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An antibody to a synthetic peptide that recognises SV40 small-t antigen

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A peptide Tyr.Arg.Asp.Leu.Lys.Leu corresponding to the carboxy-terminal six amino acids of small-t antigen predicted from the DNA sequence of SV40 was synthesised, coupled to bovine serum albumin and to ovalbumin and used to raise antibody in rabbits. The sera obtained immunoprecipitated [^{125}I]peptide. It also recognised SV40 small-t that was synthesised *in vitro* from SV40 mRNA or extracted from SV40 infected monkey cells. The immunoprecipitation of small-t was inhibited by added peptide. To demonstrate that the determinant was present at the carboxy-terminal end of the molecule, truncated versions of small-t coded for by 0.54-0.59 deletion mutants were tested. dl 890 small-t, which contains an in-phase deletion removing nine amino acids but leaving the carboxy-terminal sequences intact, was recognised by the antipeptide serum. By contrast dl 885 small-t, which has an out-of-phase deletion leading to an altered carboxy terminus coded in an alternative reading frame, was not recognised. The data confirm the location and specificity of the determinant recognised on small-t by the antipeptide serum.

Key words: antibodies/small-t/SV40/synthetic peptides

Introduction

In addition to its ability to replicate in monkey cells, SV40 can transform the growth properties of a number of rodent cell lines and can cause tumours when injected into susceptible animals (Tooze, 1980). The early region of the viral DNA responsible for the ability to transform cells codes for two proteins of partially overlapping sequence called large-T and small-t (Crawford *et al.*, 1978; Paucha *et al.*, 1978b). As yet the biochemical properties associated with the two proteins, and more particularly the role each plays in bringing about transformation, are uncertain.

Mutants of SV40 (Shenk *et al.*, 1976; Sleigh *et al.*, 1978, Feunteun *et al.*, 1978) that are deleted within the sequences coding for the carboxy-terminal half of small-t (called dl 0.54-0.59 mutants) have been used to study the functions of small-t. The mutations have little or no effect on the synthesis of large-T because the deleted region is spliced from the large-T mRNA (Crawford *et al.*, 1978; Berk and Sharp, 1978). The dl mutants are viable for replicative growth, which is often interpreted to mean that small-t has no role in the productive infection cycle (Shenk *et al.*, 1976; Sleigh *et al.*, 1978; Feunteun *et al.*, 1978). However, one report has shown that the titre of virus produced is reduced with some of the deletion mutants (Topp, 1980). Similarly, studies on the ability of the dl 0.54-0.59 mutants to transform cells in culture have been conflicting. Studies using REF cells (Sleigh *et al.*, 1978; Bouck *et al.*, 1978), NIH 3T3 cells (Sleigh *et al.*, 1978; Feunteun *et al.*, 1978; Fluck and Benjamin, 1979), or CHL cells (Bouck *et al.*, 1978) showed that the ability of the mutants to transform cells to anchorage independent growth was severely reduced, whereas later studies using CHL cells (Martin *et al.*, 1979) showed that, under many conditions, the dl mutants could transform cells as efficiently as a wild-type virus. The mutants are also able to produce tumours in new born hamsters although the latent period is somewhat longer than with wild-type virus (Lewis and Martin, 1979; Topp *et al.*, 1981).

Virtually nothing is known about the biochemical activities associated with small-t. It has been shown that its presence correlates with the loss of actin cable networks and with the synthesis of plasminogen activator in transformed cells (Topp and Rifkin, 1980, Graessmann *et al.*, 1980). Whether this is a direct effect of small-t is unclear. Small-t interacts with two host cell proteins with apparent mol. wts. of 56 000 and 32 000, but the identity of the host cell proteins is unknown (Yang *et al.*, 1979).

To conclude on the basis of the experiments reported to date that small-t plays no role in either productive or transforming infections is almost certainly premature. It is doubtful that the sequences coding for such a protein would have been retained in the SV40 viral genome or that the sequences coding for an analogous protein would have been so conserved in the related papovaviruses BK (Dhar *et al.*, 1979) and polyoma (Friedmann *et al.*, 1978; Soeda *et al.*, 1979), if the protein were unnecessary. It is more likely that the protein does play a role in the virus life-cycle but that this role is rather subtle and not easily measured by the procedures tested so far.

