities. The affinity constants of KT3 and KT1 are within the range of affinities determined for monoclonal

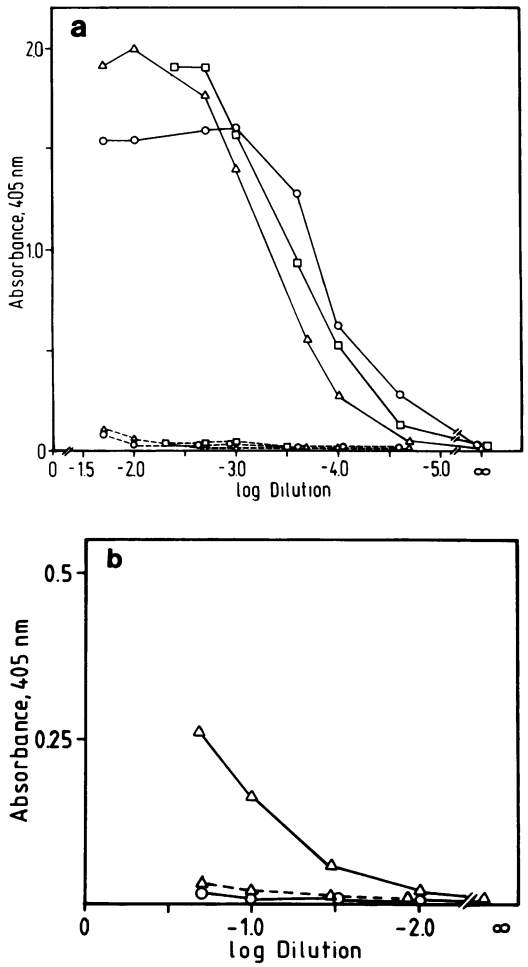

FIG. 8. (a) Titration of rabbit anti-Lys-Thr–BSA, mouse anti-Lys-Thr–BSA, and rabbit anti-SDS-T sera against Lys-Thr–OVA. Increasing dilutions (100 μl) of rabbit anti-Lys-Thr (○), mouse anti-Lys-Thr (□), and rabbit anti-SDS-T (△) were tested against Lys-Thr–OVA (——) and OVA (– – –) in ELISA. (b) Titration of hamster antitumor serum pool against Lys-Thr–OVA in the absence and presence of free Lys-Thr. Antitumor serum (100 μl) against Lys-Thr–OVA without the peptide (△——△), with 1 nmol/100 μl of the peptide (△– – –△), and against OVA (○——○).

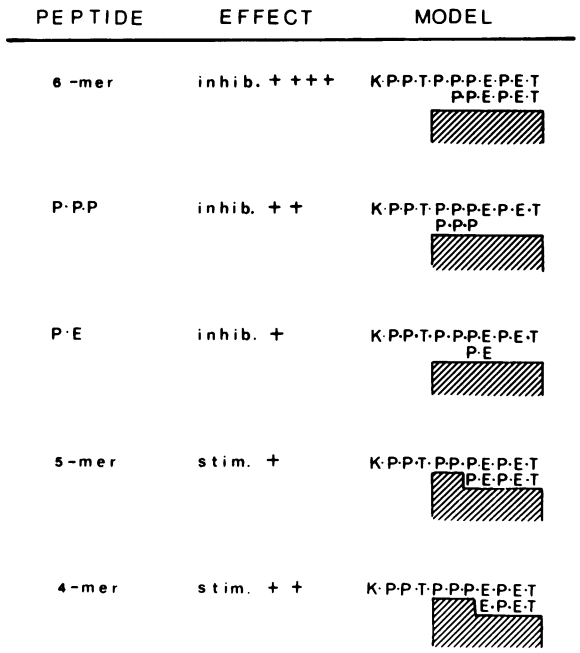

FIG. 9. Model explaining enhancement of antibody binding to Lys-Thr peptide by pentamer and tetramer. The hatched area symbolizes the antigen-combining site of the antibody. A conformational change, indicated by the altered shape of this area, leads to increased affinity of antibody to Lys-Thr peptide in the proline-rich region. 6-mer, Hexamer; 5-mer, pentamer; 4-mer, tetramer; inhib., inhibition; stim., stimulation.

antibodies to insulin (19). Although these values are somewhat low, especially for KT4, all three monoclonal antibodies precipitated large T from cell extracts. The data presented in Fig. 7 indicate that the IgG binds better to coupled peptide than to free peptide. The affinities of the antibodies for native large T may therefore be greater than for the free peptide.

When comparing the amounts of large T immunoprecipitated by the monoclonal antibodies, the difference between KT3 and KT4 appears to be less than expected from the Lys-Thr–OVA binding data. Whereas KT3 and KT4 antibodies differ 1,000-fold in their affinities for Lys-Thr–OVA as measured in the ELISA, they differ at most 5- to 10-fold in immunoprecipitation of large T.

A most interesting finding is the stimulation of binding of KT4 antibodies to the undecapeptide by the addition of free penta- and tetrapeptide (Fig. 6). One possible explanation is illustrated in Fig. 9. It is assumed in this model that the pentamer and tetramer occupy part of the antigen-combining site, leading to a conformational change and an increase in affinity in the remainder of the combining site. To our knowledge, a similar phenomenon has not been reported as yet. It remains to be seen whether it is of a general nature.

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