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Protein kinase activities associated with distinct antigenic forms of polyoma virus middle T-antigen

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The tyrosine-specific protein kinase activity previously described in T-antigens of polyoma virus immunoprecipitated with anti-tumour sera has been investigated using monoclonal antibodies. This activity is associated with middle T-antigen but it can be separated by selective antibody precipitation from the majority of this protein. The difference between active and inactive forms can be accounted for by an antigenic difference at the N terminus of middle T-antigen molecules. Moreover, the two different mol. wt. forms of middle T-antigen that can act as phosphoacceptors have been separated by antibody precipitation and therefore shown to be immunologically distinct. The binding position of the antibody used for immunoprecipitation has been observed to have a quantitative influence on the *in vitro* protein kinase reaction, in one case appearing to stimulate the activity. The detection of the *in vitro* protein kinase activity in immunoprecipitates obtained with several different monoclonal antibodies directed against the middle T-antigen indicates that the activity is a property tightly associated with this polyoma virus-coded protein.

Key words: polyoma virus/tumour antigens/protein kinase/monoclonal antibodies/phosphotyrosine

Introduction

The small DNA tumour virus, polyoma, is capable of lytically infecting mouse cells in culture, and of transforming, both *in vitro* and *in vivo*, other rodent cells (rat and hamster fibroblasts, predominantly). The 'early region' of polyoma virus DNA codes for three major polypeptides that are recognized by antibodies present in sera of animals with tumours induced by this virus (for reviews, see Ito, 1980; Türler, 1980; Eckhart, 1981). These proteins have been designated large T-antigen (100 kd), middle T-antigen (55 kd), and small T-antigen (22 kd). There is much circumstantial evidence to indicate that the middle T-antigen is the prime effector of transformation (Griffin *et al.*, 1980a, 1980b; Ito and Spurr, 1980; Ito *et al.*, 1980), and recently it has been shown that expression of this protein alone is capable of inducing most of the properties associated with transformed cells (Treisman *et al.*, 1981).

The establishment of a direct relation between middle T-antigen and cellular transformation by polyoma virus has intensified interest in the structural and catalytic properties of the protein, since they may lead to an understanding of the mechanisms involved in cellular transformation induced by the virus. Middle T-antigen has been found to be associated with cellular membranes in both lytically infected and transformed cells (Ito *et al.*, 1977b; Ito, 1979). Further, it has been shown to be associated with a protein kinase activity in immunoprecipitates, with the major phosphate-acceptor species being the antigen itself (Eckhart *et al.*, 1979; Schaffhausen and Benjamin, 1979; Smith *et al.*, 1979). Under some conditions, rat immunoglobulin heavy chain is also phosphorylated (Smith *et al.*, 1979). *In vitro*, the protein kinase activity transfers phosphate from [γ-^{32}P]ATP to tyrosine (Eckhart *et al.*, 1979). This unusual substrate specificity has so far been associated chiefly with protein kinases believed to be involved in cellular transformation or proliferation (Hunter and Sefton, 1981). The activity is severely impaired in essentially non-transforming mutants of polyoma virus, such as the *hrt* mutants (Eckhart *et al.*, 1979; Schaffhausen and Benjamin, 1979; Smith *et al.*, 1979), and the *mlt* mutants dl-23 (Smith *et al.*, 1979), MOP1033 (Templeton and Eckhart, 1982), and Py 1387-T (Carmichael *et al.*, 1982), in which the middle T-antigen is affected by the lesions.

Because of the correlation between the middle T-antigen-associated *in vitro* protein kinase activity and the transforming properties of polyoma virus, the elucidation of the properties of this activity becomes especially important. Most studies to date on T-antigen-associated protein kinase activities have used anti-tumour serum to immunoprecipitate the polypeptides prior to incubation with [γ-^{32}P]ATP. The interpretation of the resulting data has therefore been complicated by the wide and variable range of antibody activities in such sera, which allow the possibility that low level activities against proteins other than the known T-antigens have been responsible for some of the observed *in vitro* protein kinase activities. Indeed, it has previously been reported that other polypeptides are specifically immunoprecipitated by anti-tumour serum (Ito *et al.*, 1977a, 1977b; Hutchinson *et al.*, 1978; Schaffhausen *et al.*, 1978; Silver *et al.*, 1978), and that the sera from individual tumour-bearing animals differ

Table I. Properties of monoclonal antibodies used for immunoprecipitation studies

Antibody[a]	P Ab. No.[b]	T-Antigen recognized	Other polypeptides recognized
αPyC1	701	large, middle, small	–
αPyC4	702	large, middle, small	–
αPyLT1	711	large	–
αPyLT4	712	large	>200 kd
αPyLT7	713	large	–
αPyMT5	721	middle	–
αPyMT7	722	middle	–
αPyMT10	723	middle	–
αPyMT13	724	middle	80 kd
αPyMT16	725	middle	–

[a]From Dilworth and Griffin, 1982
[b]According to recommended nomenclature, Crawford and Harlow, 1982.

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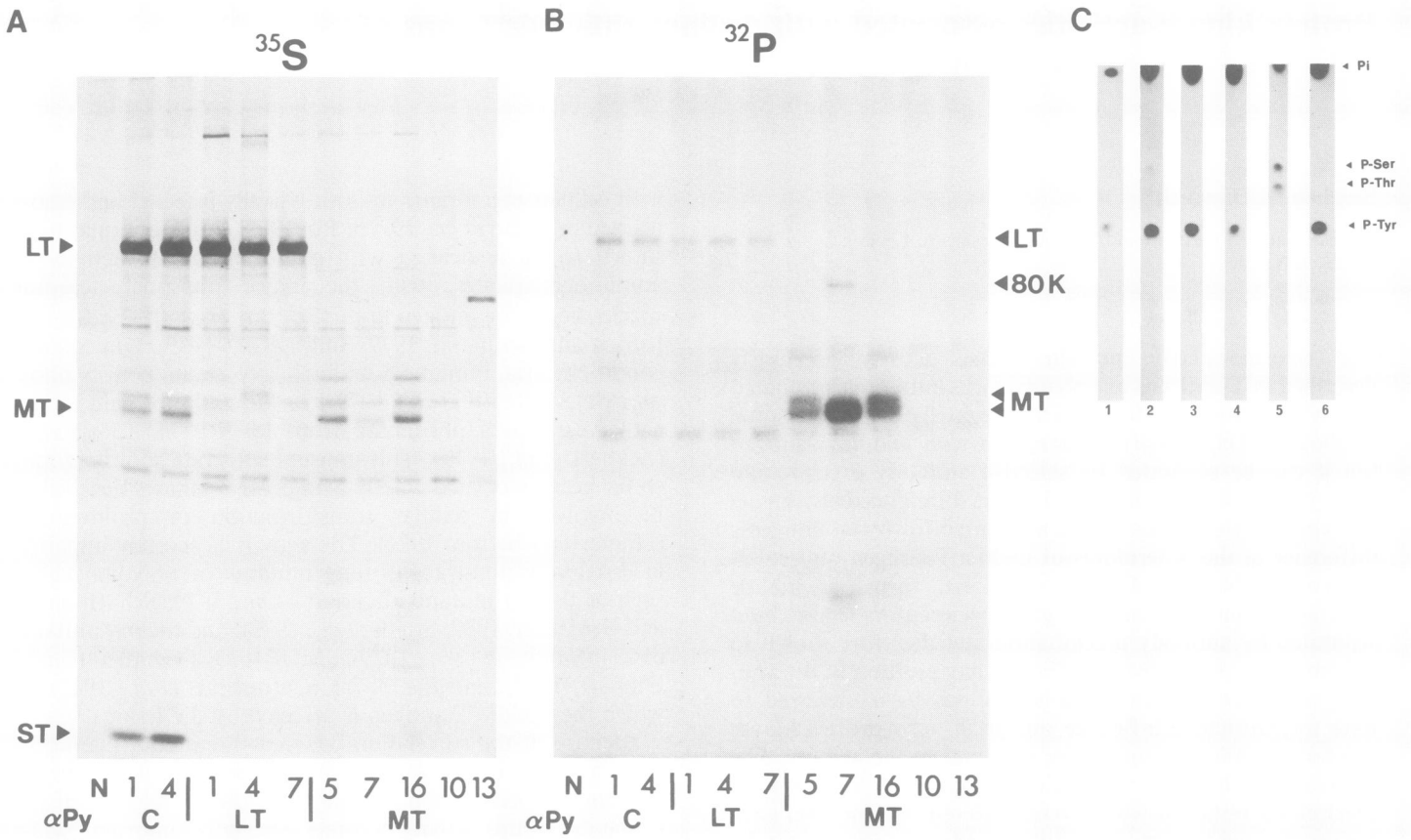

