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Differential subcellular localization of *in vivo*-phosphorylated and nonphosphorylated middle-sized tumor antigen of polyoma virus and its relationship to middle-sized tumor antigen phosphorylating activity *in vitro*

(plasma membrane/phosphotyrosine/two species of middle-sized tumor antigen/phosphatase treatment)

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ABSTRACT **A small fraction of polyoma virus middle-sized tumor (T) antigen is phosphorylated *in vivo*, resulting in a small amount of phosphotyrosine and phosphothreonine and significantly larger amounts of phosphoserine. When infected cells are separated into nuclear, plasma membrane, and low-speed supernatant fractions, 80–95% of *in vivo*-phosphorylated middle-sized T antigen is localized to the plasma membrane fraction, while 25–50% of [^{35}S]methionine-labeled middle-sized T antigen is found in the nuclear fraction and the same amount is found in the plasma membrane fraction. Immunoprecipitated T antigens contain a protein kinase activity that phosphorylates middle-sized T antigen at tyrosine residues. Eighty to 90% of this activity is located in the plasma membrane fraction. When immunoprecipitated T antigens are treated with alkaline phosphatase, middle-sized T antigen-phosphorylating activity decreases as $^{32}PO_4$ is lost from *in vivo* ^{32}P-labeled middle-sized T antigen. The possibility that *in vivo*-phosphorylated middle-sized T antigen located in the plasma membrane is an active tyrosine-specific kinase is discussed.**

Polyoma virus induces three species of tumor antigen (T antigen): large [100 kilodaltons (kDal)], middle-sized (55 kDal), and small (22 kDal). Of these three, middle-sized T antigen is essential in inducing cell transformation and tumors in animals (reviewed in refs. 1 and 2). The amino-terminal half of large T antigen (3) and possibly small T antigen may have additional effects on the phenotype of transformed cells.

When immunoprecipitated T antigens are incubated with [γ-^{32}P]ATP, middle-sized T antigen is phosphorylated (4–7) as is, in some cases, the IgG of the anti-T antigen serum (5, 7). The only amino acid that is phosphorylated in this reaction is tyrosine (4). The enzyme activity appears to require functional middle-sized T antigen (4–6). In this communication, this enzyme activity will be called MT kinase (middle-sized T antigen-phosphorylating kinase). A good correlation exists between the inability of viral mutants to transform cells and their lack of MT kinase activity (4–6). Mutant viruses altered in middle-sized T antigen that induce faster- or slower-growing transformed cells as colonies in soft agar or dense foci on monolayers have, respectively, higher or lower protein kinase activity. This suggests that the middle-sized T antigen is required in maintaining the transformed state of cells and that the MT kinase is involved in this function (5, 8, 9). In spite of this correlation, there have been some unsettled problems concerning the association of protein kinase activity with middle-sized T antigen (10). The transforming protein of avian sarcoma virus, pp60^{src}, is the most extensively studied protein having an associated protein kinase activity that phosphorylates itself at tyrosine in *in vitro* assay (11). pp60^{src} is phosphorylated *in vivo* at tyrosine as well as at serine (12). The protein itself is generally considered to be a kinase. Despite its *in vitro* tyrosine kinase activity, middle-sized T antigen is reported to be phosphorylated *in vivo* only at low levels (6), and the phosphate is not at tyrosine residues (13). Furthermore, the MT kinase activity is considerably less than that of pp60^{src} of avian sarcoma virus (11), and the overall level of phosphotyrosine in polyoma virus-transformed cells is about the same as that in untransformed cells, while the level in cells transformed by avian sarcoma virus is about 10 times higher than that of untransformed cells (14).

Middle-sized T antigen was first detected in the plasma membrane fraction of mouse cells (15), although it was not clear whether it was present exclusively in the plasma membrane. Subsequently, MT kinase was found concentrated in the plasma membrane fraction (5). The plasma membrane location of middle-sized T antigen suggests that crucial events to convert normal cells to a transformed state may occur at the plasma membrane.

