The Cellular Proteins Which Can Associate Specifically with Polyomavirus Middle T Antigen in Human 293 Cells Include the Major Human 70-Kilodalton Heat Shock Proteins

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We compared the proteins which associate with middle T antigen (MT) of polyomavirus in human cells infected with Ad5(pymT), a recombinant adenovirus which directs the overexpression of MT, with the MT-associated proteins (MTAPs) previously identified in murine fibroblasts expressing MT. MTAPs of 27, 29, 36, and 63 kilodaltons (kDa) appeared to be fairly well conserved between the two species, as judged by comigration on two-dimensional gels. Several 61-kDa MTAP species detected in MT immunoprecipitates from both cell sources also comigrated on these gels. However, no protein comigrating precisely with the murine 85-kDa MTAP could be detected in the human cells. Furthermore, two proteins of 72 and 74 kDa associated with wild-type MT in the infected human cells but not in murine fibroblasts expressing MT. It had been previously reported for murine cells that the 70-kDa heat shock protein associates with a particular mutant MT but not with wild-type MT (G. Walter, A. Carbone, and W. J. Welch, *J. Virol.* 61:405-410, 1987). By the criteria of comigration on two-dimensional gels, tryptic peptide mapping, and immunoblotting, we showed that the 72- and 74-kDa proteins that associate with wild-type MT in human cells are the major human 70-kDa heat shock proteins.

Rodent cells expressing polyomavirus middle T antigen (MT) have been utilized extensively for the identification and analysis of proteins which associate with MT. To date, cellular proteins of approximately 27, 29, 36, 51, 61, 63, and 85 kilodaltons (kDa) that specifically coimmunoprecipitate with wild-type MT in these cells have been identified (4, 8, 12-14, 18-21). One of the proteins in the 61-kDa range is known to be pp60^{c-src}, a cellular tyrosine kinase (4). The 85-kDa protein has been tentatively identified as a 3'-phosphatidylinositol kinase, since its presence in MT immunoprecipitates correlates with MT-associated 3'-phosphatidylinositol kinase activity (3, 9, 14). The molecular functions of the remainder of the MT-associated proteins (MTAPs), however, are unknown.

Human 293 cells expressing MT have been utilized more recently with the development of a recombinant adenovirus, Ad5(pymT), which facilitates the expression of MT in these cells at levels much higher than those found in polyomavirus-infected or -transformed rodent cells (1, 21). The MT synthesized in the human 293 cells appears to be the same as the MT expressed in rodent cells: it localizes to membranes, forms a complex with pp60^{c-src}, and is modified in the same way as MT produced in polyomavirus-infected mouse cells (21). It has been previously reported that when MT is obtained from human 293 cells by immunoaffinity purification, three cellular proteins of 63, 72, and 74 kDa that specifically copurify with MT can be visualized by Coomassie blue staining (21). In this report we describe further the identification and analysis of proteins which associate with MT in human 293 cells. We show that just as in rodent cells, proteins of 27, 29, 36, 61, and 63 kDa associate with MT. The 85-kDa MTAP identified in rodent cells could not readily be identified in this system, although the MT-associated 3'-phosphatidylinositol kinase activity is

known to be present (D. Kaplan and T. M. Roberts, unpublished data). We also show that the 27-, 29-, 72-, and 74-kDa proteins are abundant cellular proteins and that the 72- and 74-kDa proteins are the major human 70-kDa heat shock proteins.

MATERIALS AND METHODS

Cell culture. Murine cells which express wild-type MT and G418 resistance or only G418 resistance have been described previously (2). All rodent cell lines were maintained in Dulbecco modified eagle medium (DMEM) supplemented with 10% calf serum. Human 293 cells (7) were maintained in DMEM supplemented with 10% fetal calf serum.

Radiolabeling and extraction of cells. For metabolic labeling of rodent cells with methionine, subconfluent dishes of cells were labeled for 5 h with [³⁵S]methionine (300 µCi/ml) in DMEM lacking methionine but supplemented with 0.5% dialyzed fetal bovine serum. For metabolic labeling of 293 cells, cells were labeled with [³⁵S]methionine (100 µCi/ml) for 24 h postinfection in DMEM lacking methionine but supplemented with 2.5% fetal calf serum and 5% the normal level of methionine. Cell lysates were prepared as described previously (25).

Immunoprecipitation and protein kinase assays. Immunoprecipitates were prepared as described previously with rabbit anti-polyomavirus tumor antigen sera raised against purified small t antigen (15). Protein kinase assays were performed *in vitro* as described previously (10).