Fig. 1. (A) Polypeptides from 3T6 mouse cells infected with wild-type (A2 strain) polyoma virus, labelled *in vivo* with [^{35}S]methionine, and immunoprecipitated by 10 different monoclonal antibodies. The cells were labelled by incubation with DMEM containing only 10% of the normal methionine concentration plus 5% foetal calf serum and 1 mCi/90 mm dish of [^{35}S]methionine from 24 to 42 h post-infection at 32°C. This long labelling period was chosen to reflect the total polypeptides present. Immunoprecipitations for this experiment and that in **B** were conducted in parallel using SAC plus MRC Ox 12 antibody. The immunoprecipitated polypeptides were separated on a SDS-polyacrylamide gel and visualized by autoradiography as described in Materials and methods. Antibodies used are indicated (underneath); lane N shows immunoprecipitates with the tissue culture fluid from the growth of the myeloma parental cells, NS-1. Positions of the ^{35}S-labelled large (LT), middle (MT), and small (ST) T-antigens are indicated on the left. **(B)** Polypeptides from wild-type polyoma virus-infected 3T6 cells, labelled *in vitro* by incubation with [γ-^{32}P]ATP. Protein kinase reactions were conducted as described in Materials and methods and antibodies are indicated (underneath). The positions of the ^{32}P-labelled large (LT) and the middle (MT) T-antigens, as well as the apparent 80 000 (80 K) mol. wt. polypeptides, are indicated on the right. **(C)** An analysis of the phosphoamino acids present in phosphorylated polypeptides observed in **B**. Phosphoamino acids are from: **column (1)** the 55-kd polypeptide precipitated by αPy MT5; **(2)** 57-kd polypeptide precipitated by αPy MT5; **(3)** 55-kd polypeptide precipitated by αPy MT7; **(4)** 80-kd polypeptide precipitated by αPy MT7; **(5)** 100-kd polypeptide precipitated by αPy C1. **(6)** 57-kd polypeptide precipitated by αPy MT16. The positions of marker phosphoserine (P-Ser), phosphothreonine (P-Thr), phosphotyrosine (P-Tyr), and inorganic phosphate (Pi) are indicated.

in their ability to immunoprecipitate a protein kinase activity (Smith *et al.*, 1980).

In our experiments, we have attempted to resolve these ambiguities by making use of specific monoclonal antibodies directed against polyoma virus T-antigens (Dilworth and Griffin, 1982). Previous studies with monoclonal antibodies indicated that middle T-antigen was not a single molecular species, but could be separated into a number of antigenic forms (Dilworth and Griffin, 1982). Therefore, it was also of interest to determine which of these antigenic forms of middle T-antigen were responsible for the protein kinase activity. The data reported here suggest that the main protein kinase activity can be separated antigenically from the majority of the middle T-antigen molecules; an alteration at the N-terminal end of the polypeptide appears to be responsible for the different response. Moreover, the data indicate that the two types of middle T-antigen molecules observed by Schaffhausen and Benjamin (1981) to act as phosphoacceptors are immunologically distinct species. These polypeptides are present within a cell lysate and are not generated by different *in vitro* protein kinase reactions. The site of attachment of the bound antibody used to immunoprecipitate the middle T-antigen is shown to influence the level of the *in vitro* protein kinase reaction. Finally, these data show conclusively that recognition of the middle T-antigen itself, and not of some other polypeptide, is responsible for the main *in vitro* protein kinase reaction.

Results

Protein kinase activities in immunoprecipitates with monoclonal antibodies

A comparison of the polypeptides immunoprecipitated by 10 different monoclonal antibodies (Dilworth and Griffin, 1982; properties summarized in Table I) from wild-type polyoma virus-infected 3T6 mouse cells is shown in Figure 1. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) separation of polypeptides labelled *in vivo* with [^{35}S]methionine are shown in Figure 1A, and those labelled *in vitro* with [γ-^{32}P]ATP in Figure 1B. The conditions used for immunoprecipita-

tion of the polypeptides were identical in both cases. The αPy MT antibodies are specific for the middle T-antigen polypeptides, but probably recognize various antigenic sub-groups of the protein (Dilworth and Griffin, 1982). The middle T-antigen molecules recognized by αPy MT10 and αPy MT13 antibodies can be seen to be only a very small proportion of the available middle T-antigen molecules (Figure 1A; Dilworth and Griffin, 1982), and no phosphorylation of a polypeptide of the same mol. wt. as middle T-antigen is observed in an *in vitro* phosphorylation reaction (Figure 1B). It seems likely, therefore, that the antigenic sub-groups of middle T-antigen molecules recognized by these antibodies are not involved in the protein kinase activity expressed *in vitro*. αPy MT5 and αPy MT16 antibodies, on the other hand, immunoprecipitate large amounts of the metabolically labelled (55 kd) middle T-antigen molecules (Figure 1A). In an *in vitro* phosphorylation reaction, immunoprecipitates with these antibodies incorporate phosphate into a polypeptide with the same apparent mol. wt., by SDS-PAGE, as middle T-antigen. Phosphate is also incorporated in these immunoprecipitates into a polypeptide with a mol. wt. of 57 kd (Figure 1B). This finding is similar to that reported by Schaffhausen and Benjamin (1981) using anti-tumour serum. αPy MT7 antibody immunoprecipitates, despite containing only approximately half of the metabolically labelled middle

Table II. Quantification of ^{32}P incorporated into polypeptides

Monoclonal antibody used to effect immunoprecipitation	Apparent mol. wt. on SDS-PAGE	Cerenkov (c.p.m.)
αPyC1	100 kd	260
αPyLT1	100 kd	240
αPyMT5	55 kd	550
αPyMT5	57 kd	650
αPyMT5	80 kd	100
αPyMT7	55 kd	3500
αPyMT7	80 kd	250
αPyMT16	55 kd	750
αPyMT16	57 kd	800
αPyMT16	80 kd	90