In this communication, we report the results of detailed analysis of *in vivo*-phosphorylated middle-sized T antigen. In particular, we examined phosphate-acceptor amino acids and subcellular localization of *in vivo*-phosphorylated middle-sized T antigen and evaluated their relationship to *in vitro* MT kinase activity.

MATERIALS AND METHODS

Viruses and Cells. The A2 strain of wild-type (WT) polyoma virus (16) and the deletion mutant dl8 (17) were used. Mouse 3T6 cells were grown in Dulbecco's modified Eagle's minimal essential medium (DME medium)/5% fetal calf serum.

Immunoprecipitation. 3T6 cells were infected with WT virus or dl8 at a multiplicity of 10–100 plaque-forming units per cell. Between 24 and 27 hr later, cells were labeled with $^{32}P_i$ (1 mCi/ml; 1 Ci = 3.7×10^{10} becquerels) in DME medium lacking phosphate or with [^{35}S]methionine (100 or 200 μCi/ml) in DME medium lacking methionine and chased for 2 hr in complete medium. T antigens were extracted and immunoprecipitated as described (18, 19) except that the urea step was omitted and hamster anti-T serum (a gift of Kenneth K. Takemoto, National Institutes of Health) was used instead of rat anti-T serum. Relative amounts of T antigen in gels were determined by cutting out appropriate portions and assaying their radioactivity.

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Abbreviations: T antigen, tumor antigen; kDal, kilodalton(s); MT kinase, middle-sized T-antigen-phosphorylating kinase; WT, wild type; DME medium, Dulbecco's modified Eagle's minimal essential medium.

Phosphoamino Acid Analysis. Phosphorylated proteins were electroeluted from the appropriate gel slices, precipitated with 10% (wt/vol) trichloroacetic acid, and hydrolyzed in 6 M HCl at 105°C for 2 hr (20). The hydrolysates were lyophilized and dissolved in 5 μl of electrophoresis buffer [formic acid/acetic acid/H_2O, 25:87:887 (vol/vol, pH 1.9)] and subjected to electrophoresis as the first dimension on cellulose-coated thin-layer plates with a standard marker of phosphoamino acids. Phosphoserine and phosphothreonine were purchased from Sigma. Phosphotyrosine was synthesized by the method of Mitchell and Lunan (21) as described (4, 22). Ascending chromatography in the second dimension was carried out in isobutyric acid/0.5 M NH_4OH, 5:3 (vol/vol).

Cell Fractionation. Polyoma virus-infected 3T6 cells were fractionated according to the method of Warren (23). Briefly, virus-infected cells were swollen in 50 mM Tris·HCl (pH 7.4) containing aprotinin (Sigma) at 253 Kallikrein inhibitor units/ml and phenylmethylsulfonyl fluoride (Sigma) at 0.3 mg/ml and disrupted with a fine-fitted Dounce homogenizer. Under these conditions, no intact cells were observed when examined microscopically. The homogenates were adjusted to 10% sucrose and layered on discontinuous gradients containing 65%, 50%, 45%, 40%, 35%, 30%, and 20% sucrose. The gradients were centrifuged at 1,380 × g for 15 min. Crude plasma membrane fractions were collected from the 35–40% sucrose layer and were further purified on a second discontinuous sucrose gradient. A nuclear fraction was obtained from the 50–65% sucrose layer. In this study, further purification of the nuclei was not attempted. The 10% sucrose fraction was taken as a low-speed supernatant fraction and concentrated with 80% (wt/vol) ammonium sulfate. To immunoprecipitate T antigen, these fractions were adjusted to the extraction buffer conditions (18) and cleared by centrifugation. More than 95% of the Nonidet P-40-extractable T antigens are recovered in these three fractions.

***In Vitro* Protein Kinase Assay.** Unlabeled cell extracts from WT virus- and dl8-infected cells were prepared and immunoprecipitated as described above. Washed immunoprecipitates were suspended in 20 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$ containing 10 μCi of [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham) and incubated at 30°C for 10 min (5). After a kinase reaction, phosphorylated proteins were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis.