One-dimensional and 2D gel electrophoresis and fluorography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide) was performed as described by Laemmli (11). Two-dimensional (2D) gel analysis was performed as described previously (13). Gradients of pH 4.8 to 7.4 were obtained by mixing 400 µl of ampholine (pH 3.5 to 10; LKB Instruments, Inc.) with 150 µl of ampholine (pH 6 to 8; LKB) per 10 ml of gel solution. Gels of methionine-

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FIG. 1. Identification of MTAPs in human 293 cells. Cell lysates from 293 cells infected with either recombinant adenovirus Ad5(pymT) or wild-type adenovirus Ad5(wt) and labeled with [35 S]methionine for 24 h were immunoprecipitated with anti-tumor antigen serum (imm) or preimmune antiserum (pre) (13). The immunoprecipitates were analyzed on sodium dodecyl sulfate-10% polyacrylamide gels, and the radiolabeled proteins were visualized by fluorography and autoradiography. The positions of MT (mT), actin, and MTAPs are indicated by arrows. Proteins thought to be breakdown products of MT are not indicated.

labeled proteins were preincubated with En³Hance before exposure; gels of phosphate-labeled proteins were exposed with intensifying screens. All exposures were done on XAR-5 film (Eastman Kodak Co.) at -70°C .

Peptide mapping. Samples for maps of limit digests of methionine-labeled proteins were prepared as described previously (13). The peptides were analyzed by published procedures (5, 6). The final samples (2 μl) were spotted on plastic-backed cellulose-coated thin-layer chromatography plates (10 by 10 cm; EM Laboratories). Electrophoresis and chromatography were carried out as described previously (13).

Immunoblotting. Immunoblotting was carried out by standard procedures (23). Briefly, [35 S]methionine-labeled proteins in 2D gels were electrophoretically transferred to nitrocellulose membranes at 0.5 A for 3.5 h in an electroblotting apparatus (Hoefer). The membranes were blocked with 3% bovine serum albumin, washed, and probed for 1 h at 22°C with a 1:500 dilution of N27, a mouse monoclonal

antibody specific for both constitutive and inducible forms of hsp70 (17; W. J. Welch, personal communication). They were washed again and probed with alkaline phosphatase-conjugated second antibody. The blots were developed with an appropriate substrate and air dried, and the radiolabeled proteins were visualized by autoradiography. All antibody solutions incubated with the blots contained 0.5% bovine serum albumin to reduce nonspecific binding.

RESULTS

Identification of MTAPs in human 293 cells. To identify proteins in human cells that associate either directly or indirectly with wild-type polyomavirus MT, we analyzed immunoprecipitates of MT from Ad5(pymT) recombinant adenovirus-infected (MT-expressing) (1) and Ad5(wt) wild-type adenovirus-infected (control) human 293 cells by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1). A few bands, e.g., actin, were present at the same level in immunoprecipitates prepared from MT-expressing or control cells and therefore represent polypeptides nonspecifically sticking to protein A-Sepharose beads or antibody. Many bands, however, were present in immunoprecipitates prepared from MT-expressing cells but only found in trace amounts in immunoprecipitates prepared from control cells with immune serum (Fig. 1). To determine if any of these proteins were nonspecifically sticking proteins induced in cells expressing MT, we prepared immunoprecipitates from MT-expressing cells with preimmune serum and analyzed them in parallel with those prepared with immune serum (Fig. 1). Any MT-induced proteins nonspecifically associating with immunoprecipitates should be present at the same levels in immunoprecipitates prepared with immune and preimmune sera. However, if the proteins specifically associate directly or indirectly with MT, they should be present at significantly higher levels in immunoprecipitates prepared with immune serum. The latter possibility was the case; the proteins specific for the presence of MT were also specific for the use of immune serum. They are likely to be MTAPs. The possibility that some bands represented proteins that both cross-reacted with immune sera and were induced by MT cannot be ruled out. Bands below MT potentially could be breakdown products of MT, whereas bands above MT were all most likely cellular proteins. The numbered arrows in Fig. 1 indicate the approximate positions of proteins that we have determined are not breakdown

TABLE 1. MTAPs identified on 2D gels^a

Size of associated protein (kDa)	Approx pI	Presence (+) or absence (−) in immunoprecipitates of MT from:	
		NIH 3T3 lines expressing MT	293 cells infected with Ad5(pymT)
27	5.3	+	+
29	5.1	+	+
36	6.1	+	+
61	6.6–7.4	+	+ ^b
63	5.6	+	+
72	6.2	−	+
74	6.15	−	+
85	6.8	+	?