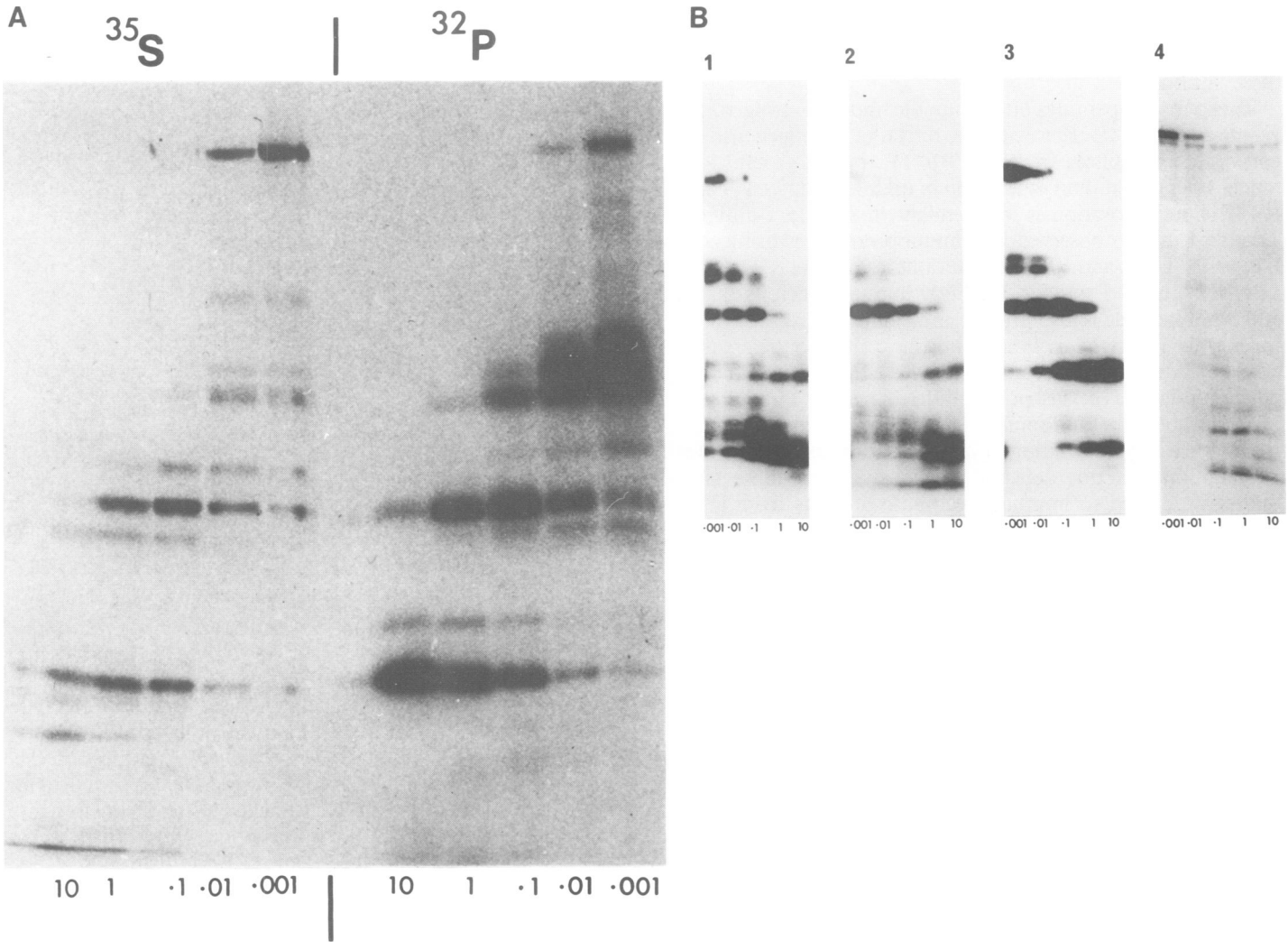

Fig. 2. (A) Fragments produced by limited proteolytic digestion of the [^{35}S]methionine and ^{32}P-phosphorylated labelled middle T-antigen 55-kd species immunoprecipitated with αPy MT7 antibody. Concentrations (in mg/ml) of *S. aureus* V8 protease used in the experiment are indicated (at bottom). **(B)** Comparison by limited proteolytic cleavage of the different phosphorylated polypeptides isolated following *in vitro* protein kinase reactions. The concentrations of *S. aureus* V8 protease (in mg/ml) are indicated underneath. Sample **1** is the 55-kd polypeptide immunoprecipitated by αPy MT5, sample **2** the 57-kd polypeptide immunoprecipitated by αPy MT5; sample **3** the 55-kd polypeptide immunoprecipitated by αPy MT7 and sample **4** (longer exposure), the 80-kd polypeptide immunoprecipitated by αPy MT7.

T-antigen (Figure 1A), incorporate large amounts of phosphate into a polypeptide with the same apparent mol. wt. as middle T-antigen when subjected to an *in vitro* phosphorylation reaction (Figure 1B). No 57-kd polypeptide is visible . Quantitation of the phosphate incorporated into each polypeptide by determination of Cerenkov counts of excised species indicated that approximately six times more phosphate is incorporated into a 55-kd polypeptide in αPy MT7 antibody immunoprecipitates than in those with αPy MT5 or αPy MT16 antibodies , Table II.

Interestingly, a significant difference between the polypeptides labelled metabolically or by a protein kinase reaction is observed in immunoprecipitates with αPy C1 or αPy C4 antibodies. Whereas these antibodies efficiently immunoprecipitate middle T-antigen labelled *in vivo*, very little phosphorylation of a polypeptide of this mol. wt. is observed in immunoprecipitates subjected to an *in vitro* phosphorylation reaction (Figure 1B). After long exposure, a phosphorylated polypeptide of the expected mol. wt. is observed, but phosphate incorporation is extremely low.

Large T-antigen specific monoclonal antibodies, αPy LT1, αPy LT4, and αPy LT7 do not react with middle T-antigen and, as expected, no polypeptide of the same mol. wt. as middle T-antigen is phosphorylated in immunoprecipitates with these antibodies.

Phospho-polypeptides other than the middle T-antigen, are visible in Figure 1B. For example, the large T-antigen also incorporates phosphate from [γ-^{32}P]ATP, but in general to a much lesser extent than that found in middle T-antigen (Table II). This incorporation is independent of middle T-antigen. That is, it is also observed in immunoprecipitates using αPy LT1, αPy LT4, and αPy LT7 antibodies. In the αPy MT5, αPy MT7, and αPy MT16 antibody immunoprecipitates, an 80-kd polypeptide is also present and, similar to results found for middle T-antigen, is found phosphorylated to the greatest extent in immunoprecipitates with the αPy MT7 antibody; phosphorylation of this polypeptide in αPy MT5 and αPy MT16 antibody immunoprecipitates occurs at a low level, and is observed by autoradiography on a long exposure. This polypeptide was not detected previously when these antibodies were used to immunoprecipitate proteins from [^{35}S]-methionine-labelled cells. It does not correspond to any known viral T-antigen, and may be a host protein.

Immunoprecipitations of proteins from the polyoma-transformed rat cell line PyREWA5/T1A1 (Ito *et al.*, 1977b) show the same phosphorylation pattern as that observed with lytically infected mouse cells (see Figure 1B), whereas experiments with non-transformed or uninfected cells reveal no significant phosphorylated polypeptides (data not shown).

Characterization of the polypeptides phosphorylated in vitro

To ensure that the protein kinase reactions observed in Figure 1B are similar to those reported previously, the polypeptides phosphorylated in these reactions have been characterized. Figure 1C shows an analysis of the amino acids phosphorylated in the polypeptides from the gel shown in Figure 1B. The phosphorylated polypeptides were eluted from the polyacrylamide gel, hydrolysed, and the amino acids separated by electrophoresis on thin layer cellulose plates. The 55-kd and 57-kd polypeptides phosphorylated in αPy MT5 antibody immunoprecipitates were found to contain predominantly phosphotyrosine. The 55-kd polypeptide phosphorylated in αPy MT7 antibody immunoprecipitates also contained phosphotyrosine, as did the 80-kd polypeptide

