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Multimerization of Polyomavirus Middle-T Antigen

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The oncogenic protein of polyomavirus, middle-T antigen, associates with cell membranes and interacts with a variety of cellular proteins involved in mitogenic signalling. Middle-T antigen may therefore mimic the function of cellular tyrosine kinase growth factor receptors, like the platelet-derived growth factor or epidermal growth factor receptor. Growth factor receptor signalling is initiated upon the binding of a ligand to the extracellular domain of the receptor. This results in activation of the intracellular tyrosine kinase domain of the receptor, followed by receptor phosphorylation, presumably as a consequence of dimerization of two receptor molecules. Similar to middle-T antigen, phosphorylation of growth factor receptors leads to recruitment of cellular signalling molecules downstream in the signalling cascade. In this study, we investigated whether middle-T antigen, similar to tyrosine kinase growth factor receptors, is able to form dimeric signalling complexes. We found that association with cellular membranes was a prerequisite for multimerization, most likely dimer formation. A chimeric middle-T antigen carrying the membrane-targeting sequence of the vesicular stomatitis virus G protein instead of the authentic polyomavirus sequence still dimerized. However, mutants of middle-T antigen unable to associate with 14-3-3 proteins, like *dl*8 and S257A, did not form dimers but were still oncogenic. This indicates that both membrane association and binding of 14-3-3 are necessary for dimer formation of middle-T antigen but that only the former is essential for cell transformation.

Proteins expressed early in the life cycle of polyomavirus, the tumor antigens (T antigens), are responsible for tumor formation in virus-infected animals and for virus-mediated transformation of cells in culture (31). Large-T antigen is a nuclear protein known to immortalize primary cells in culture (28), and middle-T antigen causes phenotypic changes associated with malignant cell growth (32). The activity of middle-T antigen results from its association with intracellular signal-transducing proteins, like members of the Src family of tyrosine kinases (c-Src, Fyn, and c-Yes) (reviewed in reference 8), the 85- and 110-kDa subunits of phosphatidylinositol 3-kinase (PI 3-kinase) (35), the catalytic and regulatory subunits of protein phosphatase 2A (22, 34), phospholipase C-γ1 (29), and the phosphotyrosine binding domain-containing protein SHC (5, 9), whose role is to activate the Ras signalling pathway (17, 25). Middle-T immunoprecipitates have also been found to contain a member of the 14-3-3 family of proteins (21). 14-3-3 proteins have been shown to dimerize and associate with a variety of cellular signalling proteins (reviewed in reference 2). Their role may be to form a protein scaffold, allowing the assembly of signalling complexes.

Signalling by middle-T antigen shares many aspects of the mechanisms used by tyrosine kinase growth factor receptors, like the platelet-derived growth factor, epidermal growth factor, and colony-stimulating factor 1 receptors. Early after ligand stimulation, these receptors dimerize (4, 14), resulting in phosphorylation of the intracellular domain of the receptor. This results in recruitment of cellular proteins into active signalling complexes. Here, we investigated whether middle-T antigen also forms dimers. We found that all middle-T mutants capable of associating with cell membranes and of binding 14-3-3 proteins efficiently dimerized. A middle-T mutant lacking the interaction site with 14-3-3 proteins was unable to dimerize but still transformed cells with high efficiency. This mutant has been shown to be highly oncogenic in cell transformation assays (13), indicating that dimerization and association with 14-3-3 are not required for middle-T oncogenicity.

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MATERIALS AND METHODS

Cell line. NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum.

Plasmids. All the plasmids used, except for pcDNA200Δ20mT, have been described before (3, 33). All T-antigen mutants were expressed in the pcDNA1neo expression vector (Invitrogen). Middle-T mutants 200Δ20 (9) and T203E (4a) do not associate with c-Src and are transformation defective. 308aa is a truncated form of middle-T antigen lacking the membrane anchor (18).

Transfection of cells. Cells were transiently transfected with Lipofectamine (Life Technologies) as previously described (11). Cell populations stably expressing various middle-T mutants were selected with G418 for 3 weeks after cotransfection with a plasmid that confers neomycin resistance.

