Characterization of Middle T Antigen Expressed by Using an Adenovirus Expression System

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The adenovirus Ad5(pymT) has been used to express middle T antigen at very high levels in 293 cells. The middle T antigen produced was localized to membranes and was modified in the same way as that expressed in polyoma virus-infected mouse cells. It was phosphorylated *in vivo* on serine residues and *in vitro* on tyrosine residues. The *in vivo* phosphorylations occurred between residues 223 and 275. The middle T antigen encoded by Ad5(pymT) was phosphorylated *in vitro* in a complex with human pp60^{c-src}. Interestingly, the extreme overexpression of middle T antigen did not cause a parallel increase in the amount of complex; most of the pp60^{c-src} remained unassociated. Immunoaffinity purification resulted in approximately 100 µg of middle T antigen from a 100-mm tissue culture dish. Several cell proteins copurified with the Ad5(pymT)-derived middle T antigen. Two of these, the 74- and 63-kilodalton species, are of particular interest because they were also purified from mouse tumors expressing middle T antigen.

Middle T antigen is a central element in polyomavirus-mediated transformation (14). A major obstacle to the elucidation of its action has been the absence of a rich source of protein. The hybrid adenovirus-polyomavirus Ad5(pymT) just described (1) provides such a source. However, the value of this Ad5(pymT) vector or any expression system depends upon how similar the protein being expressed is to that found in its original environment. One general concern is that patterns of postsynthetic modifications may differ. For Ad5(pymT), not only the very high level of expression itself but also adenovirus-mediated shutoff of host transcription and translation may affect how the cell modifies middle T antigen. This work demonstrates that middle T antigen produced in Ad5(pymT)-infected 293 human cells is qualitatively identical to that from polyomavirus-infected mouse cells. Moreover, the behavior of middle T antigen under conditions of very high-level expression provides some insight into its normal functioning.

MATERIALS AND METHODS

Cells and viruses. Polyomavirus stocks were prepared on 3T6 cells grown in Dulbecco modified Eagle medium supplemented with 5% calf serum. Wild-type polyomavirus NG59RA was prepared from *hr-t* mutant NG59 by marker rescue (8). Adenovirus stocks were prepared on 293 cells grown in Dulbecco modified Eagle medium with 10% fetal calf serum. Ad5(pymT) and Ad5(309) are described in the accompanying paper (1).

Antisera. Three sera were used for immunoprecipitation of middle T antigen. Anti-T ascites fluid was extracted from Brown Norwegian rats bearing a mixed solid-ascites tumor as described previously (19). Rabbit serum was prepared from animals immunized with small t antigen (12a). Finally, monoclonal antibody pAB 815 directed against middle T antigen was used to prepare an affinity gel by being coupled to Affigel 10 (Biorad) as described previously (11). The

anti-pp60^{c-src} monoclonal GD11 was used for immunoprecipitation of pp60^{c-src} (13).

T-antigen analysis. The methods for radiolabeling cells, T-antigen extraction, and immunoprecipitation were those previously published (15-19). For 293 cells, supernatants from each wash were saved and centrifuged at 1,000 × *g* to collect detached cells. For pulse-labeling with [³⁵S]methionine, cells were incubated in Hanks salts containing 50 µCi of [³⁵S]methionine (New England Nuclear Corp.) per ml for 90 min; for pulse-labeling with ³²PO₄ cells were incubated in phosphate-free medium at 200 to 400 µCi/ml for 90 min. T antigens were routinely extracted in Nonidet P-40-containing buffer (0.137 M NaCl-0.01 M Tris hydrochloride [pH 7.0]-0.001 M MgCl₂-0.001 M CaCl₂-10% [vol/vol] glycerol-1% [vol/vol] Nonidet P-40). Cleared extracts were incubated with antisera and protein A-Sepharose (Pharmacia, Inc.) for 1 h. For protein kinase reactions, washed immunoprecipitates were incubated in 0.02 M Tris hydrochloride (pH 7.5)-0.005 M MnCl₂ with 5 to 25 µCi of [^γ-³²P]ATP for 15 min at room temperature.