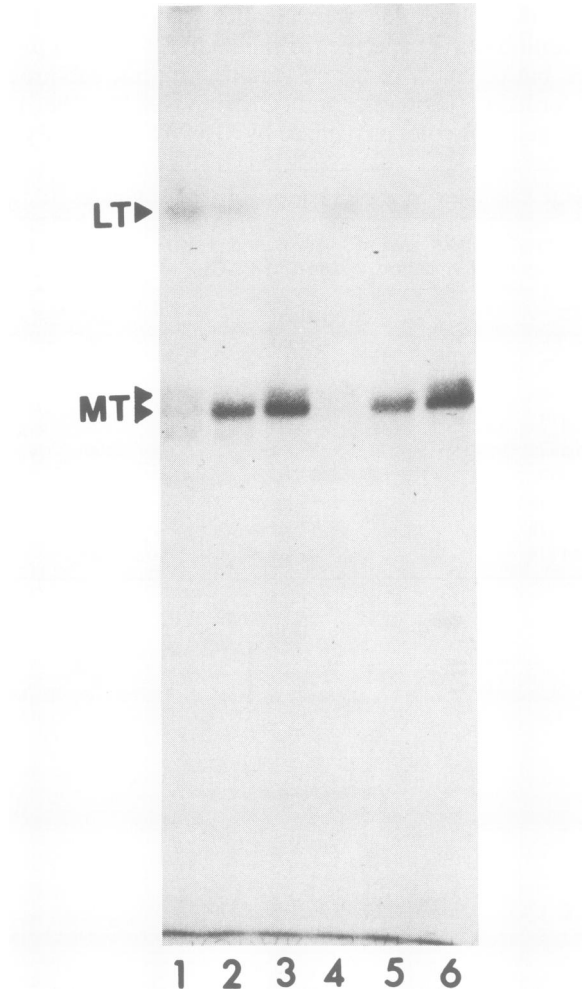

Fig. 3. The polypeptides phosphorylated in an *in vitro* protein kinase reaction after immunoprecipitation with two monoclonal antibodies and SAC plus MRC Ox 12. Autoradiography was for a shorter time than used for Figure 1B experiments, to allow variations in the level of phosphorylation to be detected. The antibodies used were : **lane 1,** αPy C4 and αPy MT5; **2,** αPy C4 and αPy MT7; **3,** αPy MT5 and αPy MT7; **4,** αPy MT5 and αPy LT1; **5,** αPy MT7 and αPy LT1; **6,** αPy MT7 and αPy MT16. The positions of large (LT) and middle (MT) T-antigens are indicated for each gel (on the left).

phosphorylated in the same reaction. The large T-antigen, and also the 55-kd polypeptide (data not shown), were phosphorylated on serine and threonine residues to low levels in αPy C1 antibody immunoprecipitates.

The proteins were further analysed by limited proteolytic cleavage, followed by separation of the polypeptides produced on SDS-PAGE. The patterns obtained from these digests are shown in Figure 2. Figure 2A shows the polypeptides obtained from middle T-antigen labelled with [^{35}S]methionine and the ^{32}P-labelled 55-kd polypeptide phosphorylated *in vitro* in immunoprecipitates with αPy MT7 antibody. The patterns can be seen to be closely related, allowing the conclusion that phosphorylation *in vitro* occurs on middle T-antigen. Figure 2B shows the patterns obtained by limited proteolytic cleavage of the various polypeptides phosphorylated in the *in vitro* protein kinase reaction. Polypeptides from both the 55-kd and 57-kd phosphorylated species from αPy MT5 antibody immunoprecipitates can be seen to be similar to those obtained from the 55-kd polypeptide phosphorylated in αPy MT7 antibody precipitates. Phosphoryla-

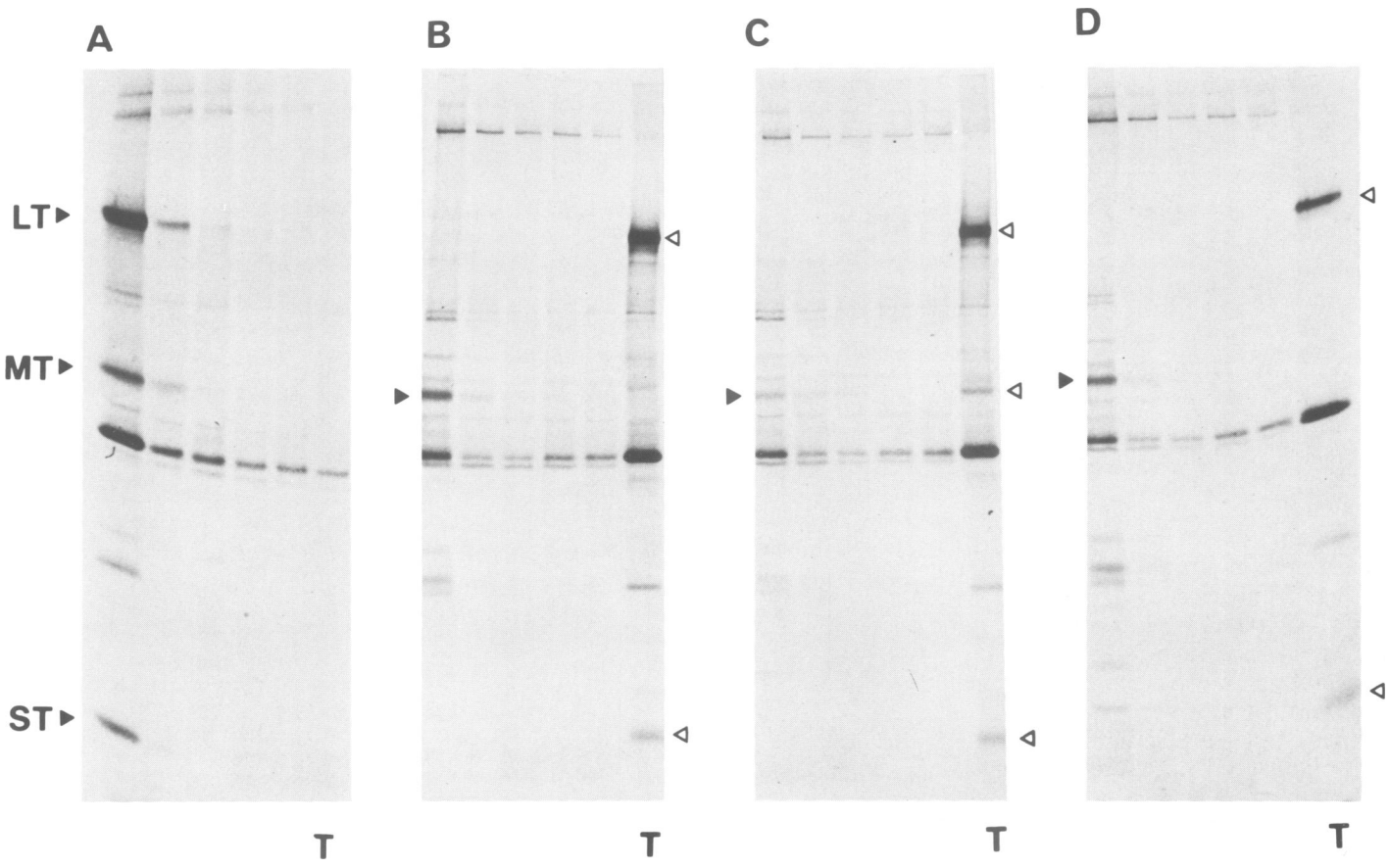

Fig. 4. 'Cascade' immunoprecipitations on [^{35}S]methionine-labelled cell extracts of wild-type polyoma virus infected 3T6 cells. Cells were labelled at 37°C with 1 mCi/90 mm dish of [^{35}S]methionine (≥600 Ci/mmol) in DMEM lacking methionine at 24–28 h post-infection and extracted as described in Materials and methods. An initial and four serial precipitations were carried out with each antibody, and anti-tumour serum (anti-T) then added to immunoprecipitate any remaining antigens, as indicated in each case in the lanes designated (T). The positions of the T-antigens precipitated by the first antibody (solid arrows) are indicated on the left, and those precipitated by the anti-T serum (open arrows) on the right. The initial antibodies used are: **A**, αPy C1; **B**, αPy MT5; **C**, αPy MT7; and **D**, αPy MT16.

tion in αPy MT5 immunoprecipitates is, therefore, also on middle T-antigen. The 80-kd polypeptide phosphorylated in these immunoprecipitates, however, does not give similar polypeptides when cleaved under the same conditions, and is, therefore, probably not related to middle T-antigen.