Phosphatase Treatment. Immunoprecipitates were mixed with calf intestine alkaline phosphatase (Boehringer) in 100 mM Tris·HCl, pH 8.5/5 mM $MgCl_2$ containing aprotinin at 253 Kallikrein inhibitor units/ml and phenylmethylsulfonyl fluoride at 0.3 mg/ml and incubated at 37°C for 30 min. The samples were washed and then subjected to an *in vitro* kinase reaction, rewashed extensively with 0.15 M NaCl/50 mM Tris·HCl, pH 7.4/50 mM EDTA/0.05% Nonidet P-40, and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis.

RESULTS

Phosphorylation of the T Antigen *in Vivo*. Fig. 1*A* shows three species of WT and dl8 polyoma virus T antigen labeled with [^{35}S]methionine, extracted, and immunoprecipitated from productively infected cells. Cells were labeled for 3 hr and chased for 2 hr in all labeling experiments described here in order to detect modified proteins more easily and to allow proteins to reach cellular organelles where they normally would be before extraction. The large- and middle-sized T antigens of dl8 (94 and 51 kDal, respectively) are smaller than the corresponding WT proteins by about 5 kDal (8). The sizes of the small T antigen of WT and dl8 virus (22-kDal protein) are identical (in the studies described here, the mutant dl8 was used in parallel with WT virus mainly to facilitate the detection of virus-coded proteins). WT middle-sized T antigen has a faint second band above the main band not seen with dl8. This second band is more prominent after chasing for more than 5 hr (unpublished results). Sometimes, however, these two components could not be well separated. These two components were most consistently detected after middle-sized T antigen was phosphorylated *in vitro* in the MT kinase reaction (see below). Under the conditions used, the main band of middle-sized T antigen migrates as a protein of 56,000 kDal while the upper component migrates as one of 59,000 kDal. These two components probably correspond to the two species of middle-sized T antigen described by Schaffhausen and Benjamin (13).

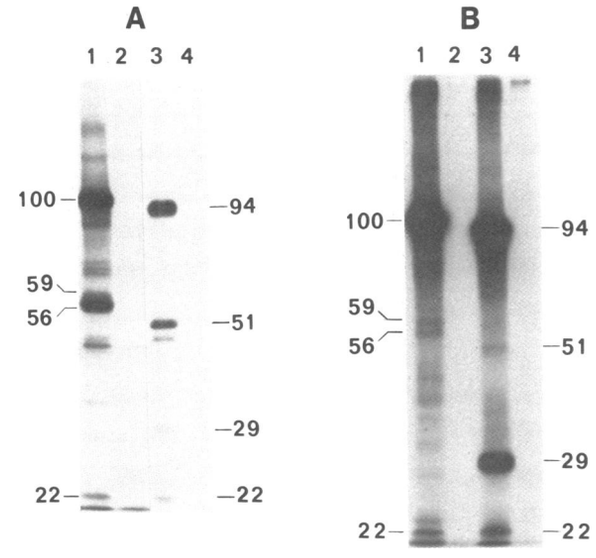

FIG. 1. T antigens of WT polyoma virus and dl8, labeled with [^{35}S]methionine (*A*) or $^{32}P_i$ (*B*) *in vivo*. 3T6 cells were infected with WT polyoma virus (lanes 1 and 2) or dl8 (lanes 3 and 4) at multiplicities of about 10 and 100 plaque-forming units per cell, respectively, and labeled. Twenty microliters of hamster anti-T (lanes 1 and 3) or normal hamster (lanes 2 and 4) serum was added to the cell extracts from about 10^6 cells and the mixtures were incubated for 1 hr at 0°C. Immunoprecipitated proteins were analyzed by 12.5% $NaDodSO_4$/polyacrylamide gel electrophoresis. Numbers on the right and left of the gels indicate molecular masses in kDal.