^a The 51-kDa MTAP identified in rodent cells is not included, since it may be a breakdown product of one of the 61-kDa proteins.

^b A subset of the 61-kDa MTAP species found in NIH 3T3 cells has been found in 293 cells.

FIG. 2. Comparison on 2D gels of the MTAPs from human and rodent cells. MT and control immunoprecipitates were prepared from ^{35}S -labeled Ad5(pymT)-infected 293 cells and from ^{35}S -labeled MT-expressing NIH 3T3 cells as described in Materials and Methods. These immunoprecipitates and untreated lysate from ^{35}S -labeled uninfected 293 cells were analyzed on parallel 2D gels. (a) MT-expressing NIH 3T3 cells, immune serum; (b) MT-expressing NIH 3T3 cells, preimmune serum; (c) Ad5(pymT)-infected 293 cells, immune serum; (d) Ad5(pymT)-infected 293 cells, preimmune serum; (e) uninfected 293 cell lysate. The positions of the comigrating MTAPs are indicated by nonstarred arrowheads in panels a to d. The starred arrowheads indicate the positions of the human 72- and 74-kDa MTAPs. The positions of actin (A) and tubulin (T) are indicated for reference.

FIG. 3. Comparison by complete tryptic peptide mapping of the 27- and 29-kDa MTAPs and their comigrating counterparts from 293 cell lysates. MT and control immunoprecipitates were prepared from ^{35}S -labeled Ad5(pymT)-infected 293 cells as described in Materials and Methods. These immunoprecipitates and untreated lysate from ^{35}S -labeled uninfected 293 cells were analyzed on parallel 2D gels similar to those in Fig. 2c to e. The 27- and 29-kDa proteins were excised and exhaustively digested with trypsin. The peptides were prepared as described in Materials and Methods and analyzed on thin-layer plates by electrophoresis (horizontal axis) followed by chromatography (vertical axis). (a) Peptides of the 27-kDa protein from MT immunoprecipitates; (b) peptides of the 29-kDa protein from MT immunoprecipitates; (c) peptides of the 27-kDa protein from 293 cell lysates; (d) peptides of the 29-kDa protein from 293 cell lysates. The peptides in panel d were electrophoresed 5 min longer than those in the other panels and are therefore spread out more in the horizontal direction.

products of MT but rather are cellular proteins that associate with MT (see below). These include proteins of 27, 29, 36, 63, 72, and 74 kDa. Two other proteins of approximately 93 and 110 kDa, indicated by arrowheads in Fig. 1, probably are also cellular proteins but have not been analyzed further.

To determine which proteins from the human cells may correspond to the rodent cell MTAPs previously identified (14) (Table 1), we analyzed MT immunoprecipitates from both cell sources in parallel on 2D gels (Fig. 2). The nonstarred arrowheads in Fig. 2a and c indicate the MTAPs from the two sources which comigrated. Comigration was determined initially by a careful comparison of the parallel 2D gels but was confirmed by an experiment in which the immunoprecipitates from the two sources were mixed prior to analysis on 2D gels (data not shown). The comigrating MTAPs included the 27-, 29-, 36-, and 63-kDa proteins and at least one of the five 61-kDa species identified previously in mouse cells (14). The starred arrowheads in Fig. 2 indicate the human 72- and 74-kDa MTAP species that appear to be specifically associated with MT in human cells but not rodent cells.

Because adenovirus shuts down host protein synthesis around 12 h postinfection (22), some 61-kDa species may be labeled less well in human cells than in rodent cells, making them difficult to detect by metabolic labeling. Therefore, since several of the 61-kDa species can be phosphorylated efficiently in MT immunoprecipitates by incubation with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (unpublished data), MT immunoprecipitates from human and mouse cells were phosphorylated *in vitro* and analyzed on one-dimensional and 2D gels (data not shown). On examination of these gels, several more 61-kDa

MTAP species from the human and mouse cells that comigrated on the 2D gels could easily be identified. In addition, the rodent 85-kDa MTAP could be seen clearly, but no precisely comigrating band could be detected in the MT immunoprecipitates from human cells (data not shown). However, on one-dimensional gels, several minor bands that migrated slightly higher or lower than the rodent 85-kDa protein were detected in MT immunoprecipitates from human cells, and one of these may represent the human equivalent of this protein.