Further analysis of the in vitro phosphorylation reactions

The failure to observe phosphorylation of middle T-antigen polypeptides after immunoprecipitation with αPy C1 or αPy C4 antibodies is surprising since these antibodies efficiently immunoprecipitate metabolically labelled middle T-antigen. A number of possibilities may account for this observation. The obvious ones are: (a) binding of the αPy C1 and αPy C4 antibodies to the middle T-antigen molecule inhibits the enzymic activity responsible for the phosphorylation reaction; (b) binding of the αPy C1 and αPy C4 antibodies fails to stimulate the enzymic activity necessary for phosphorylating the polypeptides, (whereas αPy MT5, αPy MT7, and αPy MT16 antibodies do stimulate this activity); (c) the components of the *in vitro* protein kinase reaction need to be in a specific orientation in order for the reaction to occur and αPy C1 and αPy C4 antibodies do not produce this orientation; or (d) the antigenic form of the middle T-antigen immunoprecipitated by αPy C1 and αPy C4 antibodies are not components of the *in vitro* protein kinase activity.

To investigate the above possibilities, and particularly to study the possibility of inhibition, the middle T-antigen was immunoprecipitated with two monoclonal antibodies to examine the result of interaction between antibodies. Polypeptides from lysates of lytically infected 3T6 mouse cells were immunoprecipitated using two monoclonal antibodies and subjected to an *in vitro* phosphorylation reaction. Results are shown in Figure 3. The pairs of antibodies used had previously been observed not to block the binding of one another to the same molecule (unpublished data). As a control, αPy LT1 antibodies, which do not react with any middle T-antigen molecules, were used. Reactions in immunoprecipitates using αPy LT1 with αPy MT5 antibodies (track 4) and αPy LT1 with αPy MT7 antibodies (track 5) are similar to those previously observed (Figure 1B). When αPy C4 antibodies were added together with either αPy MT5 or αPy MT7, no effect on the level of phosphorylation (tracks 1 and 2) was observed; therefore, αPy C4 antibody does not appear to inhibit either activity. When αPy MT5 (track 3) or αPy MT16 (track 6) antibodies were added together with αPy MT7 antibodies the same higher level of protein kinase activity previously observed was found; therefore, these antibodies also do not inhibit the protein kinase activity. In the presence of αPy MT5 or αPy MT16 antibodies the 57-kd polypeptide is visible, but at a low intensity; therefore, the ability of the antibodies to immunoprecipitate this polypeptide is a property of αPy MT5 and αPy MT16 and not of the αPy MT7 anti-

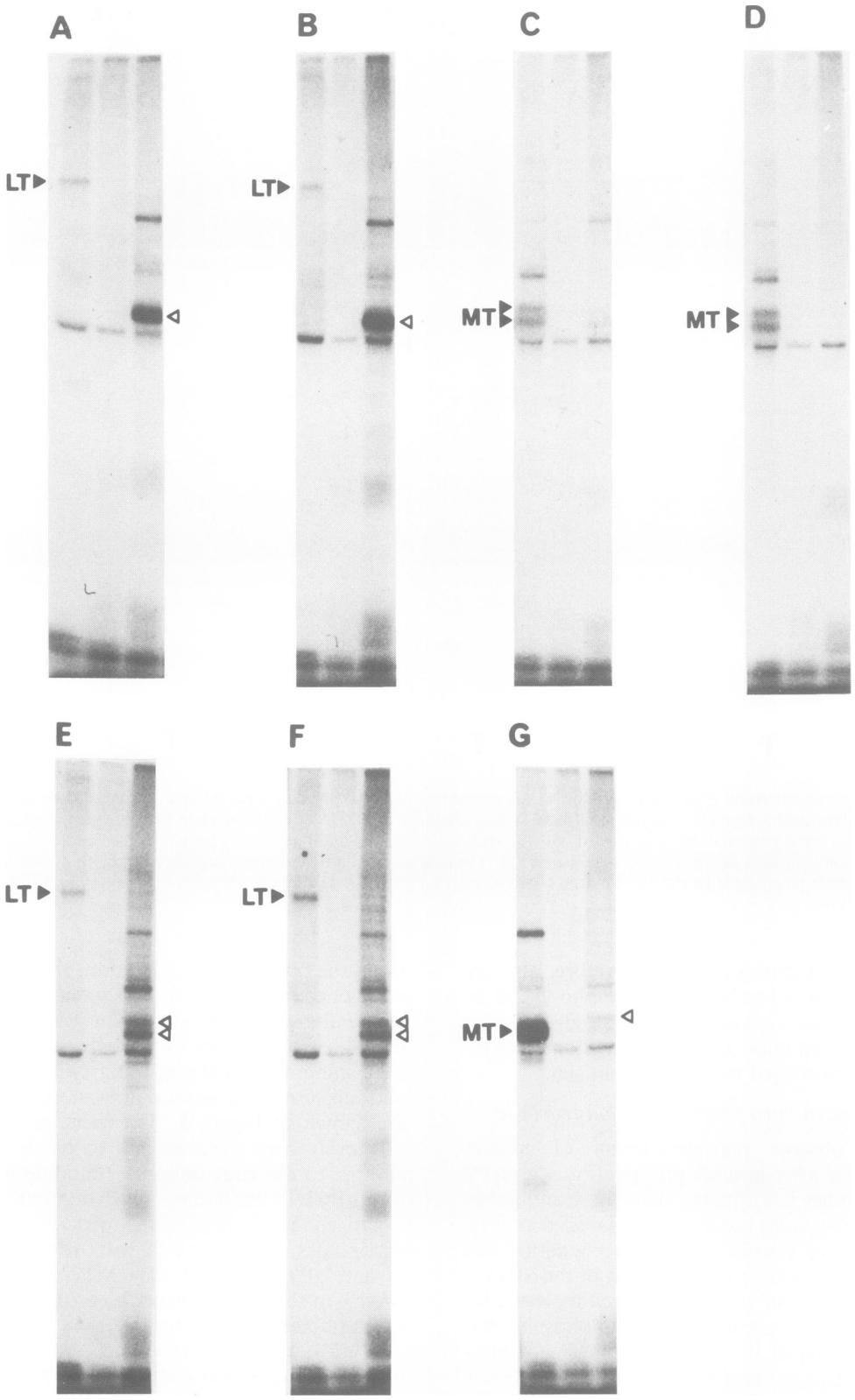

Fig. 5. 'Cascade' immunoprecipitations, followed by an *in vitro* protein kinase reaction. Serial immunoprecipitations were performed and precipitates 1 and 4, as well as the second antibody precipitation, were assayed for protein kinase activity as described in Materials and methods. The phosphorylated T-antigens precipitated by the first antibody (tracks 1 and 2 of each panel) are indicated by solid arrows and those precipitated by the second antibody (track 3) by open arrows. Note that in each case the centre track is essentially blank, indicating complete precipitation by the first antibody. The reactions shown are: **(A)**, αPy LT1 followed by αPy MT7; **(B)**, αPy C1 followed by αPy MT7; **(C)**, αPy MT5 followed by αPy MT7; **(D)**, αPy MT16 followed by αPy MT7; **(E)**, αPy LT1 followed by αPy MT16; **(F)**, αPy C1 followed by αPy MT16; **(G)**, αPy MT7 followed by αPy MT16.