Partial proteolysis has also been described previously (16, 17). Proteins to be analyzed were resolved on cylindrical discontinuous buffer sodium dodecyl sulfate (SDS)-gels containing 10% acrylamide. The cylinders were placed on a 12.5% slab gel, and digestion was carried out by overlaying the gel with enzyme solution. Electrophoresis was carried out at 50 V until the bromophenol blue reached the bottom of the gel.

Phosphoamino acid analysis. Proteins were extracted from gels and SDS was removed by using the methods of Weiner et al. (23). After extraction in 0.05% NaHCO₃-0.1% SDS-1 mM dithiothreitol, the proteins were precipitated with 9 volumes of acetone. The precipitates were washed twice with acetone and dried. Resuspended proteins were analyzed by partial acid hydrolysis and two-dimensional phosphoamino acid mapping (10). Hydrolysis was carried out for 2 to 4 h in 6 N HCl at 110°C. The hydrolysates were lyophilized twice. Samples were then electrophoresed on cellulose plates (Merck & Co., Inc.) at pH 1.9 (acetic acid, 88% formic acid, distilled water [78:25:897, vol/vol/vol]) for

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FIG. 1. Ad5(pymT) middle T antigen is localized to membranes and is associated with pp60^{c-src}. Left: 293 cells infected with Ad5(pymT) were labeled with [³⁵S]methionine for 90 min at 18 h after infection. Cells were separated into nuclear (lane N), cytoplasmic (lane C), and membrane (lane M) fractions as described in Materials and Methods. Portions of each fraction were compared with those of total protein (lane T) on a discontinuous buffer SDS-gel of 10% acrylamide. The position of middle T antigen is indicated by the arrowhead; for comparison, a cytosolic protein specific to adenovirus-infected cells is indicated by the horizontal line. Right: middle T antigen was immunoprecipitated from Ad5(pymT)-infected 293 cells with either anti-T ascites (A) or anti-pp60^{c-src} monoclonal GD11 (13) (lane B). The immunoprecipitates were labeled in vitro with [γ -³²P]ATP (15). The minor band just below middle T antigen is a proteolytic fragment lacking a portion of the C-terminus (17).

6 h at 500 V or 4 h at 800 V. Dried plates were then chromatographed with isobutyric acid–0.5 M NH₄OH(5:3, vol/vol). Phosphoamino acid standards were visualized by ninhydrin staining (0.25% in *n*-butanol).

Cell fractionation. Ad5(pymT)-infected 293 cells were extremely rounded and were easily detached. For cell collection, supernatants were saved and cells were harvested at 1,000 $\times$ *g* in a Beckman TJ6 centrifuge. In addition, cell monolayers were harvested with a rubber policeman. Cells were washed in phosphate-buffered saline and then quickly washed in reticulocyte-suspending buffer (RSB; 0.01 M Tris hydrochloride [pH 7.4], 0.01 M KCl, 0.0015 M MgCl₂). Cells were then suspended in RSB and allowed to swell. After 15 min, the cells were broken with a Dounce homogenizer until 90 to 95% were broken as judged by using the phase-contrast microscope. Nuclei and unbroken cells were removed by centrifugation at 2,000 $\times$ *g* for 2 min in an International 269 centrifuge. Membranes were then separated from the cytosolic fraction by centrifugation at 10,000 $\times$ *g* for 10 min.

RESULTS

Middle T antigen expressed in Ad5(pymT) cells is found in cell membranes and is associated with pp60^{c-src}. Middle T antigen is associated with cell membranes by a C-terminal hydrophobic sequence (3). To determine whether dramatic overproduction of middle T antigen affected its localization, simple cell fractionation was performed. Infected human 293 cells labeled with [³⁵S]methionine were lysed after hypotonic shock. Nuclei and membranes were collected by differential centrifugation. Portions of each fraction were analyzed by SDS-polyacrylamide gel electrophoresis; the identification of middle T antigen was confirmed by peptide mapping. Most middle T antigen was associated with the membrane fraction (Fig. 1, left). Immunofluorescence showed an appar-

ent perinuclear fluorescence, observed previously by others (22), which probably represents association with the endoplasmic reticulum (not shown).