Immunoprecipitation and in vitro kinase assays. Cell lysis, immunoprecipitation, and in vitro kinase assays with [γ-^{32}P]ATP were essentially performed as previously described (15). Antibodies were coupled to protein A-Sepharose (Pharmacia Biotech) with 20 mM dimethylpimelimidate for 30 min at room temperature at pH 8.3, followed by quenching with 100 mM glycine. Lysates were incubated for 2 h with coupled antibodies at 4°C. Immunoprecipitates were analyzed on sodium dodecyl sulfate (SDS)-polyacrylamide gels.

Western analysis. Immunoprecipitates were run on SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Millipore), and immunodecorated by using an alkaline phosphatase-linked antispecies antibody (Southern Biotechnology Association) and subsequently a chemiluminescence detection system (CDP-Star; Tropix). For redecoration, blots were stripped with a buffer containing 62.5 mM Tris-HCl (pH 6.8), 100 mM β-mercaptoethanol, and 2% SDS for 1 h at 50°C.

Quantification of gel data. Gels were scanned with a Umax flat-bed scanner and quantified with the ImageQuant software package (Molecular Dynamics). The relative intensities of the bands corresponding to full-length and deletion mutant middle-T antigens were determined from scanned gels (see Fig. 2A, 2C, and 4A). The intensity of the full-length protein was always set at 1.

RESULTS

We investigated whether in analogy with membrane-bound growth factor receptors, signalling by middle-T antigen requires the formation of dimers to activate downstream targets. Various mutants defective for association with cellular proteins were used (Fig. 1). In our experimental setup, a full-length and

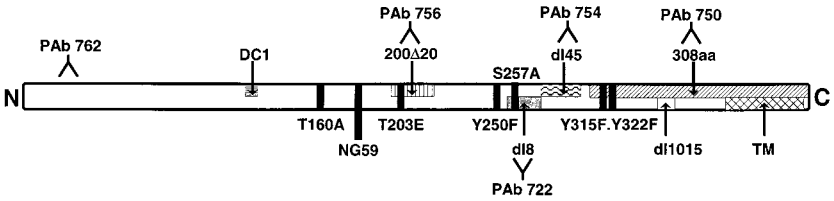

FIG. 1. Schematic representation of the middle-T mutants used in this study. Black bars indicate the positions of mutations T160A, NG59, T203E, Y250F, and S257A and the double mutation Y315F.Y322F. The deletions in DC1 (horizontal lines), 200Δ20 (vertical lines), *dl*8 (gray bar), *dl*45 (wavy lines), *dl*1015 (open bar), and 308aa (diagonal lines) are also indicated. The bar with hatched lines represents the transmembrane domain (TM). The approximate binding regions for PAbs 722, 750, 754, 756, and 762 are indicated.