Fig. 1*B* shows the T antigen of WT and dl8 metabolically labeled with $^{32}P_i$, extracted, and immunoprecipitated from productively infected cells. The large T antigen of WT virus and dl8 were heavily phosphorylated. The 56- and 59-kDal components of WT middle-sized T antigen and 51-kDal component of dl8 middle-sized T antigen were all weakly phosphorylated. The extent of phosphorylation of the 56- and 59-kDal components was comparable, suggesting that the 59-kDal component was phosphorylated to a much higher extent than the 56-kDal component. The $^{32}P/^{35}S$ ratio of the large T antigen was approximately 20 times that of the middle-sized T antigen, suggesting that far fewer phosphate groups were attached to the middle-sized T antigen than to the large T antigen or that only a small population of the middle-sized T antigen was phosphorylated *in vivo* (see below). In addition to the large and middle-sized T antigens, the 22-kDal component was also phosphorylated. This component may be small T antigen, since this protein was not detected in NG-18 infected cells, which lack small T antigen (data not shown). In addition to these T antigens, dl8 induced an additional prominent component of 29,000 kDal (Fig. 1*B*, lane 3). Peptide mapping of the protein showed that the 29-kDal protein was the amino-terminal portion of large T antigen (data not shown).

Phosphoamino Acid Analysis. The large T antigen of WT virus and dl8 contained phosphoserine and phosphothreonine but not phosphotyrosine (Fig. 2, *A1* and *B1*). A similar pattern was observed for the 29-kDal protein of dl8, consistent with it being a truncated large T antigen (Fig. 2 *B3*). The 22-kDal proteins of WT virus and dl8 had mainly phosphoserine (Fig. 2 *A4* and *B4*) and a trace amount of phosphothreonine (detectable only in the original x-ray film). The 56- and 59-kDal species of middle-sized T antigen of WT virus and the 51-kDal dl8 middle-sized T antigen all contained mainly phosphoserine and, in addition, small amounts of phosphothreonine and phosphotyrosine (Fig. 2 *A2*, *A3*, and *B2*). It is noteworthy that phosphotyrosine was detected only in middle-sized T antigen. It is not known how many phosphate acceptor sites are present in middle-sized T antigen for each of the three amino acids. Therefore, it is not clear whether the three kinds of phosphoamino acids are present in a single molecule having the relative amounts shown in Fig. 2 *A2*, *A3*, and *B2* or whether middle-sized T antigen is heterogeneously phosphorylated and some molecules are phosphorylated only at serine.

Intracellular Localization of the T Antigen. [^{35}S]Methionine- and ^{32}P-labeled 3T6 cells infected with WT virus or dl8 were separated into nuclear, plasma membrane, and supernatant fractions, and the T antigens were immunoprecipitated from the extracts of each fraction. The results are shown in Fig. 3 *A* and *B*. About 25–50% and 30–45% of ^{35}S-labeled middle-sized T antigen were detected in the plasma membrane and nuclear fractions, respectively (Fig. 3*A*). Immunoprecipitable 55-kDal protein was also present in the supernatant fraction of WT virus-infected cells in addition to the usual 56-kDal middle-sized T antigen (Fig. 3A, lane 2). In these cell fractionation experiments, it was not clear which fraction contained the 59-kDal protein (Fig. 1). Immunoprecipitable 55- and 48-kDal proteins were detected in the supernatant fraction of dl8-infected cells in addition to the 51-kDal dl8 middle-sized T antigen (Fig. 3A, lane 5). The 55- and 48-kDal proteins are probably breakdown products of large and possibly middle-sized T antigen. In contrast to the ^{35}S-labeled middle-sized T antigen, 80–90% of the *in vivo* ^{32}P-labeled middle-sized T antigen was recovered in the plasma membrane fraction (Fig. 3*B*, lanes 3 and 7). These results suggest that a subpopulation of middle-sized T antigen is not phosphorylated and that the phosphorylated form is specifically localized in the plasma membrane fraction.

