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Antibodies Against a Nonapeptide of Polyomavirus Middle T Antigen: Cross-Reaction with a Cellular Protein(s)

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Antibodies were raised against the sequence Glu-Glu-Glu-Glu-Tyr-Met-Pro-Met-Glu, which represents a part of the middle T antigen of polyomavirus that is considered to be important in inducing the phenotype of transformed cells. The antibodies reacted with native as well as denatured middle T antigens. In addition, the antibodies immunoprecipitated a cellular protein with an apparent molecular weight of 130,000 (130K) from mouse and rat cells. In some cases, a 33K protein was also immunoprecipitated. Immunoprecipitation of middle T antigen as well as 130K and 33K proteins was blocked by the peptide. The antibodies labeled microfilaments of untransformed mouse, rat, human, and chicken cells by immunofluorescence. This labeling was also blocked by the peptide. The labeling pattern and distribution under a variety of conditions were indistinguishable from those of anti-actin antibodies, although no evidence has been obtained to indicate that the anti-peptide antibodies react with actin. The 130K protein migrated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis slightly slower than chicken gizzard vinculin (130K) and slightly faster than myosin light-chain kinase of chicken smooth muscle (130K). Neither of these proteins absorbed the anti-peptide antibodies. The 33K protein does not seem to be tropomyosin (32K to 40K).

The early region of polyomavirus encodes three proteins in an overlapping manner. They are commonly called large, middle, and small T antigens (12, 16, 22, 55). Middle T antigen is a main inducer of the phenotype of transformed cells (24), and without it transformation does not occur. Middle T antigen alone can transform established lines of cells (54), although large and possibly small T antigens may also be required for the full expression of the phenotype of transformation in low amounts of mitogenic growth factors in culture medium (40). Large or small T antigens alone cannot transform cells (5, 26, 40, 45).

Middle T antigen has a molecular weight of 49,000 (49K) and migrates in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as a 55K to 60K protein (20, 23, 44). The amino-terminal half of middle T antigen is almost completely homologous to small T antigen. The carboxyl-terminal half of middle T antigen is the unique region, a large part of which is encoded in a region of the viral genome where a part of large T antigen is also encoded in a different reading frame (1, 8, 48, 52).

Several mutants have been isolated which have deletions in the region of the genome where a part of both large and middle T antigens are encoded (1, 10, 18, 32, 38). This set of mutants transforms cells, but the phenotype of these transformed cells varies depending upon the positions of the deletion. The most extreme cases are those of the mutants *dl*8 and *dl*23 (17, 18); cells transformed by *dl*8 grow faster than wild-type transformed cells as colonies in soft agar or dense foci on plastic surfaces in liquid medium, whereas cells transformed by *dl*23 grow much more slowly than wild-type transformed cells (24). Middle T antigen of *dl*23 lacks 34 amino acids from positions 302 to 335 (51). From the analysis of *dl*23 and other mutants which have deletions overlapping with that of *dl*23 (Fig. 1), it has been suggested that the removal of the sequence Glu-Glu-Tyr-Met-Pro-Met-Glu which spans from amino acid 313 to 319 of middle T molecule drastically reduces the ability of the virus to induce the full transformation phenotype (10, 38). From the effect of the deletion of *dl*23 on protein kinase activity associ-

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ated with middle T antigen (see below), the effect on transformation caused by the deletion is generally considered to be due to the alteration of the properties of middle T antigen (24). It is not known whether the deletion has any effect on the function of large T antigen.

When purified middle T antigen or an immunoprecipitate containing middle T antigen is incubated with [γ-^{32}P]ATP, middle T antigen becomes phosphorylated on tyrosine (13, 43, 47, 50, 58). In the present communication, we refer to this enzyme activity as MT kinase activity. This enzyme activity is associated mainly with the subfraction of middle T population which is present in the plasma membrane fraction. There is a good correlation between the level of MT kinase activity and transforming ability of the virus (50). Middle T antigen of *dl*8 is associated with higher MT kinase activity than that of the wild type (50). Middle T antigen of *dl*23 becomes phosphorylated at a greatly reduced level in the MT kinase reaction (50). It has been suggested that the phosphate acceptor in this MT kinase reaction is at tyrosine 315, which is included in the heptapeptide mentioned above (41).

To evaluate the importance of the region of middle T antigen around this heptapeptide in oncogenic activity of the virus, antibodies were raised against a synthetic nonapeptide, Glu-Glu-Glu-Glu-Tyr-Met-Pro-Met-Glu. The antibodies against this peptide react with native as well as denatured middle T antigens. Properties of the antibodies raised against the same nonapeptide in another laboratory with respect to MT kinase activity have been reported (42). In the present communication, we show evidence that the antibodies cross-react with a component(s) of microfilament bundles and surface ruffles and that the antibodies immunoprecipitate 130K and 33K cellular proteins.

MATERIALS AND METHODS

Viruses. The A3 strain of polyomavirus wild type (14), a prototype of the *hr*-t mutant, NG18 (2) (obtained from T. Benjamin, Harvard Medical School, Boston, Mass.), *dl*8 and *dl*23 (17) (obtained from B. E. Griffin, Imperial Cancer Research Fund, London, England), and *dl*1015 (32) (obtained from G. Magnusson, Karolinska Institute, Stockholm, Sweden) were used. The map positions of the mutants are diagrammatically shown in Fig. 1. Viruses were plaque purified in secondary mouse embryo fibroblasts in the presence of 1 μM dexamethazone (35). Stock viruses were grown in primary mouse kidney cells inoculated at the multiplicity of 0.1 to 0.5 PFU per cell.