A well characterised antibody specific for small-t would be of value in further attempts to characterise the protein. Recently, antibodies have been raised against a number of different proteins by synthesising short peptides corresponding to amino acid sequences predicted by the relevant DNA sequence and some react with the parent protein (e.g., Walter *et al.*, 1980, 1981; Shimizu et al., 1981) Here we report the production of an antibody to a hexapeptide from the carboxy terminus of SV40 small-t and show that it reacts specifically with the protein.

Results

The first, and perhaps most crucial, step in the production of an antipeptide serum that reacts with its parent protein is the choice of which peptide to use. We have analysed the predicted amino acid sequence of SV40 small-t by two computer programs; one (based on a method devised by Chou and Fassman, 1978) predicts the secondary structure and hydrophilicity of the protein, the other (based on a method devised by Hopp and Woods, 1981) predicts immunogenic regions. These analyses predicted that the carboxy-terminal region of SV40 small-t may take up a β-sheet configuration and that it is reasonably hydrophilic. Consequently, this region may be present at the surface of the molecule and it

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Table I. Amino acid composition of peptide

Amino acid	Predicted	Observed
Tyr	1	1.02
Arg	1	0.97
Asp	1	1.00
Leu	2	2.02
Lys	1	1.05

may be immunogenic. No obvious internal peptide sequences with a strong likelihood of being immunogenic were predicted in the analyses. We therefore chose to synthesise the hexapeptide Tyr.Arg.Asp.Leu.Lys.Leu corresponding to the carboxy-terminal six amino acids predicted from the SV40 DNA sequence (Reddy *et al.*, 1978; Fiers *et al.*, 1978).

The peptide was synthesised manually using the solid phase techniques (Merrifield, 1963) outlined in Materials and methods. The peptide was removed from the resin, deblocked, and purified by chromatography on Sephadex SPC 25. Two major fractions were obtained, which were characterised by amino acid composition, enzymatic digestion, and spectroscopic analysis. Both fractions had the correct amino acid composition but one still retained some side chain blocking groups. The amino acid composition of the required fraction is given in Table I. Sequence analysis by the manual dansyl Edman method confirmed the sequence to be correct.

The peptide was coupled to both bovine serum albumin (BSA) and ovalbumin *via* the amino-terminal tyrosine using benzidine (Bassiri *et al.*, 1979) to give conjugates with ~8 mol/mol and 4.5 mol/mol of coupled peptide respectively. Antibodies were raised in four rabbits and serum samples taken at weekly intervals from weeks 7 to 24 after the first injection.

Sera were first tested for the ability to recognise ^{125}I-labelled peptide using either immunoadsorption to protein A bearing bacteria or immunoprecipitation using sheep anti-rabbit Fab_2. Antipeptide activity in one rabbit injected with peptide:BSA was detected after 7 weeks, rose to a peak between weeks 9 and 13 and remained at a significant level until week 24 (Table II). A smaller antipeptide response was detected in the three other rabbits. Serum from bleeds taken between weeks 9 and 13 were used for all the experiments described here. More peptide was precipitated by using sheep anti-rabbit Fab_2 serum (Table II) suggesting that some of the antipeptide antibody molecules belong to a class not recognised by protein A.

To examine whether the serum with antipeptide activity also recognised the parent protein, SV40 large-T and small-t were synthesised in a nuclease-treated rabbit reticulocyte lysate using mRNA isolated from monkey CV1 cells infected with SV40, and the cell-free products immunoprecipitated. Figure 1 shows the proteins immunoprecipitated using protein A bacteria by a conventional antitumour cell serum raised in hamsters, and by the serum raised against the peptide:BSA conjugate. Large-T and small-t are specifically immunoprecipitated by the anti-tumour cell serum whereas only a protein co-migrating with small-t is specifically immunoprecipitated by the antipeptide serum. The SV40 capsid protein VP1 and actin are non-specifically precipitated by both control and immune serum. This is commonly a problem with proteins synthesised *in vitro* (Paucha *et al.*, 1978a) because the capsid proteins and actin are produced in large