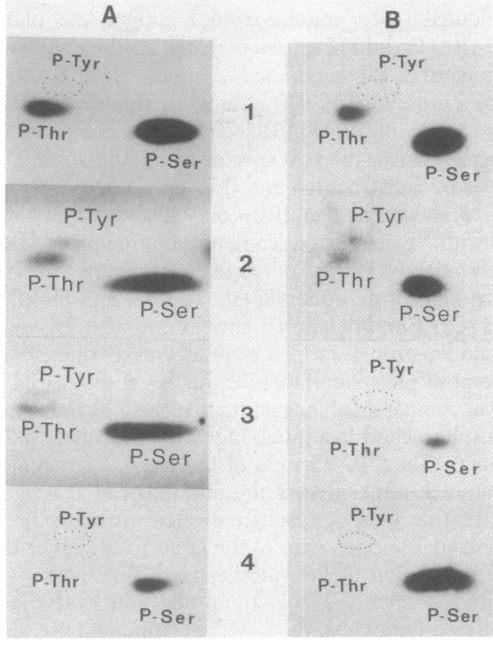

FIG. 2. Phosphoamino acid analysis of polyoma virus T antigens labeled *in vivo*. Phosphoamino acids of ^{32}P-labeled T antigens from WT virus (*A*)- and dl8 (*B*)-infected cells in Fig. 1*B* were analyzed. No spots were detected in the area marked by the dotted line. (*A1*) 100 kDal. (*A2*) 59 kDal. (*A3*) 56 kDal. (*A4*) 22 kDal. (*B1*) 94 kDal. (*B2*) 51 kDal. (*B3*) 29 kDal. (*B4*) 22 kDal. P-Tyr, phosphotyrosine; P-Thr, phosphothreonine; P-Ser, phosphoserine.

Approximately 40–50% of the ^{35}S-labeled large T antigen of WT virus and dl8 was detected in each of the supernatant and nuclear fractions (Fig. 3A, lanes 2 and 3). About 10% of the ^{35}S-labeled large T antigen was recovered in the plasma membrane fraction (Fig. 3A, lanes 1 and 4). In contrast, more than 95% of the phosphorylated large T antigen was recovered in the supernatant fraction (Fig. 3*B*, lanes 1 and 5).

More than 95% of the ^{35}S-labeled small T antigen of WT virus and dl8 and the 29-kDal protein of dl8 was detected in the supernatant fraction (Fig. 3A, lanes 2 and 5). The 22-kDal phosphoproteins of WT virus and dl8 and the phosphorylated 29-kDal protein of dl8 were also present in the supernatant fraction (Fig. 3*B*, lanes 1 and 5). The nature of several components migrating between the 43- and the 26-kDal markers in Fig. 3*B*, lane 1, is not clear. The fact that some species of proteins are present in one fraction and others are in another fraction confirmed that the cell fractionation procedure used was adequate.

Intracellular Localization of the MT Kinase. To test whether there was any relationship between the MT kinase activity and the *in vivo*-phosphorylated middle-sized T antigen, the MT kinase activity in the three fractions was examined. Eighty to 90% of the MT kinase activity was recovered in the plasma membrane fraction as determined by cutting out the bands and determining the radioactivity (Fig. 3*C*, lanes 3 and 9).

In addition to the heavily phosphorylated middle-sized T antigen, carrying out the MT kinase reaction with the plasma membrane fraction resulted in phosphorylation of the 100-,45-, and 40-kDal proteins from WT virus-infected cells and of the 94-, 45-, and 35-kDal proteins from dl8-infected cells (Fig. 3*C*, lanes 3 and 9). They appear to be all virus-coded proteins and all of them contained phosphotyrosine as well as phosphoserine and phosphothreonine (data not shown).

Phosphatase Treatment of the Immunoprecipitates. To evaluate the possibility that the *in vivo*-phosphorylated middle-sized T antigen is essential for the MT kinase activity, the effect of alkaline phosphatase treatment of the immunoprecipitates on the subsequent MT kinase reaction was examined. Under the conditions used, only 70–75% of the ^{32}P in *in vivo*-phosphorylated large and middle-sized T antigens could be removed (Fig. 4*B*), even with high concentrations of alkaline phosphatase (data not shown), probably because of the aggregate nature of the immunoprecipitated proteins. The *in vitro*-phosphorylated middle-sized T antigen was dephosphorylated virtually completely under the phosphatase treatment conditions used (data not shown). Nonradioactive immunoprecipitates were similarly incubated with alkaline phosphatase, washed extensively to remove phosphatase, and then subjected to the MT kinase assay (Fig. 4*A*); total ^{32}P incorporation into middle-sized T antigen decreased to about 42% (lane 4) or 43% (lane 8) of the level with the untreated immunoprecipitates (lanes 2 and 6, respectively). The most conspicuous change was that phosphorylation of the 59-kDal protein of WT virus became undetectable. This decrease in phosphorylated middle-sized T antigen was not due to proteolytic degradation, since the amount of ^{35}S-labeled T antigen (including the 59-kDal species of middle-sized T antigen) was not affected by the phosphatase treatment under the