a deletion mutant middle-T antigen were transiently or stably expressed in NIH 3T3 cells. Antibodies that specifically recognized epitopes missing in the shorter protein were subsequently used to immunoprecipitate the expressed T antigens. As shown in Fig. 2A, full-length middle-T antigen was specifically recognized by all the polyclonal antibodies (PAbs) used in this study. Truncated mutant proteins, on the other hand, were precipitated only by these antibodies when they were coexpressed with a full-length middle-T antigen, which is indicative of association between the two forms of middle-T antigen. In Fig. 2A, the wild-type (wt) protein (lane 1), 200Δ20 (lane 2), and both proteins (lane 3) were expressed in NIH 3T3 cells. A similar experiment was performed with mutants 200Δ20 and NG59 (Fig. 2A, lanes 4 to 6). *dl*45 middle-T antigen was cotransfected with either NG59 or wt middle-T antigen (Fig. 2A, lanes 7 to 11). In all four combinations, the short mutant form of middle-T antigen coprecipitated with the full-length protein, which was exclusively recognized by an antibody unable to bind the short mutant protein. Control experiments performed with polyclonal anti-T ascites showed that the amounts of both proteins expressed in cotransfected cells were similar (Fig. 2A). To rule out the possibility that association between various middle-T mutants was an artifact of cell lysis, we mixed separately transfected cells before lysis or lysates derived from individually transfected cells before antibody addition. After immunoprecipitation, the material was analyzed on SDS-polyacrylamide gels (Fig. 2B). Middle-T antigen did not form multimers under these conditions (Fig. 2B, upper panel, lanes 4 and 5), although similar amounts of the two forms of the protein used in this experiment were expressed, as seen for precipitations with anti-T ascites (lower panel, lanes 4 and 5). Therefore, coprecipitation of full-length and truncated middle-T antigens occurred only when the proteins were coexpressed in transfected cells, which is indicative of association in intact cells. To rule out the possibility that coprecipitation of various T-antigen mutants resulted from unspecific precipitation as a consequence of the high expression levels in transiently transfected cells, we repeated the experiment (Fig. 2B, lane 3) with stably transfected cells. A quantification of the data obtained with transiently or stably transfected cells is shown in Fig. 2D. Data for experiments performed in stable cell lines (Fig. 2D, lane a) and for experiments performed with lipofected cells (lanes b and c) are shown. Stoichiometric complexes between a full-length middle-T antigen and a deletion mutant middle-T antigen formed under both conditions.

Various mutants (Table 1) were tested for the ability to multimerize in order to identify the epitope(s) responsible for this interaction. Mutants, such as DC1 and NG59, disabled in binding any of the proteins targeted by middle-T antigen (18), as well as mutants unable to bind c-Src or lacking some of the tyrosine residues required in signalling through Ras or PI 3-kinase (5, 6, 9), associated with each other. This indicates that these T-antigen mutants are able to multimerize. The molecular mass of the middle-T complex is on the order of 250 kDa, suggesting that this complex most likely contains a dimer of the middle-T antigen together with the cellular proteins described before (8).

We used a truncated middle-T antigen (Fig. 2C, lanes 3, 4, 7, and 8) lacking the membrane anchor sequence and small-t antigen (lanes 5, 6, 9, and 10) for similar experiments. These truncated T antigens were not coprecipitated by full-length middle-T antigen (Fig. 2C, lanes 7 and 9). A quantification of the data is given in Fig. 2D, lane d. An additional mutant, mT/G (30), where the membrane-targeting sequence was replaced by a functionally similar sequence derived from the G protein of vesicular stomatitis virus (VSV), associated with membrane-bound deletion mutant middle-T antigen (Table 1) (30). Based on these results, we conclude that neither Src kinases, PI 3-kinase, protein phosphatase 2A, nor SHC is required for dimerization of middle-T antigen. The data also show that membrane association is obligatory for T-antigen multimerization and that even a membrane-targeting sequence derived from a different viral protein, the VSV G protein, allows dimerization of middle-T antigen.

We next asked whether middle-T mutants unable to bind Src are phosphorylated at tyrosine residues when they are coexpressed with a mutant competent to do so. wt middle-T antigen was expressed together with a deletion mutant, 200Δ20, that has been shown not to associate with c-Src (9) (Fig. 3A). Functional multimeric complexes that allowed in vitro phosphorylation of both proteins were formed (Fig. 3A, lanes 6 to 8). A comparison of the intensity of the truncated phosphoprotein in Fig. 3A, lane 6, with those of proteins in lanes 7 and 8 shows that Src bound to wt middle-T antigen preferentially phosphorylated the wt protein. Increased phosphorylation was seen when the T antigen competent to bind Src was itself disabled as a substrate for c-Src, such as in Y250F middle-T antigen (Fig. 3A, lane 7) or, even more pronounced, in the Y315F.Y322F mutant (lane 8). Figure 3B confirms that all middle-T mutants were expressed at equal levels. No cross-phosphorylation was observed with immunoprecipitates of pooled lysates from individually transfected cells (Fig. 3C). These data further confirm that middle-T antigen is capable of forming multimers upon coexpression in transfected cells. Most importantly, mutants unable to associate with Src were phosphorylated by Src associated with functional middle-T antigen when it was present in a heterodimer.