Identification of the 27-, 29-, 72-, and 74-kDa proteins directly in cell lysates analyzed on 2D gels. To determine whether any of the human MTAPs were major cellular proteins, we analyzed Nonidet P-40 lysates of 293 cells metabolically labeled with methionine in parallel with MT immunoprecipitates on 2D gels. The 27-, 29-, 72-, and 74-kDa proteins but not the 36-, 61-, or 63-kDa protein were found to comigrate with predominant cellular polypeptides from the lysates (compare panels c and e of Fig. 2). Each of the proteins was excised from these gels, and the identity of the immunoprecipitated and comigrating lysate species was confirmed by 2D separation of complete tryptic digests (Fig. 3 and 4). Of note, the 72- and 74-kDa proteins were not only identical to their comigrating counterparts but also appeared to be partially related to each other (Fig. 4).

The 72- and 74-kDa proteins are the major human 70-kDa heat shock proteins. We suspected that the 72- and 74-kDa proteins might be the human 70-kDa heat shock proteins because of their positions of migration on 2D gels, the fact that they were related to one another, and their abundance in cell lysates. To test this hypothesis, we analyzed cell lysates from heat-shocked and control 293 cells on 2D gels. The brackets in Fig. 5 indicate the positions of the major heat-inducible 70-kDa proteins in these cells. These were the same proteins that we found to comigrate with the 72- and

FIG. 4. Comparison by complete tryptic peptide mapping of the 72- and 74-kDa proteins and their comigrating counterparts from 293 cell lysates. MT and control immunoprecipitates were prepared from ^{35}S -labeled Ad5(pymT)-infected 293 cells as described in Materials and Methods. These immunoprecipitates and untreated lysate from ^{35}S -labeled uninfected 293 cells were analyzed on parallel 2D gels similar to those in Fig. 2c to e. The 72- and 74-kDa proteins were excised and exhaustively digested with trypsin. The peptides were prepared as described in Materials and Methods and analyzed on thin-layer plates by electrophoresis (horizontal axis) followed by chromatography (vertical axis). (a) Peptides of the 72-kDa protein from MT immunoprecipitates; (b) peptides of the 74-kDa protein from MT immunoprecipitates; (c) peptides of the 72-kDa protein from 293 cell lysates; (d) peptides of the 74-kDa protein from 293 cell lysates.

FIG. 5. Identification of the 72- and 74-kDa MTAPs as the major human 70-kDa heat shock proteins. Parallel 60% confluent dishes of human 293 cells were incubated at 37 or 43°C for 3 h and labeled with [³⁵S]methionine (100 μ Ci/ml in DMEM lacking methionine) for 1 h at the same temperatures. Cells were harvested, washed with phosphate-buffered saline, and boiled in sample buffer (1.5% sodium dodecyl sulfate, 80 mM dithiothreitol, 8% glycerol). The lysates were sonicated and treated for 20 min with DNase I (50 μ g/ml) and RNase (35 μ g/ml) at 37°C. Finally, the samples were analyzed on 2D gels, and the radiolabeled proteins were visualized by autoradiography. The brackets indicate the major 70-kDa heat shock proteins. A, Actin; T, tubulin. The numbers correspond to molecular weights in thousands.

74-kDa MTAPs and that we showed by tryptic mapping were equivalent to these MTAPs (Fig. 4). The 90-kDa heat shock protein was also visible in Fig. 5, although it did not focus well and was retarded at several positions in the gel. Other proteins could also be seen to vary with the change in temperature, but those variations were not seen consistently.

Finally, we examined the relationship between the 72- and 74-kDa MTAPs and the 70-kDa heat shock proteins immunologically. 2D gels of MT immunoprecipitates prepared with immune and preimmune sera from ³⁵S-labeled cells were immunoblotted with antisera specific for the 70-kDa heat shock proteins. Figure 6a and b show the profile of ³⁵S-labeled proteins from these immunoprecipitates after transfer to nitrocellulose membranes. The 72- and 74-kDa MTAPs are indicated by arrowheads. Figure 6c and d show the results of probing these membranes with anti-hsp70 monoclonal antibody N27 (17). The antiserum specifically recognized the 72- and 74-kDa MTAPs but none of the other proteins in the immunoprecipitates, further supporting the hypothesis that these are the major human 70-kDa heat shock proteins.