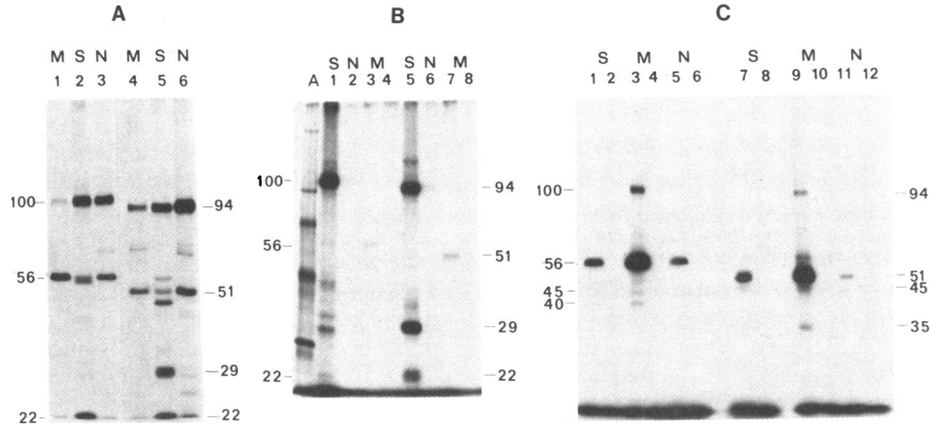

FIG. 3. Intracellular distribution of polyoma virus T antigen and MT kinase activity. Polyoma virus-infected 3T6 cells labeled with [^{35}S]-methionine (*A*) or $^{32}P_i$ (*B*) and unlabeled infected cells (*C*) were separated into plasma membrane (M), low-speed supernatant (S), and nuclear (N) fractions. Each fraction was extracted with 100 mM Tris·HCl, pH 8.0/100 mM NaCl/0.5% Nonidet P-40, and the extracts were immunoprecipitated with hamster anti-T serum (*A*, lanes 1–6; *B*, lanes 1–3 and 5–7; *C*, lanes 1, 3, 5, 7, 9, and 11) or normal hamster serum (*B*, lanes 4 and 8; *C*, lanes 2, 4, 6, 8, 10, and 12). The samples were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis directly (*A* and *B*) or after incubation with [α-^{32}P]ATP (*C*). WT virus: *A*, lanes 1–3; *B*, lanes 1–4; *C*, lanes 1–6. dl*8*: *A*, lanes 4 and 5; *B*, lanes 5–8; *C*, lanes 7–12. Lane A: marker proteins. Numbers on the right and left of the gels indicate molecular masses in kDal.

same conditions (Fig. 4*C*). The decrease was also not due to residual phosphatase as evidenced by the control experiments (Fig. 4*A*, lanes 3 and 7), in which addition of phosphatase just before washing prior to carrying out the MT kinase reaction did not affect MT kinase activity at all. A possible effect on MT kinase activity of residual phosphatase was ruled out by control experiments in which the *in vitro*-phosphorylated middle-sized T antigen was incubated with formalin-fixed *Staphylococcus aureus* (immunoadsorbent) previously incubated with phosphatase for 30 min at 37°C and was not dephosphorylated at all. These results indicate that the phosphorylated protein(s) present in the immunoprecipitates was required for the MT kinase activity.