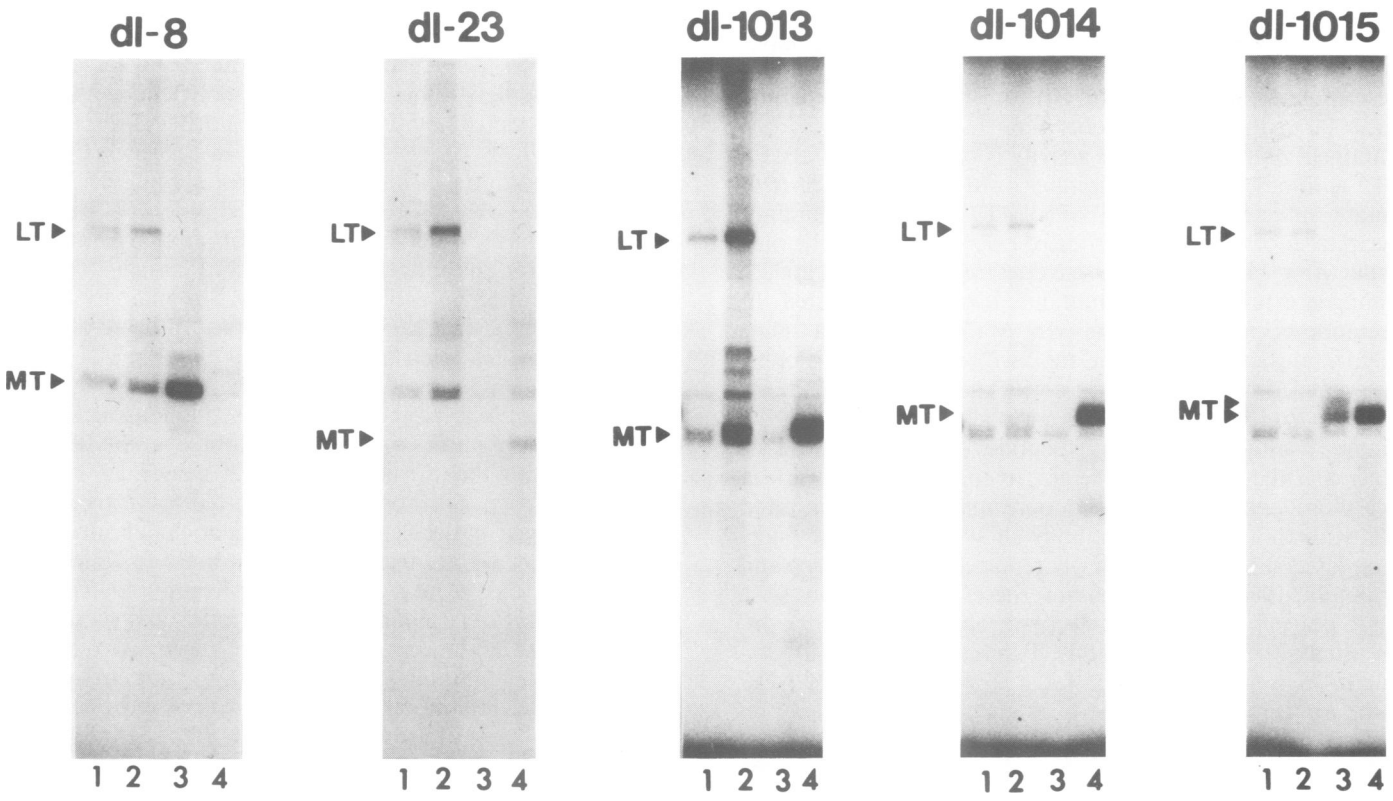

Fig. 6. A comparison of the polypeptides immunoprecipitated from a lysate of 3T6 cells infected with deletion mutants of polyoma virus by four different monoclonal antibodies, as assayed for the *in vitro* protein kinase reaction. The mutant involved is indicated at the top of each gel. The monoclonal antibodies used were: **lanes (1)**, αPy LT1; **lanes (2)**, αPy C4; **lanes (3)**, αPy MT5; and **lanes (4)**, αPy MT7. The position of the expected truncated forms of large (LT) and middle (MT) T-antigens are indicated on the left of each gel.

body. These data suggest that the position on the middle T-antigen to which the precipitating antibody binds influences both the level and nature of the polypeptides phosphorylated in the protein kinase reaction.

To investigate the possibility that αPy C1 and αPy C4 antibodies immunoprecipitate the middle T-antigen species that is normally phosphorylated *in vitro* but do not correctly stimulate, or orientate, the components of the phosphorylation reaction, the polypeptides immunoprecipitated in sequential reactions (a so-called 'cascade' method, Harlow *et al.*, 1981), were compared, after labelling *in vivo* with [^{35}S]-methionine, or *in vitro* with [α^{32}P]ATP. In this experiment, all of the polypeptides recognized by a particular monoclonal antibody are removed from the lysate by immunoprecipitation. Any remaining polypeptides are then subjected to a second antibody. The metabolically labelled polypeptides immunoprecipitated in this manner by four monoclonal antibodies are shown in Figure 4. It can be seen (panel A) that middle T-antigen is precipitated by αPy C1 antibody to such an extent that no detectable antigen is immunoprecipitated upon further treatment of cell lysates with anti-tumour (T) serum. αPy MT5 and αPy MT16 antibodies (panels B and D) are also shown to precipitate all the detectable *in vivo* labelled middle T-antigen, whereas αPy MT7 antibody (panel C) leaves a significant proportion of the middle T-antigen unprecipitated.

Different results are observed when a similar approach is followed by an *in vitro* phosphorylation reaction, however (Figure 5). The results show that αPy C1 (or αPy C4, data not shown) antibody does not appear to remove any component of the middle T-antigen associated protein kinase activity normally observed in immunoprecipitates with αPy MT7 or αPy MT5 antibodies (panels B and F). The activity remaining unprecipitated by αPy C1 antibody was indistinguishable from that observed after αPy LT1 antibody treatment (panels A and E), the latter being an antibody which does not react with middle T-antigen (Dilworth and Griffin, 1982). αPy MT5 and αPy MT16 antibodies, on the other hand, can be seen to precipitate all of the protein kinase activity normally observed in immunoprecipitates with αPy MT7 antibody (panels C and D). The αPy MT7 antibody appears to precipitate most of the 55-kd mol. wt. polypeptide phosphorylated in αPy MT5 antibody immunoprecipitates, but not the 57-kd mol. wt. polypeptide (panel G). This indicates that the αPy MT7 antibody does not bind to the 57-kd polypeptide, and it must, therefore, be an antigenically distinct polypeptide present in cell lysates. Similar results to those observed above are obtained if anti-tumour serum is used as a second antibody in the 'cascade' reactions (data not shown).

Protein kinase activities of truncated middle T-antigen

The polypeptides immunoprecipitated with four monoclonal antibodies from lysates of 3T6 cells infected with deletion mutants of polyoma virus (Griffin and Maddock, 1979; Magnusson and Berg, 1979) and phosphorylated *in vitro* are shown in Figure 6. These mutants have been shown previously (Ito *et al.*, 1980; Magnusson *et al.*, 1981) to encode truncated middle T-antigen polypeptides. Once again, it can be seen that immunoprecipitates with the αPy C4 antibodies show negligible phosphorylation of the middle T-antigen

(compare phosphorylation of middle and large T-antigens, see Figure 1B), despite the fact that these antibodies recognize the truncated middle T-antigen polypeptides expressed by these mutants (Dilworth and Griffin, 1982). Whenever the particular antibody used does not recognize the middle T-antigen, no protein kinase activity is observed (i.e., in αPy MT5 antibody immunoprecipitates of *dl-23, dl-1013, dl-1014* mutant infected cells, and αPy MT7 antibody immunoprecipitates of *dl-8* mutant infected cells). Whenever the particular αPy MT antibody used does react with the middle T-antigen expressed by the mutants, it is phosphorylated. In immunoprecipitates with the αPy MT7 antibody, a single polypeptide with the expected mol. wt. (as indicated) is phosphorylated. Immunoprecipitates with the αPy MT5 antibody from *dl-1015* infected cells show phosphorylation of two middle T-antigen related polypeptides, however, one 2 kd larger and the other the same size as those observed with *in vivo* those labelled cells. Immunoprecipitates of *dl-8* infected cells with the αPy MT5 antibody show only a single phosphorylated species, as previously reported (Schaffhausen and Benjamin, 1981); the *dl-8* mutant, therefore, only expresses a single polypeptide that can be phosphorylated *in vitro*. Interestingly, the truncated middle T-antigen expressed by mutant *dl-23* is phosphorylated (at a low level) in αPy MT7 immunoprecipitates, and the phosphate group found is present on tyrosine (data not shown). In all studies with the mutants, phosphate is also seen to be incorporated into the truncated large T-antigen, though generally at a low level, when it is immunoprecipitated.