Cells. Clone 7-3 of 3T6 cells was obtained from W. Eckhart (Salk Institute, La Jolla, Calif.) and was used as host cells for polyomavirus growth. Clone K of Swiss 3T3 cells was obtained from L. Jimenez de Asua (Friedrich Miescher Institute, Basel). Py6 polyomavirus-transformed Swiss 3T3 cells were obtained from T. Benjamin. SECA cells, a cell line established from a mouse tumor induced by polyomavirus, have been described before (6). Rat-1 cells, an established line of Fischer rat fibroblasts (24), Py/Rat-1/Z cells, the clone Z of Rat-1 cells transformed by wild-type polyomavirus, and *dl*23/Rat-1/14 cells, the clone 14 of Rat-1 cells transformed by *dl*23, were all described before (24). *dl*23/A31/F1 cells, a clone of BALB/c 3T3 cells transformed by *dl*23, have been described before (24). MT/Rat-1/2-4 cells, the clone 2-4 of Rat-1 cells transformed by an altered viral DNA which induces only middle T antigen, were obtained from R. Kamen (Genetics Institute, Boston, Mass.). Cell line 1837, a clone of Rat-1 cells expressing only large T antigen, has been described before (26). A line of human skin fibroblasts, KD cells (7, 25), was obtained from T. Kakunaga, National Cancer Institute, Bethesda, Md. All of these cells except 3T3 K cells were kept in culture in Dulbecco modified Eagle minimum essential medium (DMEM) supplemented with 5% fetal bovine serum. 3T3 K cells were kept in DMEM containing 10% fetal bovine serum.

Peptide synthesis. The nonapeptide was synthesized by the Merrifield solid-phase method (34), using a

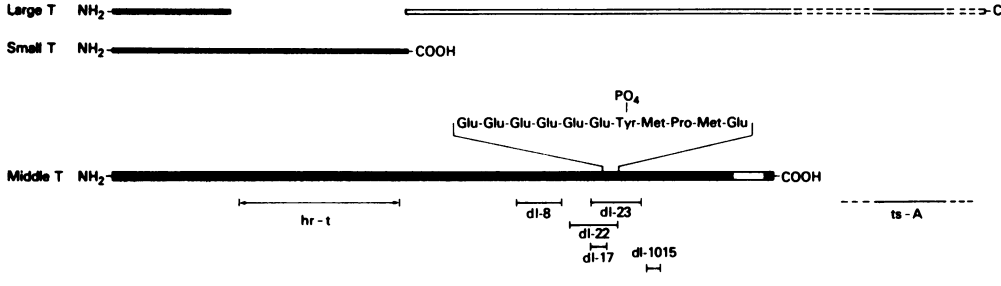

FIG. 1. Schematic diagram of the map positions of the mutants of polyomavirus used in the present studies. The data were obtained from Soeda and Griffin (53) and Hattori et al. (19) for *hr*-t, from Smolar and Griffin for *dl*8 and *dl*23 (51), and from Magnusson et al. for *dl*1015 (33). *dl*22 and *dl*17 (10) were not used in the present studies but are indicated here to explain the sequence shown enlarged (see the text). The tyrosine residue present in the enlarged sequence is at position 315, which has been suggested to be a phosphate acceptor in in vitro protein kinase reaction mediated by middle T antigen-associated kinase activity (MT kinase, see the text) (41). The blank area at the carboxy-terminal end of middle T antigen indicates the position of the cluster of hydrophobic amino acids (52).

Vega 250 peptide synthesizer. The crude product was purified successively by gel filtration (Sephadex G-25) and DEAE-Sephadex ion exchange. The major fraction obtained was judged pure by reverse-phase chromatography, amino acid composition, and sequence.

Conjugation of the peptide to BSA. The peptide was coupled to thiolated bovine serum albumin (BSA) by the use of *N*-succinimidyl-3-iodoacetamidopropionate. The use of this reagent will permit coupling of the peptide via its amino terminus to the thiolated protein. Since this reagent is heterobifunctional, one can optimize the extent of coupling by the number of thiols added to the BSA carrier. The extent of coupling was monitored by amino acid analysis. About 15 mol of peptide was coupled per mol of BSA (J. K. Inman and E. Appella, unpublished data).

Immunization. One milligram of peptide-conjugated BSA was emulsified with complete Freund adjuvant and injected into rabbits intradermally at multiple sites. The same immunization was repeated every 4 weeks. At 5 to 7 days after each immunization, the antibody titer was tested by examining the binding of ^{125}I-labeled peptide to serially diluted antibodies. At 5 weeks after the initial immunization, a significantly high anti-peptide antibody titer was obtained. The titer increased with further immunization. The highest titer was scored with the serum obtained 3 months after the initial immunization. All of the results described in this paper were obtained by using this bleed.

Immunoprecipitation and SDS-PAGE. Labeling of cells with [^{35}S]methionine, extraction of T antigens, immunoprecipitation, and SDS-PAGE were performed as described earlier (21), except that the amount of Formalin-fixed protein A-producing *Staphylococcus aureus* used per given amount of rabbit serum was fivefold more than that used for rat serum.

Affinity purification of anti-peptide antibodies. One gram of 6-aminohexanoic acid-activated Sepharose 4B (Sigma Chemical Co.) was washed with 1 mM ice-cold HCl. Twelve milligrams of the peptide was dissolved in 2 ml of 0.2 M $NaHCO_3$ (pH 8.0) and incubated with washed Sepharose at room temperature for 18 h. After incubation, 200 μl of ethanolamine was added to the mixture, and it was further incubated for 1 h at 25°C. Uncoupled peptide was washed away with 0.1 M $NaHCO_3$, a high-pH buffer (0.05 M Tris-hydrochloride [pH 8.0], 0.5 M NaCl), and low-pH buffer (0.05 M formate [pH 4.0], 0.5 M NaCl). Two-hundred milligrams of BSA was coupled to 6-aminohexanoic acid-activated Sepharose 4B as above. Three milliliters of anti-peptide serum was diluted twofold with phosphate-buffered saline (PBS) and applied to a column of Sepharose 4B coupled with BSA. Flow-through was then applied to a column of the peptide-coupled Sepharose 4B. Flow-through of this peptide-coupled Sepharose 4B column did not contain anti-peptide antibody activity as monitored by the binding assay, using ^{125}I-labeled peptide. After the column was extensively washed with PBS, anti-peptide antibodies were eluted with 3.5 M $MgCl_2$. Fractions obtained were immediately dialyzed against PBS.