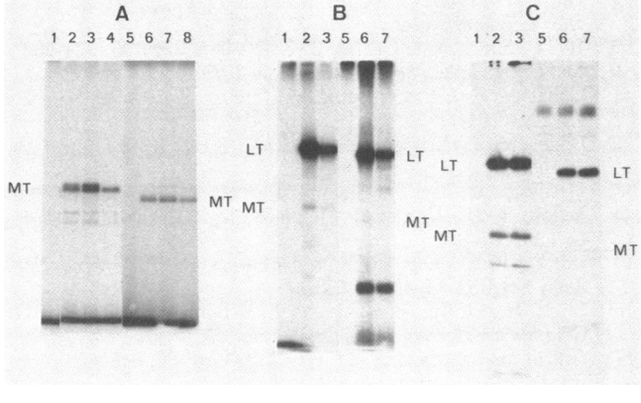

FIG. 4. Effect of phosphatase treatment on MT kinase activity. Cell extracts from unlabeled (*A*), $^{32}P_i$-labeled (*B*), or [^{35}S]methionine-labeled (*C*) WT virus (lanes 1–4) and dl*8* (lanes 5–8)-infected cells were immunoprecipitated with hamster anti-T (lanes 2–4 and 6–8) or normal hamster (lanes 1 and 5) serum. Immunoprecipitates were suspended in phosphatase buffer with (*A*, lanes 4 and 8; *B* and *C*, lanes 3 and 7) or without (*A*, lanes 1–3 and 5–7; *B* and *C*, lanes 1, 2, 5, and 6) 10 units of phosphatase and mixtures were incubated for 30 min at 37°C. (*A*) After incubation, samples were washed extensively with 0.15 M NaCl/50 mM EDTA/50 mM Tris·HCl, pH 7.4/0.05% Nonidet P-40 and subjected to the MT kinase reaction. Ten units of phosphatase was added just before washing without incubation to check the effect of the residual phosphatase on the subsequent kinase reaction (lanes 3 and 7). After the reaction, the phosphorylated materials were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. (*B* and *C*) After incubation with or without phosphatase, respectively, the samples were electrophoresed directly. LT, large T antigen.

DISCUSSION

We analyzed the *in vivo*-phosphorylated middle-sized T antigen with respect to (*i*) phosphoamino acids, (*ii*) subcellular localization, and (*iii*) possible relationship with the MT kinase.

Under some conditions, the middle-sized T antigen migrated as a doublet with a major component of 56 kDal and a minor component of 59 kDal. Detection of two, rather than one, species of middle-sized T antigen was, however, variable. Apart from the observation that the 59-kDal species was a much more efficient phosphate acceptor both *in vivo* and *in vitro*, the nature and functional significance of the 59-kDal species are not clear. Both components are phosphorylated *in vivo*, and each contains small amounts of phosphotyrosine and phosphothreonine and significantly larger amounts of phosphoserine. Although the amounts of phosphotyrosine are small, it is significant that phosphotyrosine has not been found in several other *in vivo*-phosphorylated proteins present in immunoprecipitates, notably including the large T antigen, in spite of the fact that the large T antigen is far more extensively phosphorylated than the middle-sized T antigen. This result suggests that MT kinase may be functional under physiological conditions *in vivo*.

Practically all of the *in vivo* ^{32}P-labeled middle-sized T antigen was found in the plasma membrane fraction, while as much as 50% of the ^{35}S-labeled middle-sized T antigen was present in the nuclear fraction. The results suggest that only middle-sized T antigen in the plasma membrane becomes phosphorylated or that phosphorylated middle-sized T antigen migrates to the plasma membrane preferentially. Precise location of middle-sized T antigen in the nuclear fraction is not known.

Cell fractionation studies also showed that a majority of the MT kinase activity was present in the plasma membrane. Treatment of immunoprecipitated T antigen with alkaline phosphatase reduced the MT kinase activity as it reduced the number of phosphate groups from *in vivo* ^{32}P-labeled middle-sized T antigen, suggesting the importance of the phosphorylated protein in MT kinase activity.

Two models can be proposed to explain the results obtained. (*i*) Nonphosphorylated middle-sized T antigen has no or only low levels of protein kinase activity. Phosphorylation of middle-

sized T antigen activates middle-sized T antigen to be a tyrosine-specific kinase. (*ii*) The enzyme that phosphorylates middle-sized T antigen at tyrosine *in vitro* is cellularly coded but present in immunoprecipitates, and this cellular protein must be phosphorylated to become an active kinase.