Recently, members of the 14-3-3 family of proteins have been found to bind middle-T antigen (21), but no biological function has been ascribed to this interaction. Such association seems to be mediated by a phosphorylated serine residue well conserved in a variety of proteins known to bind 14-3-3 proteins (20). Middle-T antigen also contains a serine residue in

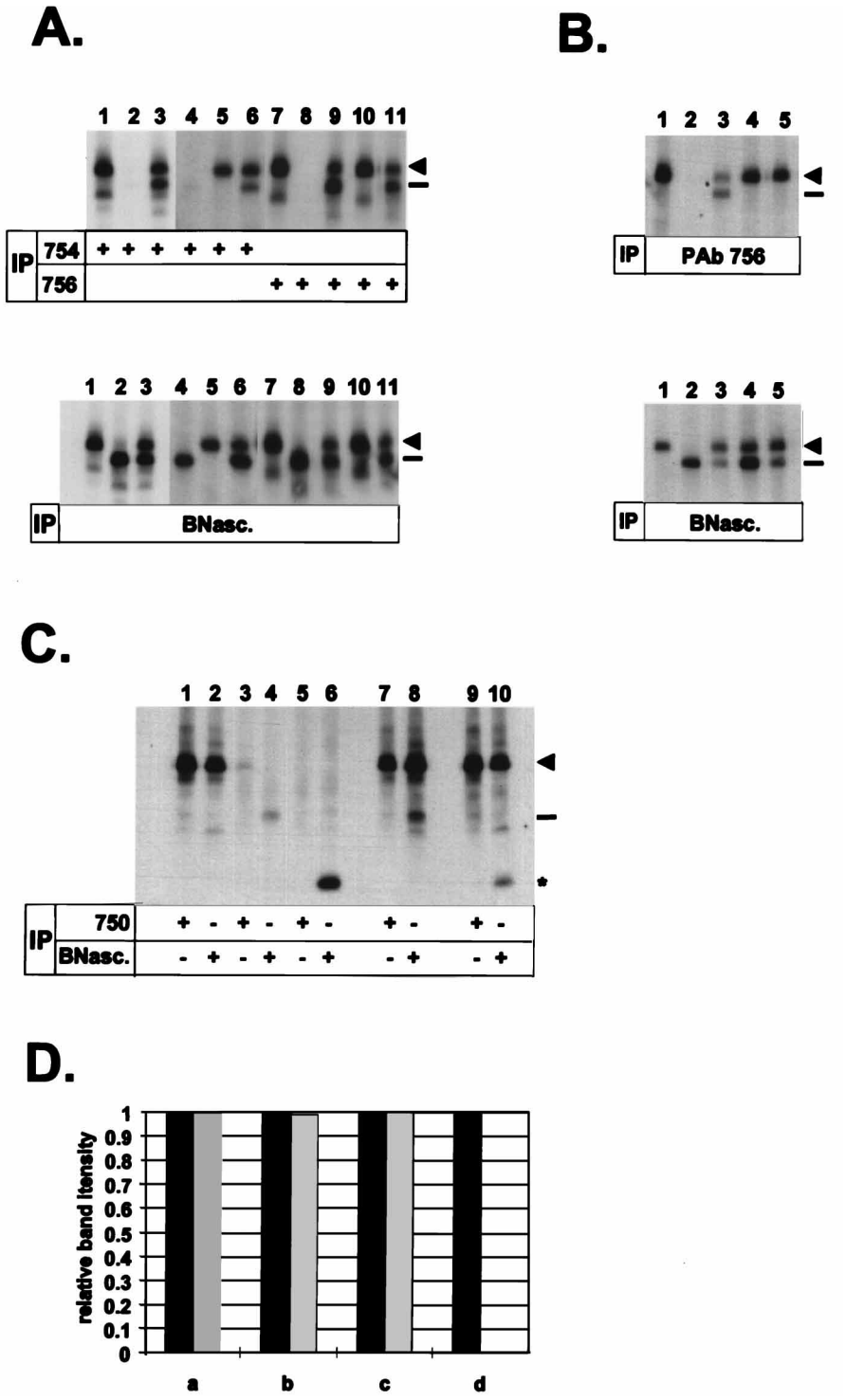

FIG. 2. Coprecipitation of full-length and deletion mutant middle-T antigens. NIH 3T3 cells were transfected with pcDNA1neo plasmids by the Lipofectamine method. Immunoprecipitations (IP) were carried out with cells coexpressing various middle-T mutants. They were incubated with either PAb 754 (which does not recognize *dl*45), PAb 756 (which does not recognize 200Δ20), or PAb 750 (which does not recognize 308aa and small-t antigen). To check for expression, half of each lysate was incubated with BN anti-T ascites (BNasc.). Western analysis was performed with PAb 762. Arrowheads show the positions of the full-length wt and NG59 middle-T antigens. Bars show the positions of *dl*45, 200Δ20, and 308aa middle-T antigens. An asterisk indicates the position of small-t antigen. (A) Heterodimerization of polyomavirus middle-T antigen. Cells were transfected with wt middle-T antigen (lanes 1 and 7), 200Δ20 (lanes 2 and 4), wt middle-T antigen plus 200Δ20 (lanes 3), NG59 (lanes 5 and 10), NG59 plus 200Δ20 (lanes 6), *dl*45 (lanes 8), wt middle-T antigen plus *dl*45 (lanes 9), and NG59 plus *dl*45 (lanes 11). The lower-molecular-mass bands in lanes 1, 3, 7, and 10 represent proteolytic fragments of middle-T antigen. (B) Dimerization occurs only in cotransfected cells. Immunoprecipitations were carried out with cells transfected with wt middle-T antigen (lanes 1), 200Δ20 (lanes 2), and wt middle-T antigen cotransfected with 200Δ20 (lanes 3). In lanes 4, wt middle-T-antigen-transfected cells were mixed before lysis with 200Δ20-transfected cells; in lanes 5, a lysate from wt middle-T-antigen-transfected cells was mixed with a lysate from 200Δ20-transfected cells. Immunoprecipitations were performed with PAb 756 or anti-T ascites. Western blotting was performed with PAb 762. (C) T antigens lacking the membrane anchor do not dimerize. Cells were transfected with wt middle-T antigen (lanes 1 and 2), 308aa (lanes 3 and 4), small-t antigen (lanes 5 and 6), wt middle-T antigen plus 308aa (lanes 7 and 8), and wt middle-T antigen plus small-t antigen (lanes 9 and 10). Even-numbered lanes contained anti-T ascites immunoprecipitates; odd-numbered lanes contained PAb 750 immunoprecipitates. (D) Quantification of the association between full-length and deletion mutant middle-T antigens. The relative intensities of bands corresponding to full-length (black bars) and deletion mutant (grey bars) middle-T antigens are shown; the intensity of the full-length protein was always set at 1. Lanes: a and b, the wt protein coexpressed with 200Δ20; c, the wt protein coexpressed with *dl*45; and d, the wt protein coexpressed with small-t antigen. Stably transfected cell cultures (lane a) and transiently transfected cells (lanes b to d) were used.

TABLE 1. Summary of different combinations of middle-T constructs tested in dimerization assays

Antigen	Dimerization with[a]:		
	wt middle-T antigen	*dl*45	200Δ20
wt middle-T		+	+
DC1			+
T160A			+
NG59		+	+
T203E		+	
Y250F			+
S257A			±
*dl*8	−		
308aa	−		
Y315F.Y322F		+	+
*dl*1015			+
Small-t	−		
mT/G			+

[a] +, association between indicated middle-T constructs; ±, weak association; −, no association.