Table II. Percentage of [^{125}I]peptide immunoprecipitated by antipeptide sera

Week no.	peptide:BSA rabbit A	peptide:ovalbumin rabbit B
Experiment 1		
0	2.1	2.1
7	12.6	4.1
8	12.4	4.2
9	14.1	3.7
11	18.4	3.9
13	13.1	2.9
14	12.6	3.8
15	10.9	3.3
16	11.2	2.1
17	9.7	2.7
18	10.2	3.4
20	12.3	2.7
22	7.9	3.0
24	8.3	3.3
Experiment 2		
0	0.5	0.5
7	20.3	6.7
8	17.1	5.9
9	24.1	5.7
11	26.6	6.1
13	14.2	6.5

10 μl samples of serum from two rabbits were reacted with [^{125}I]peptide (25 000 c.p.m.) for 1 h at 22°C and the immune complexes collected using either protein A bearing bacteria (experiment 1) or sheep anti rabbit Fab_2 (experiment 2). Results are expressed as the percentage of added counts recovered in the precipitate.

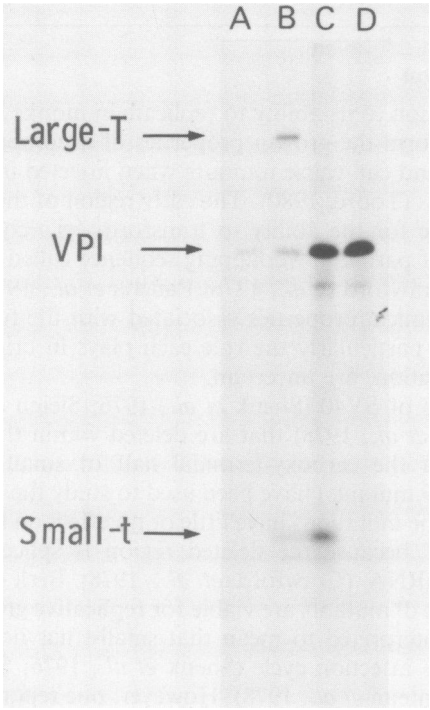

Fig. 1. Immunoprecipitation of proteins synthesised *in vitro* in response to mRNA from SV40-infected monkey cells using, **(A)** control hamster serum, **(B)** hamster anti-tumor cell serum, **(C)** rabbit antipeptide:BSA serum, **(D)** control rabbit serum.

amounts and they are not sequestered into the nucleus or cytoskeleton as they would be in cells. The data shown in Figure 1 suggest that *in vitro* synthesised small-t is immunoprecipitated by the antipeptide serum and that the determinant recognised is present in small-t sequences that are not shared with large-T.

Small-t synthesised *in vitro* may or may not take up its native conformation. To investigate whether the native protein is recognised by the antipeptide serum, CV1 cells were infected with SV40, labelled with [^{35}S]methionine 48 h after infection and extracts made after incubation for a further 4 h. Figure 2 shows that a band migrating with small-t is efficiently precipitated from the labelled extracts by the antipeptide serum.

To demonstrate that the determinant recognised corresponds to the synthetic peptide, [^{35}S]methionine-labelled extracts were immunoprecipitated by both anti-tumour cell serum and antipeptide:BSA serum in the presence of peptide and of BSA. Figure 2 shows that the immunoprecipitation of the protein co-migrating with small-t by the antipeptide serum is inhibited by added peptide but not by BSA. By contrast, the immunoprecipitation of small-t by conventional anti-tumour cell serum is unaltered by addition of either. Similar results were obtained when the cellular extracts were immunoprecipitated with an antipeptide serum raised against peptide coupled to ovalbumin in the presence of peptide and of ovalbumin. Although the latter serum had less antipeptide activity than the antibody raised against a peptide:BSA conjugate when screened using ^{125}I-labelled peptide (Table II), its anti small-t activity was comparable (Figure 2).