Ad5(pymT) middle T antigen can be phosphorylated in vitro but has a reduced specific activity judged by ³⁵S/³²P ratios (1). The kinase activity phosphorylating middle T antigen presumably comes from pp60^{c-src} (2, 5–7). Figure 1, right, demonstrates the middle T antigen-pp60^{c-src} complex directly. Both immunoprecipitates made with anti-T ascites or anti-pp60^{c-src} monoclonal GD11 (13) showed middle T antigen when phosphorylated in vitro. Mapping the phosphorylated products precipitated by anti-pp60^{c-src} with *Staphylococcus aureus* V8 protease showed the characteristic 24- and 18-kilodalton (kDa) C-terminal V8 fragments of middle T antigen (Fig. 2, left, lanes C and D). The reduced specific activity of Ad5(pymT) middle T antigen in the in vitro kinase reaction could arise from lack of sufficient pp60^{c-src}. Figure 2 shows that this is not the case. pp60^{c-src} is phosphorylated in vitro on tyrosine, giving rise to a characteristic C-terminal V8 peptide of 26 kDa (Fig. 2, left, lane B). (5). Phosphorylation in the middle T antigen-pp60^{c-src} complex does not result in this phosphorylation (Fig. 2, left, lane A), so that its presence measures unassociated pp60^{c-src}. The amount of the 26-kDa peptide was largely unaffected by infection with Ad5(pymT), indicating that most of the pp60^{c-src} remained uncomplexed with middle T antigen. This was confirmed by the observation that very little pp60^{c-src} could be detected in association with middle T antigen precipitated with anti-T serum (Fig. 2, right). Since there is considerable evidence that interaction of the two proteins is regulated (2, 4, 6; B. Bockus, manuscript in preparation), it is more likely that whatever system regulates the interaction between the two proteins is saturated.

Middle T antigen is found in a major 56-kDa species and a minor 58-kDa form. Chymotryptic mapping (Fig. 3) readily distinguishes between them: the 56-kDa form gives rise to 37- and 31-kDa species, and the 58-kDa form yields 39- and

FIG. 2. In vitro phosphorylation of middle T antigen and its association with pp60^{c-src}. Left: mapping of middle T antigen and pp60^{c-src} labeled in vitro with [γ -³²P]ATP. pp60^{c-src} was immunoprecipitated from mock-infected 293 cells (lane B) with anti-pp60^{c-src} antibody GD11. Middle T-pp60^{c-src} complex was immunoprecipitated from Ad5(pymT)-infected 293 cells with either anti-T (lane A) or anti-pp60^{c-src} at 16 (lane C) or at 20 h postinfection (lane D). The immune complexes were labeled in vitro with [γ -³²P]ATP and mapped with *S. aureus* V8 protease as described in the text. The 26-kDa peptide from pp60^{c-src} and the 24/18-kDa peptides from middle T antigen are indicated by the arrowheads. Right: Immunoprecipitation of pp60^{c-src} from 293 cells infected with Ad5(pymT). Cells were labeled with ³²P_O₄ for 90 min. pp60^{c-src} and middle T antigen were immunoprecipitated from the extracts with either anti-T ascites (lane A) or anti-pp60^{c-src} (lane B). The positions of middle T antigen and pp60^{c-src} are indicated by the arrowheads.

33-kDa fragments (16, 17). Digestions of in vitro ^{32}P -labeled middle T antigen from Ad5(pymT) or polyomavirus-infected 3T6 cells were qualitatively identical. This is consistent with the V8 mapping and indicates that same tyrosine sites are phosphorylated. However, the relative amount of the 58-kDa form present in Ad5(pymT)-infected cells was substantially reduced judged by the labeling of the 39/33-kDa and 37/31-kDa peptides. Previous work has shown that the level of the 58-kDa relative to the 56-kDa form is similarly reduced in polyomavirus-transformed cells (16) as well as for middle T antigen expressed in CV1 cells by using simian virus 40 vectors (24). It was not clear whether the reduced levels of the 58-kDa form reflect the intracellular situation or the presence of phosphatase activities in cell extracts. Because of the high level of expression, cells could be treated directly at 100°C with solutions containing SDS and middle T antigen could be identified without immunoprecipitation. Although this should have reduced problems with phosphatases, chymotryptic mapping nonetheless demonstrated principally the 56-kDa form of middle T antigen and little of the 58-kDa form (not shown).