In model *ii*, the *in vivo*-phosphorylated middle-sized T antigen either has nothing to do with MT kinase or at most it is a good phosphate acceptor. The fact that antibodies raised against a synthetic polypeptide corresponding to the COOH-terminal portion of middle-sized T antigen (24) also precipitate the MT kinase activity suggests that the putative cellular kinase predicted by model *ii* must be bound to middle-sized T antigen. If this putative tyrosine-specific kinase is bound to middle-sized T antigen *in vivo*, *in vivo* phosphorylation of middle-sized T antigen should occur at tyrosine to the same extent as in *in vitro* phosphorylation. However, middle-sized T antigen is phosphorylated at tyrosine only at very low levels *in vivo* (Fig. 2 *A2*, *A3*, and *B2*). We directly tested whether we could remove this putative cellular kinase by high concentrations of NaCl. Washing the immunoprecipitates with buffer containing NaCl at concentrations as high as 2 M prior to the MT kinase assay did not alter the MT kinase activity at all (data not shown). It is unlikely, therefore, that a cellular protein responsible for the MT kinase is associated with middle-sized T antigen by ionic bonds. Therefore, model *ii* seems to be improbable. The most likely sequence of events would therefore involve the phosphorylation of middle-sized T antigen at serine only or serine and threonine residues by a cellular enzyme probably present in the plasma membrane to activate middle-sized T antigen as a tyrosine-specific kinase. The phosphorylated middle-sized T antigen then phosphorylates middle-sized T antigen itself (inter- or intramolecularly) and probably some cellular proteins specifically (*in vivo*) or even nonspecifically (*in vitro*).

Our results also suggest that at least a part of the reason why middle-sized T antigen is not phosphorylated extensively *in vivo* is due to the fact that not all of the middle-sized T antigen is in the plasma membrane. Since it is not known how many phosphate acceptor sites are present in middle-sized T antigen molecules for each of the three acceptor amino acids, it is not clear whether all of the middle-sized T antigen present in the plasma membrane is phosphorylated *in vivo*. If we assume, however, that polyoma virus large T antigen has four phosphate acceptor sites, as reported for simian virus 40 large T antigen (25), which shares extensive amino acid sequence homology with polyoma virus large T antigen (reviewed in ref. 1), we can estimate the degree of phosphorylation of middle-sized T antigen based on the data shown in Fig. 3 *A* and *B*. Amounts of phosphate in *in vivo*-labeled T antigen in the supernatant fraction relative to those in *in vivo*-labeled middle-sized T antigen in the plasma membrane fraction were determined by cutting out gel slices and determining the radioactivity. It was found that the large T antigen in the supernatant fraction has approximately 10 times more ^{32}P radioactivity than the middle-sized T antigen in the plasma membrane per molecule (large and middle-sized T antigen contain 15 and 17 methionine residues, respectively). On the average, therefore, approximately 40% of the middle-sized T antigen in the plasma membrane fraction would have a single phosphate group. If some molecules have more than one phosphate group, the proportion of phosphorylated middle-sized T antigen would decrease accordingly. If our assumption is correct, this observation may be another reason for the observed low level of MT kinase activity compared with that of $pp60^{src}$ of avian sarcoma virus.

The abundance and higher specific activity of the MT kinase activity in the plasma membrane fraction suggest that this is the site where middle-sized T antigen exerts its function, possibly to convert normal cells to transformed states, and that the low level of the MT kinase activity is sufficient for transformation. The low level of the MT kinase may account for the observation that the overall level of phosphotyrosine was not higher in polyoma virus-transformed cells than in untransformed cells (14).

Significant amounts of large T antigen were consistently detected in the plasma membrane fraction in the present studies. It has been reported that large T antigen is not detected in the plasma membrane when cells infected with tsa (an early mutant) are used as a source of T antigen (18). The difference is most likely due to the fact that the large T antigen of tsa is thermolabile (19) and, therefore, large T antigen of tsa radiolabeled at the nonpermissive temperature either did not reach the plasma membrane before it was degraded or lost the ability to associate stably with the plasma membrane. It is of interest to see if large T antigen in the plasma membrane has any physiological role.

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