The data presented thus far establish that a protein co-migrating with authentic small-t can be immunoprecipitated *via* a defined determinant from extracts of virus infected cells or from the products synthesised *in vitro*. To establish that the protein is authentic small-t and that the peptide determinant is present at the carboxy terminus of small-t, we have investigated the ability of the serum to immunoprecipitate abnormal forms of small-t coded for by dl 0.54 – 0.59 mutants (Shenk *et al.*, 1976).

dl 890 has an in-phase deletion that converts isoleucine 112 to a methionine, deletes amino acids 113 – 121 and retains the normal carboxy-terminal sequence of small-t (Volckaert *et al.*, 1979). dl 885 is an out-of-phase deletion that codes for a small-t altered after amino acid 139 by addition of six amino acids coded for by another reading frame (Seif *et al.*, 1980). We would therefore predict that the antipeptide serum would recognise the truncated small-t coded for by dl 890 but not that coded by dl 885 (Figure 3A).

In addition to their effect on the size of small-t, deletions in the 0.54 – 0.59 region alter the pattern of splicing of the early region transcripts. We have shown earlier that both the amount of abnormal small-t present in infected cells and the amount of abnormal small-t mRNA isolated from them are very small compared with the amounts of large-T and its mRNA (Paucha and Smith, 1978). To generate adequate amounts of the truncated small-t species we previously showed that it is possible to synthesise the proteins *in vitro* using a template complementary RNA (cRNA) transcribed *in vitro*

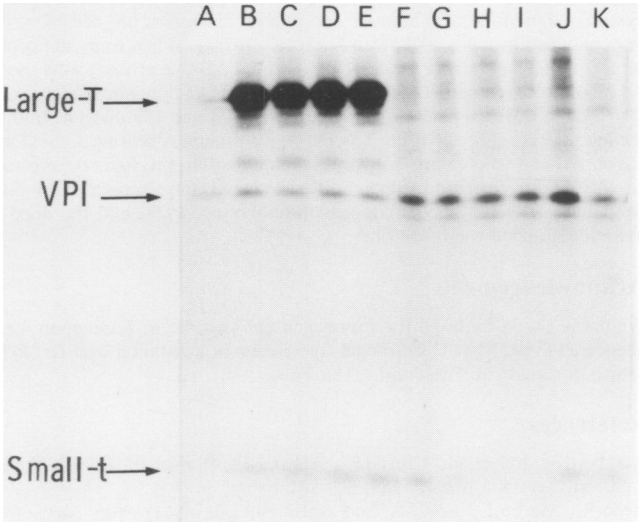

Fig. 2. Immunoprecipitation of extracts of SV40 infected monkey cells using, **(A)** control hamster serum; **(B – E)** hamster anti-tumour cell serum; **(I)** control rabbit serum; **(F – H, J)** rabbit antipeptide:BSA (week 9) serum; and **(K)** rabbit antipeptide:ovalbumin (week 9) serum in the presence of **(C** and **G)** 0.1 μg peptide; **(D** and **H)** 1 μg peptide; and **(E** and **J)** 5 μg BSA.