In vivo $^{32}\text{PO}_4$ labeling of Ad5(pymT)-infected cells. The major 56-kDa and minor 58-kDa forms of middle T antigen are differentially phosphorylated in vivo. This phosphorylation is principally on serine residues, although limited amounts of phosphothreonine and phosphotyrosine have also been observed (20). Ad5(pymT)-infected 293 cells and polyomavirus-infected 3T6 mouse cells were labeled with $^{32}\text{PO}_4$. Approximately 40-fold more phosphorylated middle T antigen was immunoprecipitated from 293 cells than from an equivalent number of polyomavirus-infected 3T6 cells (Fig. 4a). This parallels the observed increase in [^{35}S]methionine (1). The phosphate was principally esterified to serine, but some phosphothreonine was also observed (Fig. 4c). A very limited amount of phosphotyrosine may also be present. By contrast, Ad5(pymT) middle T antigen labeled in vitro is phosphorylated on tyrosine (not shown).

In vivo serine phosphorylation of middle T antigen in mouse cells or in Ad5(pymT) cells occurs between amino acids 225 and 275, proximal to the major tyrosine phosphorylation sites at amino acids 315 and 322. Hydroxylamine

FIG. 3. Chymotryptic digestions of middle T antigen labeled in vitro with [γ - ^{32}P]ATP. Middle T antigen from wild-type polyomavirus-infected 3T6 cells (left) or Ad5(pymT)-infected 293 cells (right) was immunoprecipitated with anti-T serum and labeled in vitro. The positions of undigested middle T antigen (56- and 58-kDa species) are shown by the vertical lines; the positions of the diagnostic 39/33-kDa and 37/31-kDa peptides are indicated by the arrowheads.

FIG. 4. Analysis of Ad5(pymT)-derived middle T antigen labeled in vivo with $^{32}\text{PO}_4$. (a) The sequence of middle T antigen showing the in vitro tyrosine phosphorylation sites (vertical lines) and the region of in vivo serine phosphorylations (4, 16). The positions of the major V8 and hydroxylamine fragments are indicated below the sequence. (b) $^{32}\text{PO}_4^{3-}$ labeling in vivo of middle T antigen. 3T6 cells infected with wild-type polyomavirus or 293 cells infected with Ad5(pymT) were labeled with $^{32}\text{PO}_4$ for 90 min starting at 22 h after infection, and T antigens were extracted and immunoprecipitated. Lanes: 1, middle T antigen from one 60-mm dish of 3T6 cells; 2, middle T antigen from 1/10 as many 293 cells as 3T6 cells; 3, middle T antigen from an equal number of 293 cells. (c) Phosphoamino acid analysis of middle T antigen from Ad5(pymT)-infected 293 cells. The phosphoamino acids in vivo $^{32}\text{PO}_4$ -labeled middle T antigen were analyzed as described in Materials and Methods. The positions of the unlabeled phosphoamino standards are indicated by the circles. (d) Hydroxylamine mapping of middle T antigen phosphorylated in vivo in Ad5(pymT)-infected 293 cells. Lanes: A, in vitro [γ - ^{32}P]ATP-labeled middle T antigen from infected 3T6 cells; B, Ad5(pymT)-derived middle T antigen labeled in vivo with $^{32}\text{PO}_4$ in 293 cells; C, [^{35}S]methionine-labeled middle T antigen from polyomavirus-infected 3T6 cells. The N-terminal (N) and C-terminal (C) fragments of 56-kDa middle T antigen as well as the C-terminal fragment (C') derived from the 58-kDa form of middle T antigen are indicated by the arrowheads. (e) V8 digests of in vitro [γ - ^{32}P]ATP-labeled middle T antigen from infected 3T6 cells (lane PY) and in vivo $^{32}\text{PO}_4$ -labeled middle T antigen from Ad5(pymT)-infected 293 cells (lane AD). The C-terminal 24- and 18-kDa V8 fragments are indicated by the arrowheads.

cleavage of [^{35}S]methionine-labeled middle T antigen at residue 223 gives N-terminal (N) and C-terminal (C) half-molecule fragments. The 58-kDa species yields an altered C-terminal fragment (C'). Middle T antigen from Ad5(pymT)-infected cells labeled with $^{32}\text{PO}_4$ in vivo gave pri-