MT kinase assay. Immunoprecipitates were extensively washed with NET (0.15 M NaCl, 50 mM EDTA, 50 mM Tris-hydrochloride [pH 7.4], 0.05% Nonidet P-40, 100 kallikrein inhibitor units per ml of aprotinin [Sigma Chemical Co.]) and incubated with [γ-^{32}P]ATP for 15 min at 30°C in a buffer containing 25 mM Tris-hydrochloride (pH 7.5) and 5 mM $MgCl_2$. After the reaction, immunoprecipitates were washed extensively again and analyzed by SDS-PAGE as described earlier (47).

Indirect immunofluorescent labeling. Cells were fixed in 3.7% formaldehyde in PBS for 20 min at room temperature, rinsed, and treated with 0.3% Nonidet P-40 in PBS for 5 min at room temperature. The cells were incubated with affinity-purified antibodies (1:10 dilution) for 40 min at 37°C, washed, and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (IgG) antibodies (1:30 dilution) (Cappel Laboratories) for 40 min at 37°C. Acetone treatment of formaldehyde-fixed cells resulted in the same fluorescent pattern as that obtained by Nonidet P-40 treatment. Samples were photographed with a Leitz fluorescence microscope, using an oil emersion lens (100×, numerical aperture = 1.32) and Kodak Tri-X film. Exposure time was approximately 30 s in most cases.

RESULTS

Immunoprecipitation of middle T antigen with anti-peptide antibodies. To examine whether anti-peptide antibodies would react with detergent-extracted native middle T antigen, immunoprecipitation was performed for the extracts of cells infected with the virus and labeled with [^{35}S]methionine. To facilitate the detection of virus-coded middle T antigen, a set of deletion mutants was used. These deletion mutants included NG18 (2), *dl*8 (18), *dl*23 (18), and *dl*1015 (32). T antigens of these mutants that immunoprecipitated with conventional anti-T serum are shown in Fig. 2A. NG18 induced normal size large T antigen. Middle and small T antigens of NG18 were very small fragments and undetectable in Fig. 2A. *dl*8 induced 95K large and 50K middle T antigens as well as normal size small T antigen (24). Large T antigen of *dl*23 was a 95K protein. Middle T antigen of *dl*23 is, unlike *dl*8, apparently 43K (24). Small T antigen of *dl*23 is the same as that of wild type. *dl*1015 induces 97K large T antigen, 52K middle T antigen, and normal size small T antigen (33).

Anti-peptide antibodies are expected to react only with middle T antigen of wild type, *dl*8, and *dl*1015. Figure 2B shows that anti-peptide antibodies immunoprecipitated proteins from extracts of cells infected with wild-type, *dl*8, or *dl*1015 but not from mock-infected, NG18-infected, or *dl*23-infected cell extracts which comigrate with respective middle T antigens. This immune reaction was blocked by the addition of the peptide at 10^{-4} M. The results strongly suggest that the anti-peptide antibodies specifically react with native middle T antigen. To test this specificity further, [^{35}S]methionine-labeled middle T antigen immunoprecipitated by anti-tumor serum and separated by SDS-PAGE was eluted and reacted with the anti-peptide antibodies. As expected, SDS-PAGE-purified middle T