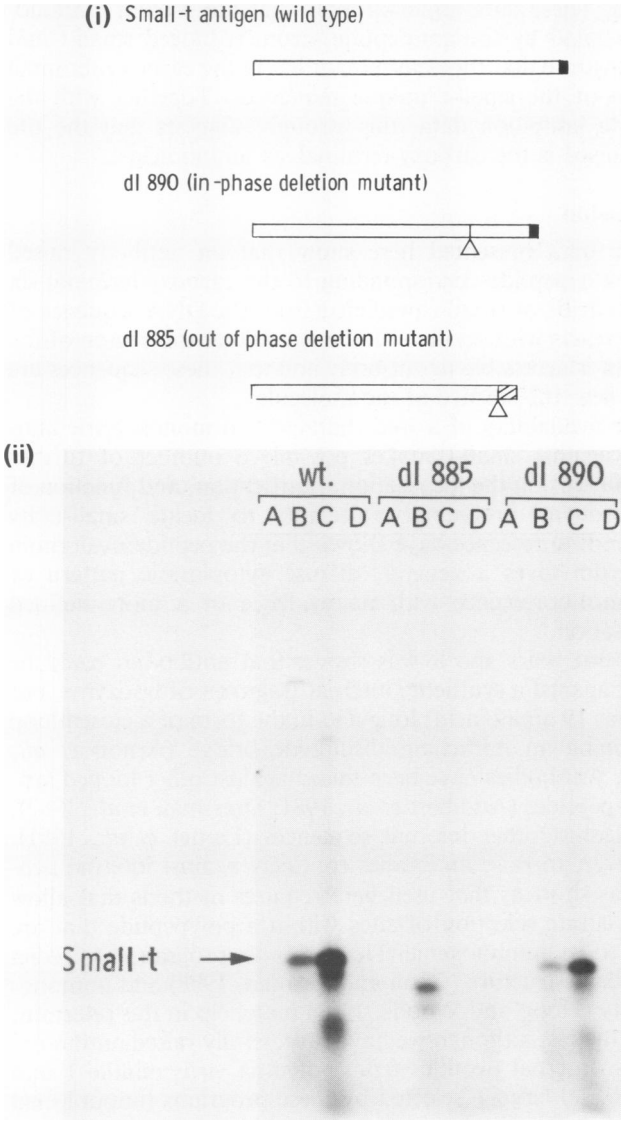

Fig. 3. Immunoprecipitation of different forms of small-t coded for by wild-type and dl mutant cRNAs. **(i)** Schematic representation of different small-t molecules; the shaded area indicates the location of the hexapeptide. **(ii)** Immunoprecipitation of the products made in response to cRNA by **(A)** control hamster serum, **(B)** hamster anti-tumour cell serum, **(C)** rabbit antipeptide:BSA serum, **(D)** control rabbit serum.

using *Escherichia coli* RNA polymerase and wild-type and deletion mutant DNA (Paucha and Smith, 1978). The cell-free product contains small-t in good yield. In addition, a variety of shortened versions of large-T and small-t are present. We have previously characterised these proteins using peptide fingerprinting and deduced that they originate from internal initiation events occurring at several AUG codons within the unspliced early transcripts, and that they terminate at the termination codons for either large-T or small-t.

Figure 3B shows the proteins immunoprecipitated from the cell-free product synthesised in response to cRNA. The major product immunoprecipitated from the wild-type cRNA reaction by both anti-tumour cell serum and the antipeptide serum is small-t. A number of shortened forms of small-t are also precipitated by the antipeptide serum whereas the large-T related molecules are not. The mutant form of small-t present in the cell-free product primed by dl 890 cRNA is immunoprecipitated by both antisera whereas the shortened form coded for by dl 885 is recognised only by the anti-tumour cell serum. These data argue strongly that the protein immunoprecipitated by the antipeptide serum is indeed small-t and demonstrate that the determinant lies in the carboxy-terminal region of the small-t unique sequences. Together with the peptide inhibition data, this strongly suggests that the site recognised is the carboxy-terminal six amino acids.

Discussion

The data presented here show that an antibody raised against a peptide corresponding to the carboxy-terminal six amino acids of small-t predicted from the DNA sequence of SV40 reacts with small-t. This suggests that this region of the protein is accessible to antibody and that these sequences are at or near the surface of the molecule.

The availability of a well characterised monospecific antibody against small-t makes possible a number of further experiments on the localisation, purification, and function of the protein. Preliminary attempts to locate small-t by immunofluorescence have shown that the peptide:ovalbumin antiserum gives a general, diffuse cytoplasmic pattern of immunofluorescence with no evidence of a more defined localisation.

Several years ago it was shown that antibodies could be raised against a synthetic, internal fragment of lysozyme, but this was 19 amino acids long and in the form of a closed loop containing an intrachain disulphide bridge (Arnon *et al.*, 1971). Antibodies have been raised against other looped synthetic peptides (Audibert *et al.*, 1981; Dressman *et al.*, 1982), and against other internal sequences (Lerner *et al.*, 1981). However, to raise antibodies routinely against internal peptides as short as that used here requires methods that allow the accurate selection of sites within a polypeptide that are likely to be immunogenic. The computer programs to predict secondary structure (Chou and Fasman, 1978) and immunogenicity (Hopp and Woods, 1981) may help in this selection. Nevertheless, although we have successfully raised antibodies against internal peptides from polyoma virus middle-T and from SV40 large-T selected by these programs (unpublished results), the antibodies interact with their parent proteins less readily than does the serum reported here. From this and other similar findings (Lerner *et al.*, 1981), it would appear that if an antibody is required merely to identify an unknown protein it is advisable to use a sequence from an end of the protein under investigation.