this amino acid context, serine 257, that is essential for 14-3-3 association (7a). This site is absent in the T-antigen mutant *dl*8; we therefore used it to determine whether 14-3-3 association is essential for dimerization of middle-T antigen (Fig. 4, lanes 13 to 18). The ability of *dl*8 to dimerize was dramatically reduced, as shown by quantification of the data (Fig. 4B, lane c). The antibody used for this experiment, PAb 722, specifically recognizes amino acids 241 to 281 (10) but still weakly precipitated *dl*8 middle-T antigen. We therefore subtracted the signal that resulted from precipitation of *dl*8 middle-T antigen by PAb 722 in singly transfected cells (Fig. 4A, lane 14) from the amount coprecipitated with full-length middle-T antigen (lane 15). In three separate experiments, we found that four- to fivefold-less *dl*8 middle-T antigen was coprecipitated with the full-length protein compared with the amounts of deletion mutant proteins which map outside this epitope. As a control, a similar experiment was performed with wt and 200Δ20 middle-T antigens (Fig. 4A, lanes 1 to 6). An additional mutant, S257A middle-T antigen, recently shown to be unable to associate with 14-3-3 proteins was expressed together with 200Δ20 middle-T antigen (Fig. 4A, lanes 7 to 12). As seen by quantification of the data (Fig. 4B, lane b), this mutant protein showed reduced binding to full-length middle-T antigen, indicating that association with 14-3-3 proteins is, at least in part, responsible for dimer formation of middle-T antigen. Both *dl*8 and S257A middle-T antigens did not associate with 14-3-3 proteins (data not shown) (7a). Taken together, these data suggest (although they do not formally prove) that 14-3-3 proteins are required for dimer formation of polyomavirus middle-T antigen.

DISCUSSION

Tyrosine kinase growth factor receptors have been shown to dimerize upon ligand binding. This is an obligatory step for signalling to downstream targets. Polyomavirus middle-T antigen has many features in common with tyrosine kinase growth factor receptors and maybe considered to behave as a constitutively activated form of a membrane-bound receptor that lacks a modulatory extracellular ligand binding domain. We investigated whether dimerization is also required for signalling by polyomavirus middle-T antigen. In our experiments, a full-length middle-T antigen and a deletion mutant middle-T antigen were cotransfected into NIH 3T3 cells. The full-length protein was immunoprecipitated by an antibody that recognizes an epitope absent in the deletion mutant protein. Therefore, coprecipitation of a deletion mutant middle-T antigen with a full-length middle-T antigen was indicative of association between the two proteins.

Our data show that membrane-bound forms of middle-T antigen were capable of associating with each other. The apparent molecular mass of the middle-T antigen complex on glycerol gradients has been shown to be approximately 250 kDa (26). This suggests that no more than two copies of middle-T antigen are engaged in a multimeric complex with the associated cellular proteins described before (reviewed in reference 8). The data also demonstrate that only membrane-bound middle-T antigen dimerizes and that the T-antigen-specific membrane-targeting sequence is not essential for dimer formation since it can be replaced by a similar peptide present in the VSV G protein (30). Interestingly, although it still associates with Src kinases, this chimeric mutant protein has been shown to be transformation defective, perhaps as a consequence of improper localization in cellular membranes. Earlier work showed that another middle-T mutant, *dl*23, did not form dimers with wt middle-T antigen (7). This may be the consequence of aberrant folding of this mutant protein, which has also been shown to be defective in cell transformation (13).

To define the exact epitope(s) required for dimerization of middle-T antigen, we used a series of deletion mutants that map to various regions, *dl*1015, *dl*45, 200Δ20, and *dl*8. All mutants except for *dl*8 formed dimers. Recently, it has been shown that *dl*8 lacks the sequence responsible for binding 14-3-3 proteins (7a). 14-3-3 proteins are known to dimerize and to act as scaffolding proteins in the formation of multimeric protein complexes (1, 19, 36). Therefore, their role in middle-T-antigen signalling may be to regulate association with cellular