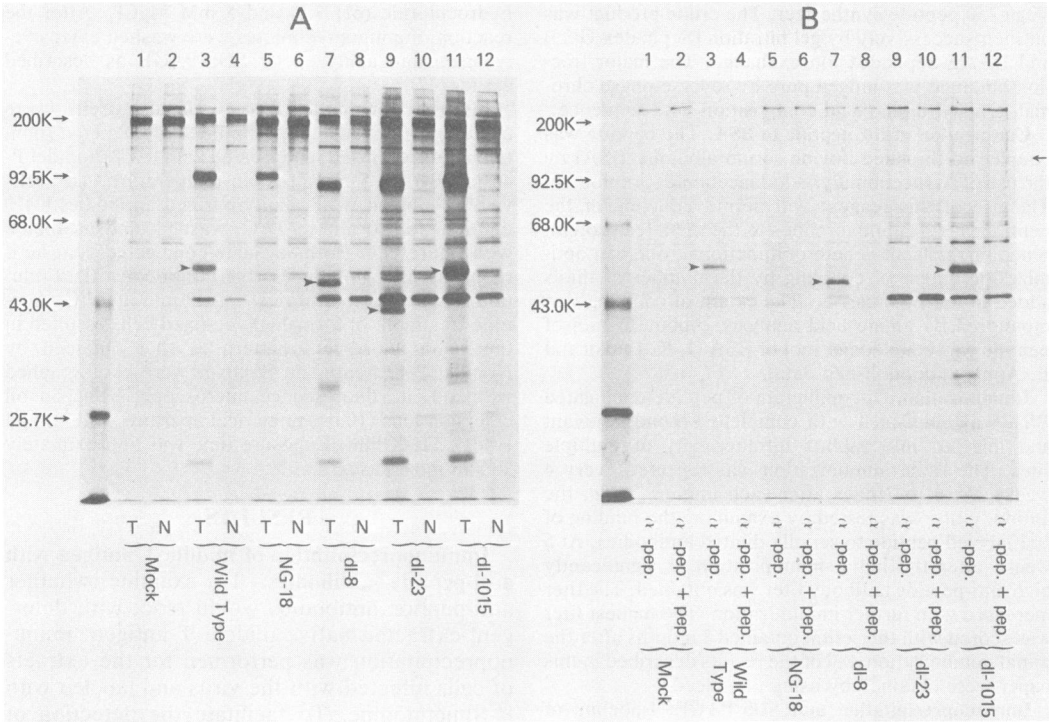

FIG. 2. Immunoprecipitation of polyomavirus T antigens with anti-tumor (T) serum or anti-peptide antibodies. About 2×10^6 3T6 cells infected with wild-type or mutant viruses at 20 to 50 PFU per cell were labeled with [^{35}S]methionine (150 μCi/ml, 800 Ci/mmol; Amersham Corp.) in methionine-free DMEM from 30 to 35 h after infection at 37°C. Extracts made from these cells were preabsorbed with nonimmune rat serum. The extracts were divided into four aliquots. (A) Each of two aliquots was incubated with 20 μl of rat anti-T serum (T) (lanes 1, 3, 5, 7, 9, and 11) or nonimmune rat serum (N) (lanes 2, 4, 6, 8, 10, and 12) for 1 h at 0°C. Immune complexes were isolated and analyzed by 9.5% SDS-PAGE as described previously (21). (B) Each of two aliquots was incubated with 5 μl of affinity-purified anti-peptide antibodies alone (lanes 1, 3, 5, 7, 9, and 11) or with 5 μl of anti-peptide antibodies and final 10^{-4} M concentration of the peptide (lanes 2, 4, 6, 8, 10, and 12) for 1 h at 0°C. Isolation and analysis of immune complexes were as described for (A) above. Mock infected (lanes 1 and 2); wild-type infected (lanes 3 and 4); NG18-infected (lanes 5 and 6); *dl*8 infected (lanes 7 and 8); *dl*23 infected (lanes 9 and 10); and *dl*1015 infected (lanes 11 and 12). Arrowheads indicate the middle T antigen of wild-type and mutant viruses. The arrow at the extreme right indicates the position of the cellular 130K protein.

antigen reacted well with the anti-peptide antibodies (data not shown). However, unexpected results were obtained when middle T antigen that phosphorylated in in vitro MT kinase reaction, using hamster anti-T serum, was electroeluted from SDS-PAGE and reacted with the anti-peptide antibodies. As shown in Table 1, SDS-PAGE-purified in vitro-phosphorylated middle T antigen reacted with anti-peptide antibodies well. This result was somewhat unexpected since the tyrosine residue in the middle of the nonapeptide has been considered to be a phosphate acceptor in in vitro-phosphorylated middle T antigen (41), and, therefore, the phosphorylated middle T antigen would not be expected to react with the anti-peptide antibodies. This result (Table 1) will be thoroughly discussed in separate studies (manuscript in preparation). In any event, the results shown in Fig. 2 and Table 1 establish that the 55K protein immunoprecipitated from wild-type infected cells with anti-peptide antibodies is a virus-coded middle T antigen and that the anti-peptide antibodies react with both native and SDS-denatured middle T antigens.

Cellular protein(s) cross-reactive with anti-peptide antibodies. In addition to middle T antigen, Fig. 2B shows an additional protein which reacted with anti-peptide antibodies and migrated between the 200K and 92.5K markers (indicated by the arrow at the right in Fig. 2B). This protein comigrates with β-galactosidase (data not shown) and, therefore, will be called the 130K protein; 10^{-4} M of the peptide competes with this protein for immunoprecipitation. This 130K protein was present in uninfected 3T6 cells (Fig. 2B, lane 1) and, therefore, was a cell-coded protein. Cross-reactive cellular proteins can be

TABLE 1. Immunoprecipitation with anti-peptide antibodies of in vitro-phosphorylated middle T antigen eluted from SDS-PAGE[a]

Eluted protein	Input (^{32}P cpm)	Bound (^{32}P cpm)	% Binding
Middle T antigen	1,556	433	27.8
Large T antigen	860	1	0.1
VP1	738	6	0.8

[a] When immunoprecipitates obtained by hamster anti-T serum from polyomavirus-infected 3T6 cells are incubated with [γ-^{32}P]ATP, middle T antigen becomes the major phosphorylated protein. Under some conditions, proteins comigrating with large T antigen and the major capsid, VP1, are also phosphorylated, albeit at a much lower level (unpublished data). The in vitro-phosphorylated middle T antigen, large T antigen, and VP1 were electroeluted from SDS gels and incubated with anti-peptide antibodies for 3 h at 0°C. Immune complexes were isolated by Formalin-fixed protein A-producing *S. aureus*, washed, and eluted. Radioactivity in the eluted samples is shown.

seen more clearly in Fig. 3 and 4. In Fig. 3, a protein of apparently 33K is evident in addition to middle T antigen and the 130K protein. In this case, the cell extract contained less middle T antigen relative to the 130K protein as compared with the results shown in Fig. 2. The 130K protein was reproducibly detected in a variety of cells including mouse 3T3 cells, three lines of tumors induced by polyomavirus in mice, and Rat-1 cells. The 130K protein in one mouse tumor line, SECA, is shown in Fig. 4. Detection of the 33K protein was variable. The 33K protein was not detected in Fig. 4.

Since the anti-peptide antibodies apparently react with a component(s) of actin-containing microfilaments (see below), attempts were made to examine whether any known microfilament protein corresponds to either the 130K or 33K protein. Proteins composed of or closely associated with microfilaments of between 100K and 200K include α-actinin (105K), vinculin (130K), myosin light-chain kinase (130K), and myosin (200K). The 130K protein recognized by the anti-peptide antibodies migrated in SDS-PAGE considerably more slowly than α-actinin (Fig. 3, lane 6), slightly more slowly than vinculin from chicken gizzard, and slightly faster than myosin light-chain kinase from chicken smooth muscle (data not shown).

The mobility of the 33K protein in SDS-PAGE was similar to that of several species of tropomyosin from nonmuscle cells. As discussed below, however, the proteins recognized by the anti-peptide antibodies are not likely to be vinculin, myosin light-chain kinase, or tropomyosin.

Anti-α-actinin immunoprecipitated a 43K protein, presumed to be actin, in addition to α-actinin (Fig. 3, lane 6), probably because actin is bound to α-actinin. The anti-peptide antibodies did not immunoprecipitate actin (Fig. 2 to 4).

Immunofluorescent labeling of microfilament with anti-peptide antibodies. In attempting to examine the intracellular localization of middle T antigen by using the anti-peptide antibodies, the anti-peptide antibodies were found, by indirect immunofluorescence, to react with filamentous structures of untransformed and uninfected mouse cells (Fig. 5A). This activity of the anti-peptide antibodies was removed by preincubation of the antibodies with the peptide (Fig. 5B). The antibodies eluted from the BSA-conjugated Sepharose column did not label the filamentous structure of untransformed cells. The labeling pattern shown in Fig. 5A is indistinguishable from that of actin in microfilament bundles.