Materials and methods

Peptide synthesis

The peptide was synthesised manually on 0.7 g polystyrene resin containing 350 μmol coupled leucine using sequentially, α-N-*t*-butoxycarbonyl (BOC)-ε-N-carbobenzoxy-L-lysine, N-*t*-BOC-L-leucine, N-*t*-BOC-L-aspartic acid β benzyl ester, α-N-*t*-BOC-ω-N-*p*-tosyl-L-arginine and N-*t*-BOC-O-benzyl-L-tyrosine in 6-fold excess and coupling reagent (N,N-dicyclohexylcarbodiimide) in 3-fold excess (Merrifield, 1963). The peptide was cleaved from the resin using hydrogen bromide in trifluoroacetic acid containing anisole. After drying, the peptide was desalted and the tosyl side chain blocking group removed from the arginine residue using sodium in liquid ammonia. After desalting, the peptide was purified on a 1.5 cm x 74 cm Sephadex SPC 25 resin using 0.05 M sodium phosphate buffer at pH 4.0 and a gradient of 0.1 – 0.5 M NaCl. Two major peaks were obtained. These were identified by spectroscopic analysis, amino acid analysis on a Beckman 121 MB analyser, and enzymatic digestion using amino peptidase M. The required peptide eluted at ~0.35 M NaCl and was judged to be ~65% pure by analysis on h.p.l.c. using an ODS C18 resin. The yield was 30 mg. The major contaminant was the correct peptide retaining side chain blocking groups.

Coupling

The peptide was coupled to BSA and to ovalbumin using benzidine (Bassiri *et al.*, 1979). Peptide: carrier molar ratios of 49:1 and 33:1 were used and gave 16% and 14% coupling, as determined using [^{14}C]peptide as tracer.

Antibody production

1 mg amounts of peptide: carrier conjugates in complete Freunds adjuvant were injected i.m. into female adult lop strain rabbits on days 0 and 7, and after 28 days a third dose was administered by i.p. and multiple-site s.c. injections. Serum samples were taken weekly after a further two weeks.

Labelling of peptide

50 μg samples of peptide were labelled with 100 μCi ^{125}I using iodogen (Fraker and Spek, 1978). The peptide was purified by gel filtration on Sephadex G10. The specific activity of the peptide was ~0.1 – 1 Ci/mmol.

Cell-free protein syntheses

Conditions for the isolation of mRNA from SV40 infected CV1 cells (Paucha *et al.*, 1978a, 1978b) and for the preparation of SV40 cRNA (Paucha and Smith, 1978) have been described in detail. Cell-free protein synthesis was in a nuclease treated rabbit reticulocyte lysate as described by Paucha *et al.* (1978b). Typically, 30 μl incubations contained 1 μg mRNA or 1 μg cRNA; 15 μl samples were taken for immunoprecipitation.

Extraction of small-t from infected cells

The growth, labelling, and extraction of [^{35}S]methionine-labelled proteins from SV40 infected CV1 cells was exactly as described previously (Smith *et al.*, 1978).

Immunoprecipitation

Conditions for immunoprecipitation of cellular extracts and of the products synthesised *in vitro* using hamster anti-tumour cell serum has been described in detail (Smith *et al.*, 1978; Paucha *et al.*, 1978a). When using antipeptide serum 10 μl/25 μl cellular extract or 15 μl/15 μl cell-free product were used in 0.5 ml of buffer containing 150 mM NaCl, 10 mM sodium phosphate pH 7.0, 1% sodium deoxycholate 1% NP 40, 0.1% SDS, and 1% trasylol. Immune complexes were collected using 2.5 volumes of protein A bearing *Staphylococcus aureus* or 3 volumes of sheep anti rabbit Fab_2. The precipitates were washed three times using the same buffer. Proteins were separated on SDS-containing 15% polyacrylamide gels (Smith *et al.*, 1978) and the dried gel autoradiographed for 1 – 14 days.

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