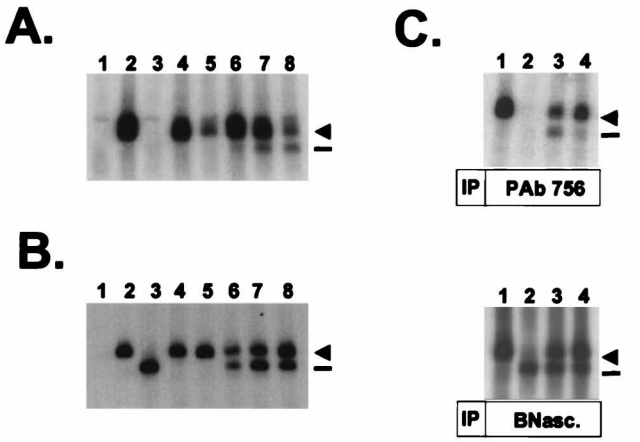

FIG. 3. (A) Cross-phosphorylation of middle-T antigen. Immunoprecipitations were carried out with lysates from NIH 3T3 cells (lane 1) or from cells transfected with wt middle-T antigen (lane 2), 200Δ20 (lane 3), Y250F (lane 4), Y315F.Y322F (lane 5), wt middle-T antigen plus 200Δ20 (lane 6), Y250F plus 200Δ20 (lane 7), and Y315F.Y322F plus 200Δ20 (lane 8). Arrowheads show the positions of full-length middle-T proteins (wt middle-T antigen, Y250F, and Y315F.Y322F); bars show the position of 200Δ20. Immunoprecipitates obtained with PAb 762 were phosphorylated with [γ-^{32}P]ATP and analyzed on SDS–12.5% polyacrylamide gels, followed by autoradiography. (B) Expression of various middle-T mutant proteins. To check the expression of different middle-T proteins, Western analysis was performed with PAb 762. Lane contents were the same as those in panel A. (C) No cross-phosphorylation was observed with immunoprecipitates (IP) of individually transfected cells. NIH 3T3 cells were transfected with Y315F.Y322F (lanes 1), 200Δ20 (lanes 2), and Y315F.Y322F plus 200Δ20 (lanes 3), or separately Y315F.Y322F- and 200Δ20-transfected cells were mixed before cell lysis. BNasc., anti-T ascites.