Discussion

The tyrosine-specific protein kinase activity associated with the middle T-antigen of polyoma virus is to date the only known enzymic activity that can be correlated with the transforming property of the virus. This activity has been studied *in vitro* using immunoprecipitates from infected cell lysates, and it must be borne in mind that the properties of the protein under these conditions are not necessarily a true reflection of its properties *in vivo*. In the case of the pp60 *src* protein of Rous sarcoma virus, for example, there is evidence that the observed *in vitro* protein kinase activity may be significantly different from the *in vivo* activity (Collett *et al.*, 1980; Hunter and Sefton, 1982). In this regard, it is interesting that middle T-antigen isolated from infected cells phosphorylated *in vivo* does not appear to contain phosphotyrosine (Schaffhausen and Benjamin, 1981), although it contains small amounts of phosphoserine and phosphothreonine.

In order to examine the protein kinase activity associated with polyoma virus middle T-antigen in immunoprecipitates, phosphorylation *in vitro* using the available monoclonal antibodies (Dilworth and Griffin, 1982) has been studied. The findings can be summarised as follows. (1) The monoclonal antibodies αPy C1 and αPy C4 are shown to immunoprecipitate all the detectable [^{35}S]methionine *in vivo* labelled middle T-antigen polypeptides in cell extracts, but to precipitate none of the components of the *in vitro* protein kinase reaction. (2) Antibodies that recognize the viral middle T-antigen, but not the large and small T-antigens (αPy MT5, αPy MT7, and αPy MT16), immunoprecipitate proteins that are able to transfer phosphate from ATP to tyrosine residues. The phosphorylated polypeptides are related to metabolically labelled middle T-antigen, and have the same apparent mol. wt. (55 kd) on SDS-polyacrylamide gels. αPy MT5 and αPy MT16 antibody immunoprecipitates also incorporate phosphate into a polypeptide an apparent 2 kd larger, but related to middle T-antigen. This 57-kd polypeptide has been described previously by Schaffhausen and Benjamin (1981). (3) Quantitatively, there is a difference in the level of phosphate incorporation in immunoprecipitates with αPy MT5, αPy MT7, and αPy MT16 antibodies, considerably more incorporation being observed in αPy MT7 antibody immunoprecipitates. (4) The antigenic sub-groups of middle T-antigen molecules defined by antibodies αPy MT10 and αPy MT13 (Dilworth and Griffin, 1982) do not appear to be involved in the *in vitro* protein kinase activity.

Since αPy MT5, αPy MT7, and αPy MT16 are separate monoclonal antibodies, the fact that protein kinase activity is observed with immune complexes of all three antibodies supports the notion that the activity is closely associated with the middle T-antigen and does not require the presence of any other T-antigen molecule. Although monoclonal antibodies can cross-react with antigens other than those to which they were raised (for a review, see Lane and Koprowski, 1982), it seems unlikely that a similar cross-reaction would occur with three separate antibodies. The absence of *in vitro* protein kinase activity in immunoprecipitates from lysates of cells infected with deletion mutants of polyoma virus in cases where the antibody used does not react with the altered middle T-antigen, also implies a specific need for middle T-antigen to be precipitated in order for the reaction to occur. Antibody activities to proteins other than the middle T-antigen are therefore probably not responsible for the presence of a protein kinase activity in immunoprecipitates. In the light of these data, the phosphorylation of an 80-kd polypeptide in the immunoprecipitates with antibodies specific for middle T-antigen is of interest, although its relevance is not clear. The fact that phosphorylation of this species also occurs on a tyrosine residue suggests a close association between it and the middle T-antigen. Whether the 80-kd polypeptide is a substrate for a middle T-antigen protein kinase activity, or the reverse, or whether both are phosphorylated by some other protein kinase activity remains to be determined.

The failure of αPy C1 and αPy C4 antibody immunoprecipitates to phosphorylate any middle T-antigen polypeptides is noteworthy. Since the protein kinase activity is not altered when αPy C4 antibody (or αPy C1, data not shown) is added together with either αPy MT5 or αPy MT7 antibodies (Figure 3), the αPy C4 antibody is probably not acting as an inhibitor of the activity. The inability of the latter to remove the protein kinase activity observed in both αPy MT7 and αPy MT16 antibody immunoprecipitates in a serial precipitation experiment (Figure 5) suggests, on the other hand, that αPy C1 or αPy C4 antibodies fail to recognize the components of the *in vitro* protein kinase activity, and this property is responsible for no activity being observed in their immunoprecipitates. Since these antibodies recognize all three T-antigens, they must necessarily bind near the N terminus of these polypeptides. Therefore, there is some antigenic difference at the N terminus between the majority of the metabolically labelled middle T-antigen molecules, and the minority of molecules that are responsible for the *in vitro* protein kinase reaction. A number of possibilities exist which could give rise to the observed results. (a) The N-terminal region of middle T-antigen may be post-translationally modified in such a way as to alter the binding of the αPy C1

and αPy C4 antibodies, and it is the unbound form that is the active form in the *in vitro* protein kinase reaction. (b) The middle T-antigen molecules could exist in different tertiary or quaternary conformational forms, and the structure responsible for the *in vitro* protein kinase activity could have the N terminus of the molecule masked by protein interaction in such a way that the binding of the αPy C1 and αPy C4 antibodies does not occur. (c) The middle T-antigen molecules could require to be proteolytically cleaved in order to take part in the *in vitro* protein kinase reaction. In support of this hypothesis, it is known that the auto-phosphorylation associated with many purified protein kinases is often stimulated by a limited proteolysis (for a review, see Flockhart and Corbin, 1982). Moreover, immunoprecipitates using αPy MT antibodies have been found to contain a number of middle T-antigen related polypeptides of varying size, with apparently common C termini; these polypeptides lack the N-terminal region of middle T-antigen and so are not precipitated by the αPy C antibodies (unpublished data). (d) A cellular protein kinase could be involved in the phosphorylation reaction and associated with the middle T-antigen molecules in such a way as to prevent the binding of αPy C1 and αPy C4 antibodies. (The binding of αPy MT antibodies, however, would need not to be inhibited by this protein.) Further studies are necessary in order to distinguish among these alternatives. It is of interest in this context that antibodies raised against a C-terminal peptide of middle T-antigen also immunoprecipitate an *in vitro* protein kinase activity (Walter *et al.*, 1981).

The two forms of middle T-antigen molecules previously described (Schauffhausen and Benjamin, 1981) have been separated during immunoprecipitation. The ability of the αPy MT7 antibody to distinguish between these two forms in a cell lysate indicates the existence of an antigenic difference between these two forms of middle T-antigen molecules. The binding site for the αPy MT7 antibody (Dilworth and Griffin, 1982) may be located in the region of the middle T-antigen molecule responsible for the apparent 2 kd difference in mol. wt. observed on SDS-polyacrylamide gels (Schaffhausen and Benjamin, 1981). The position in which the antibody used to accomplish immunoprecipitation binds to the middle T-antigen molecules is also seen to have considerable influence on the level of activity observed in the *in vitro* protein kinase reaction. The 6-fold difference in the level of phosphorylation observed in αPy MT7 antibody immunoprecipitates relative to those observed with αPy MT5 and αPy MT16 antibodies, appears to be due to a stimulation of the activity by the former rather than to any inhibition of the enzymic activity by αPy MT5 or αPy MT16 antibodies, since the same increased levels of phosphorylation are observed with αPy MT7 when used together with the other two antibodies (Figure 3).