DISCUSSION

We have analyzed the proteins that can associate specifically with MT in human 293 cells infected with Ad5(pymT),

a recombinant adenovirus which expresses MT at high levels in these cells. The proteins identified are for the most part the same as those found associated with MT in rodent cell lysates. Proteins of 27, 29, 36, 61, and 63 kDa appear to be fairly well conserved between human and rodent cells, as judged by comigration on 2D gels. Therefore, the Ad5(pymT)-human 293 cell system may be a good source of these proteins for biochemical analysis and for use as immunogens. Initial attempts by our laboratory to purify the 36- and 63-kDa MTAPs from these cells with an MT immunoaffinity column followed by preparative 2D gels have yielded microgram amounts of these proteins (unpublished data). A protein precisely comigrating with the rodent 85-kDa MTAP could not be detected, raising the possibility that the human equivalent of this protein, if it exists, might not be as conserved as the other MTAPs. Lack of detection, however, certainly does not prove its absence. Among other explanations, it is possible that the shutdown of host protein synthesis around 12 h postinfection with adenovirus (22) reduces its abundance in these cells.

Two additional proteins of approximately 72 and 74 kDa which have not previously been shown to associate with wild-type MT in rodent cells were found associated with MT in Ad5(pymT)-infected human 293 cell lysates (21; Fig. 1). These proteins were first reported by Schaffhausen and co-workers to copurify with MT when MT was immunoaffinity purified from these same cells (21). We have now identified the 72- and 74-kDa proteins as the major human 70-kDa heat shock proteins by three criteria: comigration on 2D gels (Fig. 2 and 5), 2D analysis of complete tryptic digests (Fig. 4), and immunoblotting (Fig. 6). The 72-kDa protein probably corresponds to hsc70, also called hsp70 or 72K, while the 74-kDa protein probably corresponds to hsc70, also called 73K (for a summary of the nomenclature, see reference 16). Both of these proteins are present at high basal levels in growing cells, and the 72K protein is inducible by the adenovirus E1A protein, which 293 cells express (for a review, see reference 16). This is consistent with the high level of these proteins found in 293 cells grown at 37°C (Fig. 5).

In contrast to the results with human 293 cells, neither the 73K nor the 72K protein is found specifically associated with immunoprecipitates of wild-type MT from cultured mouse cells expressing MT (14, 24). The absence of the 72K protein is expected because there is no direct rodent equivalent of this protein (16). However, the rodent equivalent of the 73K protein is present at a high level in cell lysates (16) and yet is not specifically associated with MT (24). There are a number of possible explanations for this result. One such explanation is suggested by the fact that the 73K protein does associate in mouse cells with a transformation-defective mutant of MT, NG59 MT (24), which is deficient in binding most of the MTAPs (14). It is possible that the heat shock proteins bind to MT when MT is not associated with its normal array of cellular proteins. This would be consistent with the model postulated by Pelham (16) which suggests that the 70-kDa heat shock proteins have an affinity for "abnormal proteins" and bind tightly to hydrophobic surfaces such as those that might be present at protein-protein binding sites. A site that can interact with the 70-kDa heat shock proteins might be available only when certain MTAPs are not bound to MT. In the case of NG59 MT, the NG59 mutation prevents the mutant MT from binding the 36-, 60- and 61-, and 63-kDa MTAPs, allowing, perhaps, the less stringent binding of the heat shock proteins. In the case of the 293 cells, in which 10 to 100 times more MT is synthesized than in rodent cells, the

FIG. 6. Immunoblotting of MT immunoprecipitates with monoclonal antibody to 70-kDa heat shock proteins. Cell lysates from 293 cells infected with recombinant adenovirus Ad5(pymT) and labeled with [^{35}S]methionine for 24 h were immunoprecipitated with preimmune antiserum (a and c) or anti-tumor antigen serum (b and d) (13). The immunoprecipitates were analyzed on 2D gels, electrophoretically transferred to nitrocellulose membranes, and probed with N27, a monoclonal antibody specific for the 70-kDa heat shock proteins. The radiolabeled proteins on the membranes were visualized by autoradiography (a and b). Immunoblots are shown in panels c and d. The positions of the 72- and 74-kDa MTAPs are indicated by arrowheads.

MT may have saturated some of the associated proteins, leaving some fraction of MT free to bind the 70-kDa heat shock proteins. Attempts to vary the amount of MT in the 293 cells to determine if some of the associated proteins are saturated with MT and if the association of 70-kDa heat shock proteins correlates with saturation have thus far been unsuccessful. There are alternative explanations as well. In HeLa cells coinfecting with the MT-expressing recombinant adenovirus and wild-type helper virus, the association of MT with 70-kDa heat shock proteins can also be detected, indicating that this association is not unique to the 293 cell line (unpublished data). However, both 293 cells and the wild-type helper virus express the adenovirus E1A and E1B proteins, and the effects of these proteins on 293 or HeLa cells might in some way induce the binding of heat shock proteins to MT.

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