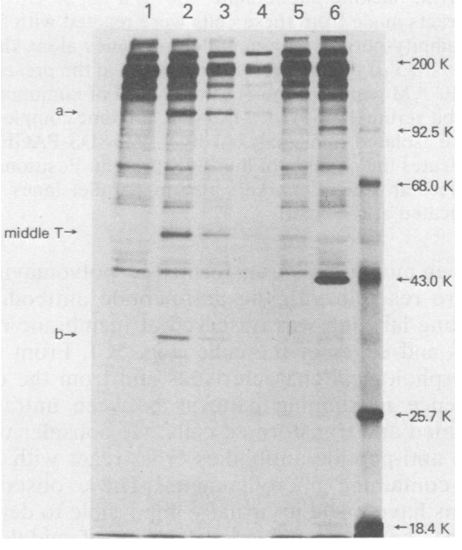

FIG. 3. Immunoprecipitation of cross-reactive cellular proteins with anti-peptide antibodies. Extracts from about 2×10^6 3T6 cells infected with wild-type polyomavirus and labeled with [^{35}S]methionine were made as described in the legend to Fig. 1. The extract from about 3.3×10^5 cells was used for the samples in lanes 1, 2, 5, and 6. One-third and one-sixth of this amount were used for the samples in lanes 3 and 4, respectively. They were incubated with 3 μl of nonimmune rabbit serum (lane 1), 5 μl of affinity-purified anti-peptide antibodies alone (lanes 2, 3, and 4), 5 μl of anti-peptide antibodies and a final 10^{-4} M concentration of the peptide (lane 5), and 5 μl of anti-α-actinin rabbit serum (lanes 6) for 1 h at 0°C. Immune complexes were isolated and analyzed by 9.5% SDS-PAGE. a and b indicate the positions of the 130K and 33K proteins, respectively. Molecular weight markers are indicated at the extreme right. In lane 6, α-actinin migrates slightly more slowly than the 92.5K marker. The strong band which comigrated with the 43K marker in the same lane is probably actin (see the text).

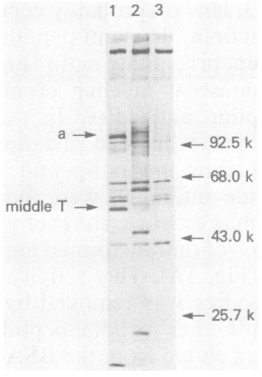

FIG. 4. Immunoprecipitation of middle T antigen and a cross-reactive cellular protein from mouse cells derived from a polyomavirus-induced tumor. About 2 × 10^6 SECA cells were labeled with [^{35}S]methionine (150 μCi/ml, 800 Ci/mmol; Amersham Corp.) in DMEM lacking methionine for 3 h at 37°C. The extracts made from these cells were reacted with 5 μl of affinity-purified anti-peptide antibodies alone (lane 1), with 5 μl of anti-peptide antibodies in the presence of 10^{-4} M peptide (lane 2), or with 3 μl of nonimmune rabbit serum (lane 3) for 1 h at 0°C. Immune complexes were isolated and analyzed by 7.5% SDS-PAGE. a indicates the position of the 130K protein. Positions of molecular weight markers run in parallel lanes are indicated at the right.

When mouse cells transformed by polyomavirus were reacted with the anti-peptide antibodies, strong labeling was observed at membrane ruffles and edges of the cells (Fig. 5C). From the morphological characteristics and from the difference in staining patterns between untransformed and transformed cells, we consider that the anti-peptide antibodies cross-react with actin-containing microfilaments. These observations have made it virtually impossible to determine the intracellular localization of middle T antigen with these anti-peptide antibodies.

Figure 6 shows the labeling pattern of mouse cells at various stages of movement. The particular cell line shown in Fig. 6 is *dl*23/A31/F1, but similar patterns could be seen in wild-type transformed as well as untransformed cells. Close examination of the pattern shown in Fig. 5 and 6 as well as those shown later (see Fig. 7 and 8) indicated that strong labeling was located at microvilli, filopodia, and ruffles as well as microfilament bundles, while there was apparently little or no labeling in the cytosol. Microfilament bundles were labeled smoothly without periodicity. The only known protein which has this distribution is actin (30, 63). This actin-labeling pattern distinguishes it from those of myosin (60, 62), tropomyosin (27, 28), α-actinin (29), myosin light-chain kinase (9), filamin (59), vinculin (15), fodrin (31), calmodulin (61), fimbrin (4), and villin (3). In the experiments not shown, affinity-purified antibodies to actin, myosin, vinculin, myosin light-chain kinase, and anti-peptide antibodies were compared in the same untransformed cells (Swiss 3T3 mouse fibroblasts), and the patterns of anti-peptide antibodies corresponded only with those seen with anti-actin.

The ability of the anti-peptide antibodies to label microfilament bundles, however, was not absorbed by actin from rabbit muscles (data not