In agreement with previous studies with anti-tumour serum (Smith *et al.*, 1979), data with monoclonal antibodies show that the level of phosphorylation of the truncated middle T-antigen molecules coded for by the deletion mutant *dl-8* appears to be greater than that observed in the corresponding wild-type protein. αPy MT7 antibody does not bind to the truncated middle T-antigen of *dl-8* and no protein kinase activity is observed in αPy MT7 immunoprecipitates. However, in αPy MT5 and αPy MT16 antibody immunoprecipitates, phosphate is incorporated into a single truncated middle T-antigen polypeptide at a significantly higher level than found in an equivalent immunoprecipitate from a wild-type infected cell lysate. The truncated polypeptide coded for by the polyoma mutant *dl-23* was previously shown to incorporate only low levels of phosphate in an *in vitro* protein kinase reaction (Smith *et al.*, 1979). In the present work, this result was confirmed with the αPy MT7 antibody. It is interesting that although the *dl-23* middle T-antigen polypeptide lacks the tyrosine residue (Smolar and Griffin, 1981) previously shown to be phosphorylated in the protein kinase reaction (Schaffhausen and Benjamin, 1981), it is still phosphorylated on a tyrosine residue. This suggest that there is more than one site of tyrosine phosphorylation on the middle T-antigen molecule, or that an alternative site can be used in the absence of the normal phosphorylation site.

The correlation of the protein kinase activity with cellular transformation emphasizes the need for further understanding of this reaction. This will probably require purified middle T-antigen protein for future study of detailed aspects of this reaction. The monoclonal antibodies are currently being used for this purpose. However, the data presented here indicate that various forms of middle T-antigen with varying enzymic activities may exist within cells and, in this context, caution will be needed to ensure that all of the T-antigen protein functions are being studied.

Materials and methods

Cells and viruses

All cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) foetal calf serum (GIBCO). Viral stocks were grown at 32°C on whole mouse embryo cells infected at low multiplicity (0.1 p.f.u./cell) and freeze-thawed twice before use.

Protein kinase reactions

3T6 Swiss Albino mouse cells were infected with polyoma virus at ~50 p.f.u/cell and incubated at 32°C for 40 h. The cells were then washed with ice-cold phosphate buffered saline, scraped from the dish, and lysed in lysis buffer (100 mM Tris HCl, pH 8.5, 100 mM NaCl, 0.5% Nonidet P-40, 0.2 Trypsin Inhibitory Units (TIU)/ml aprotinin (SIGMA)), 1 ml/90 mm dish, at 0°C for 30 min. Nuclei and cell debris were removed from the cell lysate by centrifugation in an Eppendorf microfuge for 1 min. To remove non-specific binding proteins, 50 μl of a 20% (v/v) suspension of fixed *Staphylococcus aureus* Cowan I (SAC) (Kessler, 1975) were added to the lysate. After 30 min at 0°C, the mixture was centrifuged for 5 min and the supernatant recovered. 50 μl of this lysate were then added to 10 μl of tissue culture fluid (TCF) from various monoclonal antibody secreting lines (Dilworth and Griffin, 1982), and the solution incubated at 0°C for 1 h. 10 μl of SAC with 1 μg of MRC Ox 12 monoclonal antibody (an anti-rat K light chain antibody) were added and incubated at 0°C for 30 min. The SAC precipitates were then collected by centrifugation and washed three times with Tris buffered saline (TBS; 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.05% Nonidet P-40). Each pellet was then suspended in 20 μl of 20 mM Tris acetate, pH 7.4, 5 mM manganous acetate, containing 5 μCi of [γ-^{32}P]ATP (>5000 Ci/mmol; Radiochemical Centre, Amersham) and incubated at 30°C for 15 min. Ice cold NET (150 mM NaCl, 50 mM Tris, pH 7.4, 5 mM EDTA, 0.05% Nonidet P-40) (1 ml) was then added and the SAC precipitate isolated. The pellet was resuspended in 50 μl of sample buffer (2% SDS, 10% glycerol, 80 mM Tris, pH 6.8, 5% 2-mercaptoethanol) and incubated at room temperature for 15 min, the suspension was then centrifuged and the supernatant removed and boiled for 5 min. The resulting sample was separated by SDS-PAGE with a 10% acrylamide, 0.27% bis-acrylamide separating gel, and a 5% acrylamide, 0.13% bis-acrylamide stacking gel, using a discontinuous pH buffer system as described by Laemmli (1970). The gel was then dried and autoradiographed for 1–3 days.

[^{35}S]Methionine labelling

Infected cells were labelled by incubating with [^{35}S]methionine (>600 Ci/mmol; Radiochemical Centre, Amersham) at 32°C in DMEM containing only 10% of the normal methionine concentration plus 5% foetal calf serum, between 24 and 42 h post-infection. The cells were then lysed and immunoprecipitated as above, omitting the protein kinase reaction.

Analysis of phosphoamino acids

The identification of the amino acids phosphorylated *in vitro* in the

presence of [γ-^{32}P]ATP was carried out essentially as described by Hunter and Sefton (1980). The phosphorylated polypeptide was eluted from the gel by overnight shaking of the appropriate gel slice with 50 mM $NaHCO_3$, 0.1% SDS containing 25 μg/ml carrier bovine serum albumin (Armour Pharmaceuticals). The polypeptide was precipitated with 20% trichloroacetic acid, hydrolysed with 6 M HCl for 2 h at 110°C under nitrogen and the resulting amino acids separated by electrophoresis in buffer containing pyridine: acetic acid: H_2O in the ratio 5:50:950 (pH 3.5) on thin layer cellulose plates (MN300 coated, 0.1 mm thick; Camlab) for 1 h at 750 V. Radioactive phosphoserine, phosphothreonine, and phosphotyrosine were identified by co-migration with cold standards stained with ninhydrin. Phosphoserine and phosphothreonine were purchased from SIGMA. Phosphotyrosine was a gift of K.Leppard.

Analyses of proteolytic fragments

Polypeptide analysis by limited proteolysis was performed by the method of Cleveland *et al.* (1977), as modified by A.E.Smith (personal communication). A gel fragment (previously dried for identification purposes) containing the ^{32}P-labelled polypeptide was rehydrated in 100 μl of 10 mM $NaHCO_3$, 0.1% SDS, 0.1% 2-mercaptoethanol, 20% sucrose, and homogenised. The resulting slurry was divided into five aliquots and incubated at 37°C for 1 h with 5 μl of *S.aureus* V8 protease (MILES) at a concentration of 10, 1, 0.1, 0.01, and 0.001 mg/ml, respectively. Sample buffer (25 μl, as above) was then added to each aliquot, which was loaded onto a SDS-polyacrylamide gel, using a 15% acrylamide, 0.24% bisacrylamide separating gel, and electrophoresed as above. The gel was then dried and autoradiographed.

'Cascade' immunoprecipitations

'Cascade' immunoprecipitations were performed in a manner similar to that previously described (Harlow *et al.*, 1981). 50 μl of an infected cell lysate were treated with 10 μl of a monoclonal antibody-containing TCF and incubated for 1 h at 0°C. 25 μl of SAC treated with 1 μg of MRC Ox 12 monoclonal antibody were then added and incubated for 30 min at 0°C. The SAC was then pelleted and the procedure repeated on the supernatant a further three times. After the fourth treatment, the supernatant was treated again with SAC plus MRC Ox 12 antibody to remove the last traces of the initial monoclonal antibody, then 10 μl of a different monoclonal antibody-containing TCF, or a rat anti-tumour serum, were added to the supernatant and immunoprecipitated as before. For [^{35}S]methionine-labelled extracts, all five precipitates were washed and separated as above. For protein kinase reaction cascades, the first, fourth, and last precipitates were washed and subjected to a protein kinase assay.

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