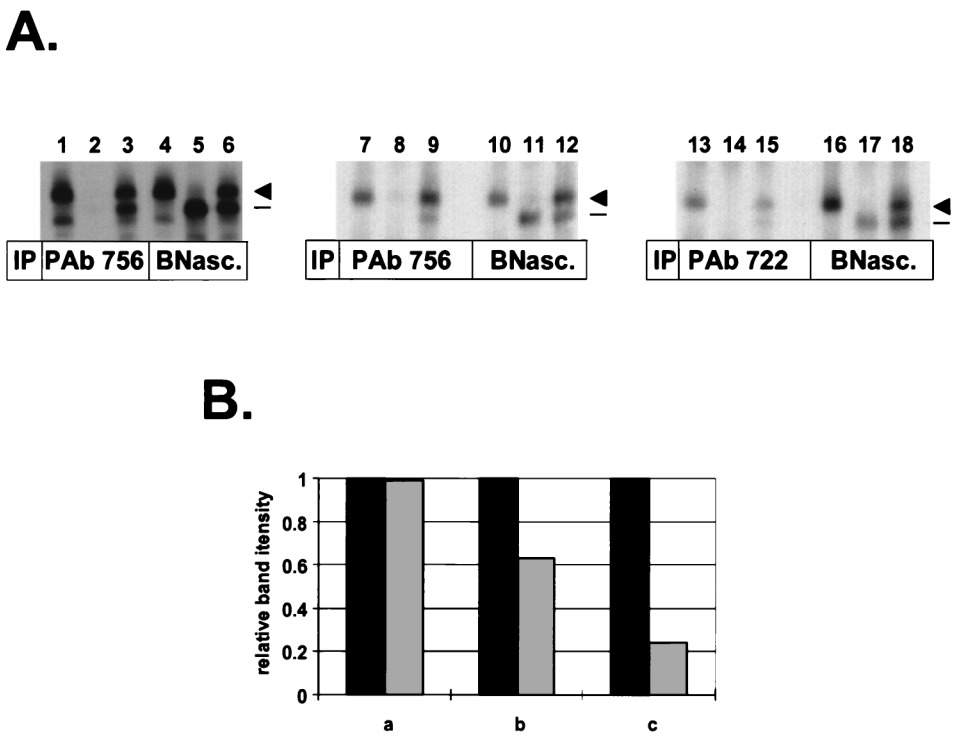

FIG. 4. Middle-T antigen unable to associate with 14-3-3 proteins does not form dimers. (A) Coprecipitations of 200Δ20 with wt middle-T antigen (lanes 1 to 6), S257A with 200Δ20 middle-T antigen (lanes 7 to 12), and *dl*8 with wt middle-T antigen (lanes 13 to 18). Expression levels of wt middle-T antigen (lanes 1, 4, 13, and 16), 200Δ20 (lanes 2, 5, 8, and 11), wt middle-T antigen plus 200Δ20 (lanes 3 and 6), S257A (lanes 7 and 10), S257A plus 200Δ20 (lanes 9 and 12), *dl*8 (lanes 14 and 17), and wt middle-T antigen plus *dl*8 (lanes 15 and 18) are shown. Immunoblotting was performed with PAb 762. IP, immunoprecipitation; BNasc., anti-T ascites. Arrowheads show the positions of full-length middle-T proteins; bars show the position of 200Δ20. (B) Quantification of dimerization efficiency by various middle-T mutants, as described in Materials and Methods and the legend to Fig. 3. The relative intensities of bands from panel A corresponding to full-length (black bars) and deletion mutant (grey bars) middle-T antigens are shown; the intensity of the full-length protein was always set at 1. Lanes: a, wt and 200Δ20 middle-T antigens; b, S257A and 200Δ20 middle-T antigens; and c, wt and *dl*8 middle-T antigens. The intensity of the band corresponding to shortened protein in lane 15 was corrected for the background in lane 14.

signalling molecules. A recently described mutant of middle-T antigen, S257A, that lacks the specific phosphorylation site for interaction with 14-3-3 upon phosphorylation by various kinases was also defective for dimer formation with full-length middle-T antigen. Both *dl*8 and S257A middle-T antigens have been shown to transform cells in vitro; the efficiency of the former was even higher than that of the wt protein. Foci formed on cell monolayers by *dl*8 middle-T-antigen-transformed cells are less adherent and tend to float off dishes (13, 24). Similarly, this mutant is more oncogenic in animals and tumors derived from virus-transformed cells appear earlier after injection. This, together with our data, suggests that dimer formation and association with 14-3-3 proteins are not required for cell transformation by middle-T antigen.

It was proposed earlier that dimerization allows for functional complementation between defective mutants of middle-T antigen (33). However, a more careful evaluation of the data showed that the mechanism underlying complementation was most likely recombination between the plasmids that express these mutants in cotransfected cells. Experiments performed with the T-antigen mutants described above showed a much lower degree of complementation than that originally described.

Based on the fact that *dl*8 and S257A middle-T antigens are oncogenic, we propose that dimerization represents a mechanism to attenuate T-antigen-mediated signalling. Viral oncogenicity is of no immediate benefit for virus replication and may even prevent efficient spreading of the virus in the natural host population. The role of 14-3-3 proteins in attenuating signalling by middle-T antigen may therefore be to allow for more efficient virus replication and spreading. Besides its mitogenic function in primary cells transfected with large-T antigen, middle-T antigen has been shown to increase the efficiency of virus encapsidation, presumably by maintaining high activity levels of kinases of the mitogen-activated protein kinase family (27, 33). An accumulation of activated mitogen-activated protein kinases in the nucleus has been suggested to phosphorylate VP1, resulting in increased encapsidation of the virus (12, 16). Dimerization and binding of 14-3-3 proteins to middle-T antigen may be means for fine-tuning the interactions of this viral protein with the cellular signalling network. Both proteins are phosphorylated by cell cycle-regulated kinases (2, 23, 24). This may be essential for coordination of the viral replicative cycle with cell cycle progression of the infected host cell.

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