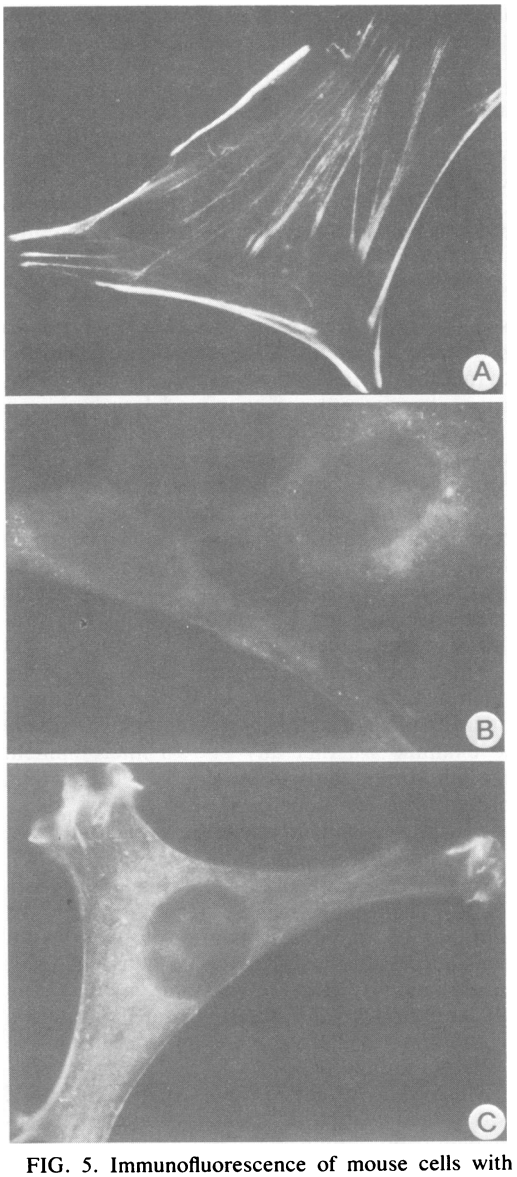

FIG. 5. Immunofluorescence of mouse cells with anti-peptide antibodies. (A) 3T3 K cells labeled with anti-peptide antibodies. (B) 3T3 K cells labeled with anti-peptide antibodies which were preincubated with a final 10^{-4} M concentration of the peptide for 2 h followed by centrifugation at 15,000 × *g* for 5 min. (C) Py6 cells labeled with anti-peptide antibodies.

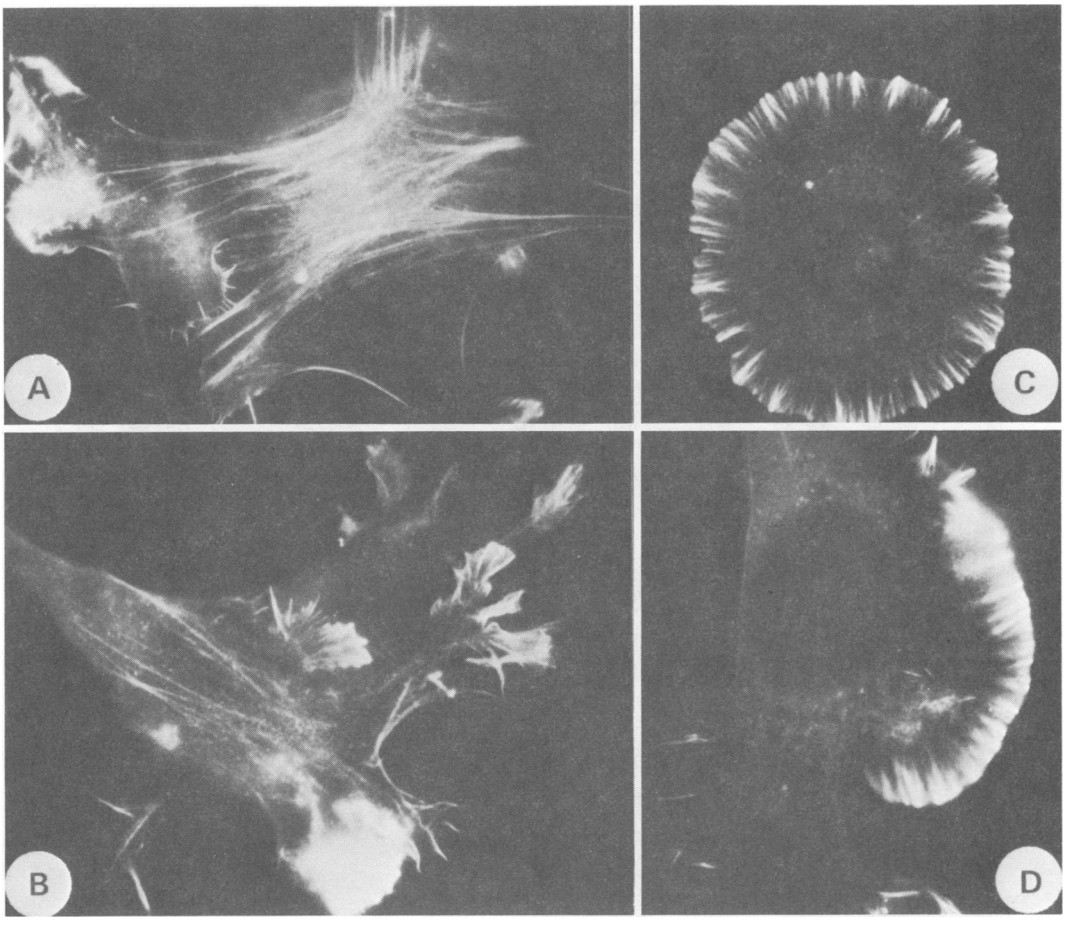

FIG. 6. Immunofluorescence of *dl*23/A31/F1 cells with anti-peptide antibodies. (A and B) Strong labeling of ruffles. (C and D) Labeling of spreading lamellae.

shown). Moreover, actin was not immunoprecipitated with the anti-peptide antibodies (Fig. 2 to 4). One possibility is that the epitope in the actin molecule which is recognized by the antibodies is revealed or created only after the treatment of actin with formaldehyde. This possibility was excluded since cells fixed only with acetone also show labeled microfilaments (data not shown). At the moment, there is no evidence that actin is the protein labeled (see Fig. 5 to 8). By absorption test, the following proteins were also unlikely to be recognized by the antibodies: myosin from rabbit muscle, myosin light-chain kinase from chicken smooth muscle, vinculin from chicken gizzard, α-actinin from chicken gizzard, and tropomyosin from rabbit muscle.

Fluorescent labeling of cells transformed by wild-type and mutant viruses. To examine the significance of microfilament labeling by the anti-peptide antibodies, immunofluorescence with the anti-peptide antibodies was studied by using cells expressing various T antigens.