FIG. 5. Immunoaffinity purification of middle T antigen from 293 cells. Middle T antigen from Ad5(pymT)-infected 293 cells (lane A) or tumor cells (lane B) was purified by chromatography on Affigel 10 to which monoclonal pAB 815 against middle T antigen was bound (11). The bound protein was released with buffer containing 3.5 M KSCN, run on a 10% acrylamide discontinuous buffer SDS-gel, and stained with Coomassie blue. Cellular bands of 63, 72, and 74 kDa always observed to copurify with middle T antigen are indicated by the arrowheads. Aliquots of the affinity gel shown in lanes A and B were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in vitro. Notice that the activity of the Ad5(pymT)-derived protein (lane C) is much lower than that derived from the tumor (lane D).

marily fragment C, the C-terminal peptide characteristic of the 56-kDa form. The extremely small amount of C' again indicates that little of the 58-kDa form is present. V8 mapping gives overlapping 24- and 18-kDa C-terminal fragments readily observed in the control labeled in vitro (Fig. 4e, left lane). Because these fragments are only slightly phosphorylated when Ad5(pymT) is labeled in vivo, the principal in vivo phosphorylations must be proximal to the acidic cluster at residues 273 to 276 from which the 24K peptide is derived. V8 and hydroxylamine mapping therefore positions Ad5(pymT) in vivo phosphorylation between residues 225 and 277, in agreement with previous results with mouse cells.

Immunoaffinity purification of middle T antigen. Immunopurification was performed with monoclonal antibody against middle T antigen (11). The Coomassie blue-stained middle T antigen obtained from approximately 1.5×10^5 Ad5(pymT)-infected 293 cells is shown in Fig. 5, lane A. Besides middle T antigen, three other bands, a doublet at approximately 72- and 74-kDa and a fainter 63-kDa band, were consistently observed. Since the bands were not seen when extracts from 293 cells infected with wild-type adenovirus or affinity gel lacking antibody was used (not shown), these polypeptides are likely to be specifically associated with middle T antigen. Another reasonable source of middle T antigen is tissue from nude mice carrying tumors expressing middle T antigen as a cDNA from the metallothionein promoter (11). Coomassie blue staining showed that the tumor tissue was less rich in middle T antigen and the extent of purification was much lower. However, the same 74- and 63-kDa bands were observed. Although their significance must be confirmed by biochemical and genetic studies, it is encouraging that the same bands are observed from very different starting material. It is worth noting that others (9) have also observed a radiolabeled band of about 60 kDa.

Comparisons of the Ad5(pymT) and tumor-derived middle

T antigen showed the tumors are a better source of middle T antigen-pp60^{c-src} complex. The specific activity of middle T antigen from the tumor was much higher for in vitro phosphorylation (Fig. 5, lanes C and D). The lower specific activity of the Ad5(pymT) middle T antigen is entirely consistent with the results of the radiolabeling experiments described above.

DISCUSSION

The availability of the Ad5(pymT) vector greatly improves prospects for biochemical analysis of middle T antigen. Middle T antigen protein produced in Ad5(pymT)-infected cells is qualitatively identical to that produced during polyomavirus infection of mouse cells. Previously, biochemical studies have been limited to investigations of radiolabeled protein. Efforts to express middle T antigen in *Escherichia coli* were technically successful (18) but the bacterial protein was unmodified in vivo and was only minimally phosphorylated in vitro when pp60^{c-src} was added (unpublished data). Simian virus 40 vectors have been used to overproduce middle T antigen in CV1 cells (24). The expression of genetically engineered tumors in nude mice provides an abundant but dilute source of material which is particularly valuable for isolation of the middle T antigen that can be phosphorylated in vitro (11). None of these systems, or the adenovirus expression system recently reported (12), provides a source as rich as the Ad5(pymT)-infected 293 cells. With these cells, simple purification procedures yield roughly 100 μg of middle T antigen per 100-mm dish. Scaling up the production to milligram quantities of protein should present no difficulty.

Finally, although there is at least one report of polyomavirus transformation of a human cell line (21), the Ad5(pymT) virus did not transform human cells. Infection of human WI38 cells at multiplicities of infection ranging from 0.5 to 50 did not result in any foci, even though middle T antigen synthesis and its association with pp60^{c-src} were easily demonstrable. The failure to transform is not related to some adventitious mutation in middle T antigen, since the same viral stocks transformed rodent cells (data not shown).

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