As for mouse cells, microfilament bundles of untransformed rat cells were labeled by the anti-peptide antibodies (Fig. 7A). The labeling pattern of rat cells transformed by wild-type polyomavirus was very similar to that of polyomavirus-transformed mouse cells (Fig. 7B). The labeling pattern of cells expressing only middle T antigen (54) was indistinguishable from that of the wild-type transformed cells (Fig. 7C). Rat cells expressing only large T antigen and morphologically indistinguishable from untransformed cells have fully organized microfilament bundles (Fig. 7D). Rat-1 cells transformed by a mutant, *dl*23, whose middle T antigen lacks the nonapeptide, largely retain microfilament bundles (Fig. 7E), in agreement with previous observations (18). Since the anti-peptide antibodies react with middle T antigen of polyomavirus, strong labeling observed around the stretched ends of wild-type transformed cells and cells expressing only middle T antigen might be due to the concentration of middle T antigen at these

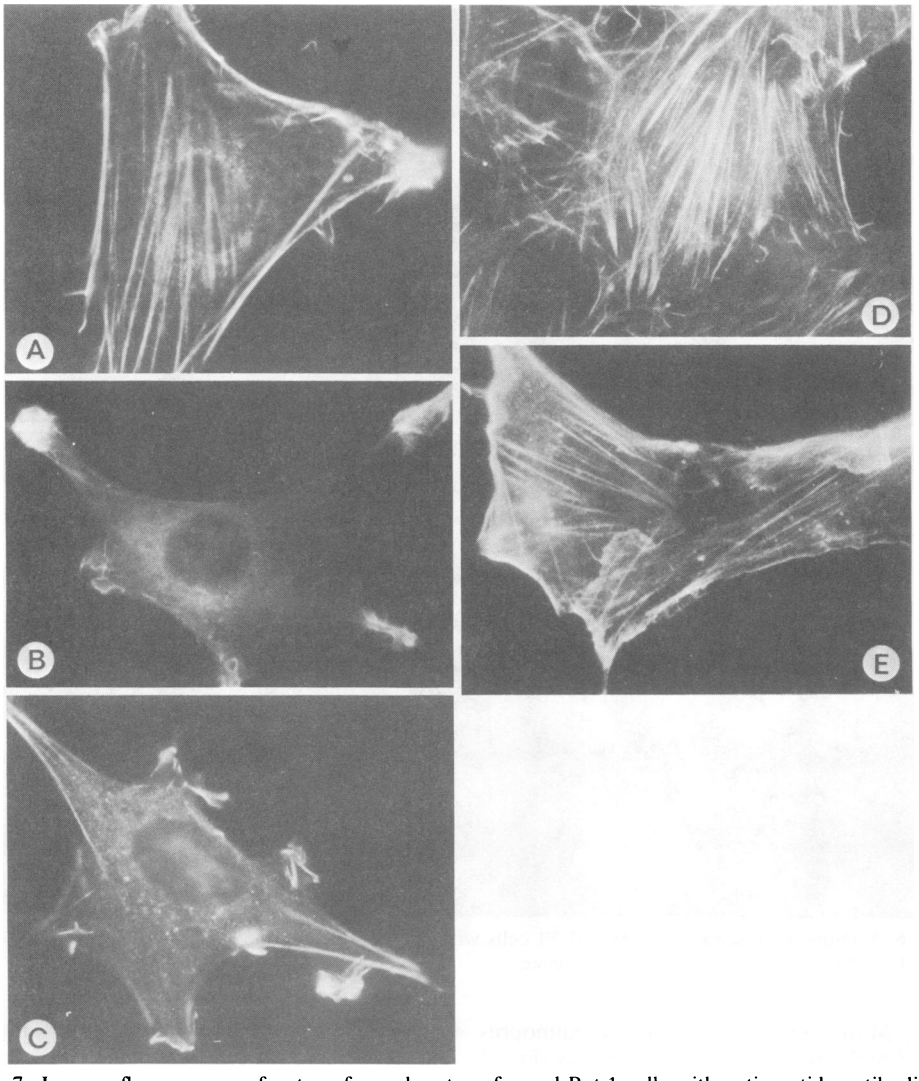

FIG. 7. Immunofluorescence of untransformed or transformed Rat-1 cells with anti-peptide antibodies. (A) Rat-1 cells, (B) Py/Rat-1/Z cells. (C) MT/Rat-1/2-4 cells, (D) 1837 cells, (E) *dl*23/Rat-1/14 cells.

sites (Fig. 7B and C). However, similar strong labeling could be seen in *dl*23-transformed cells (Fig. 7E), and, therefore, this pattern is not likely to be due to the reaction of anti-peptide antibodies with middle T antigen.

The fact that *dl*23 induces normal small T antigen (24) rules out the possibility that small T antigen is mainly responsible for the morphological changes seen. The results shown in Fig. 7, therefore, strongly suggest that middle T antigen is mainly responsible for the changes in morphology and microfilament bundle patterns in polyomavirus-transformed cells.

Fluorescent labeling of microfilaments of untransformed human and chicken cells. As shown in Fig. 8, microfilament bundles of both human and chicken cells are also recognized by the anti-peptide antibodies. This suggests that the sequence or the structure of the microfilament protein(s) which is recognized by the antibodies is well conserved among a wide range of animal species and, therefore, is likely to be functionally important.

DISCUSSION

Antibodies were raised against the synthetic nonapeptide Glu-Glu-Glu-Glu-Tyr-Met-Pro-Met-Glu. The antibodies react with native as well as denatured middle T antigen. By immunofluorescence, the antibodies label actin-containing microfilament bundles of untransformed cells. The antibodies react with microfilaments

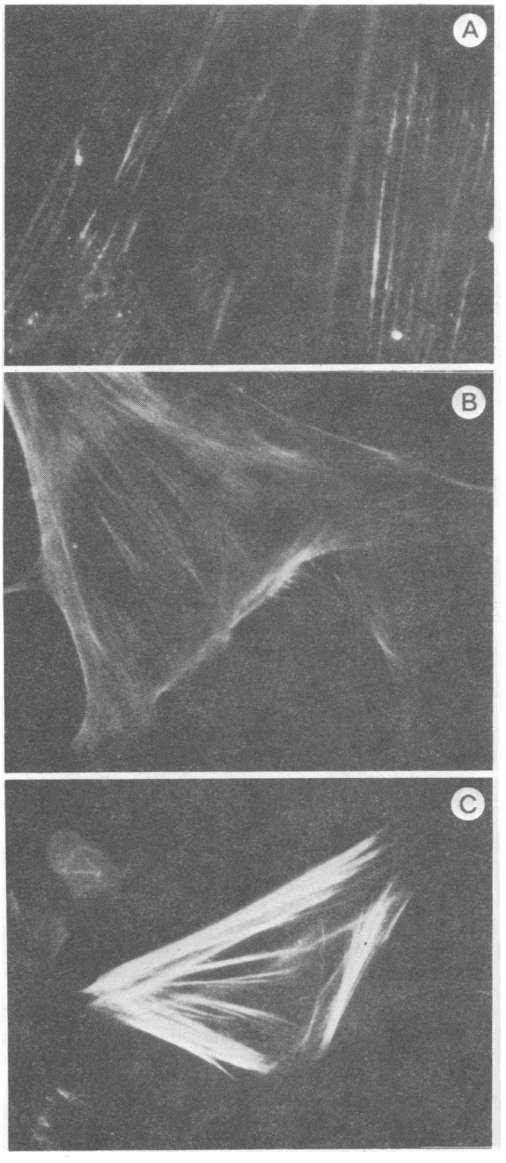

FIG. 8. Immunofluorescence of untransformed human and chicken fibroblasts with anti-peptide antibodies. (A) KD cells; (B and C), primary chicken embryo fibroblasts.

of mouse, rat, human, and chicken cells. The labeling pattern observed under various conditions was indistinguishable from that of the known pattern of anti-actin antibodies and different from those of antibodies against other known microfilament-associated proteins. However, we have not been able to obtain evidence that the antibodies react with actin. In addition, β- and γ-actin present in nonmuscle cells do not have sequences homologous to the peptide sequence, except for the first three successive Glu residues of γ-actin (56). If the antibodies are reacting with actin, they must be recognizing the amino terminus of γ-actin or some highly ordered structure of either β- or γ-actin rather than the primary sequence in such a way that the reaction does not occur when actin is extracted in Nonidet P-40-containing buffer. Alternatively, the antibodies may be reacting with hitherto unknown protein(s). If a new protein(s) is recognized by the antibodies, it would be interesting to identify and characterize the protein and examine its relationship to middle T antigen.

The antibodies immunoprecipitated the 130K and 33K proteins. The former is not myosin light-chain kinase (130K), vinculin (130K), or α-actinin (105K). The latter does not seem to be tropomyosin (30K to 35K). We do not have evidence, at the moment, of whether the 130K or the 33K proteins are microfilament-associated proteins.

It has been shown that monoclonal antibodies and antibodies against synthetic polypeptides often cross-react with entirely unrelated proteins (11, 37). Antibodies against the carboxy-terminal hexapeptide of pp60^{src}, a transforming protein of avian sarcoma virus, have been shown to cross-react with such cytoskeletal proteins as myosin, tubulin, and vimentin (36). This cross-reaction is considered to be fortuitous. A cellular homolog of this pp60^{src} which is also known to have the same tyrosine kinase activity as viral pp60^{src} has been shown not to react with the antibodies against the carboxy-hexapeptide of viral pp60^{src} (46), suggesting that the epitope recognized by the antibodies has nothing to do with the tyrosine kinase activity. It has also been reported that the antibodies against the carboxy-hexapeptide of middle T antigen of polyoma-virus cross-react with some cellular proteins (57). The importance of this hexapeptide in the function of middle T antigen is not clear. However, as studied here, the nonapeptide represents a functionally important sequence. Furthermore, at least one of the cross-reactive proteins is associated with actin microfilaments (39), and the epitope recognized by the anti-peptide antibodies is present in microfilament-associated protein(s) from chicken, rodents, and human cells.

Cells expressing only large T antigen often have well-organized microfilament bundles. Cells expressing only middle T antigen do not have organized microfilament bundles. Cells transformed by *dl*23 largely retain bundles. Since normal small T antigen is expressed in cells transformed by *dl*23 (24), it can be concluded that large T or small T alone is not responsible for the loss of organized bundles of microfilaments. Instead, middle T antigen appears to be primarily responsible for this dissolution of bun-

dles, and middle T antigen of *dl*23 has reduced level of this activity.

It is likely, of course, that the rearrangement of microfilaments into bundles reflects earlier changes in adhesiveness and other properties and that these effects of middle T antigens mentioned above would be quite indirect. Nevertheless, it is interesting to note that cells transformed by *dl*23 still had organized microfilament bundles which reacted with the antibodies against the nonapeptide and that the sequence represented by the nonapeptide was missing in the middle T antigen of *dl*23. As mentioned earlier, *dl*23 does transform cells. The frequency of transformation by *dl*23 does not seem to be significantly different from that of wild-type virus (18, 24). Therefore, one must conclude that the presence of the nonapeptide within the middle T protein is not required to determine the frequency of transformation by polyomavirus. On the other hand, absence of the nonapeptide in middle T protein appears to drastically reduce the ability of middle T antigen to induce the full morphological transformation phenotype (10, 24, 43). Middle T antigen of *dl*23 is associated with only very weak MT kinase activity (41, 49). It would be possible to hypothesize that a lack of the ability of *dl*23 middle T antigen to alter morphology is due to inability of *dl*23 middle T antigen to induce high enough levels of MT kinase activity. If this were the case, the primary event to alter morphology takes place probably at the plasma membrane, since MT kinase appears to be active only at the plasma membrane (47, 50). In this case, perhaps a putative microfilament protein which shares the sequence or the structure with the peptide of middle T antigen is involved in phosphorylation reaction. We have attempted to study phosphorylation of the 130K and the 33K proteins. We could not obtain clear-cut results indicating that the 130K and the 33K proteins were phosphorylated. However, it does not seem to be feasible to examine the phosphorylation of these proteins by using the anti-peptide antibodies, since the phosphate acceptor site may be within the epitope recognized by the antibodies, and, therefore, phosphorylated proteins may not react, as has been suggested for phosphorylated middle T antigen (42).

In considering the significance of the present observations, it is important to establish whether the same antibodies react with middle T antigen, microfilament protein(s), the 130K protein, and the 33K proteins. This is because the anti-peptide antibodies are probably not monoclonal, and, therefore, there is a possibility that different subpopulations of the antibodies react with different proteins, although the antibodies are directed against a very short peptide. If it is proved that the same antibodies can react both with middle T antigen and cellular protein(s), it would then be worth rigorously studying the possibility that the cross-reaction we observe may have some serious biological meaning.

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