Polyomavirus middle-T antigen lacking a membrane anchor sequence accumulates in the nucleus

A. Messerschmitt, C. Disela, S. Dilworth,† A. G. Marti and K. Ballmer-Hofer*

Friedrich Miescher-Institute, PO box 2543, CH 4002-Basel, Switzerland

Three proteins expressed early in the replicative cycle of polyomavirus also play an essential role during virus-mediated tumorigenesis. One of the proteins, middle-T antigen, has been shown to bind cellular proteins involved in cell signalling such as c-Src, phosphatase 2A, phosphatidylinositol 3-kinase and SHC. Association of middle-T antigen with cellular membranes has been shown to be essential for middle-T-mediated cell transformation. A mutant virus encoding a truncated form of middle-T lacking a carboxy-terminal hydrophobic sequence mediating membrane association is not oncogenic. This mutant middle-T still binds phosphatase 2A through amino-terminal sequences common to small- and middle-T and is localized in the nucleus, although the protein does not contain a classical nuclear targeting sequence. Mutations introduced into the amino-terminal domain affecting the ability of truncated middle-T to bind phosphatase 2A prevented accumulation of the protein in the nucleus and led to localization in the cytoplasm. This suggests that nuclear localization of truncated middle-T may be a consequence of binding to phosphatase 2A.

Introduction

Polyomavirus transforms cells in culture and induces tumours in virus-infected animals. Three proteins expressed early in the virus life-cycle, the tumour antigens (T antigens), are responsible for these activities (Tooze, 1980). Large-T antigen is a nuclear protein known to immortalize primary cells in culture (Strauss et al., 1990). Middle-T antigen causes phenotypic changes associated with malignant cell growth such as altered cytoskeleton organization, reduced adhesion on a solid substratum and the ability to grow in low serum and under anchorage-independent conditions (Kaplan et al., 1989; Dilworth, 1995).

The activity of middle-T is thought to result from its association with intracellular signal-transducing proteins such as members of the Src family of tyrosine kinases (c-Src, Fyn, c-Yes; Courtneidge, 1989; Dilworth, 1995), the 85 kDa subunit of a phosphatidylinositol 3-kinase (PI 3-kinase; Whitman et al., 1985), the catalytic and regulatory subunits of protein phosphatase 2A (PP2A; Pallas et al., 1990; Walter et al., 1990) and the SH2-containing protein SHC (Dilworth et al., 1994; Campbell et al., 1994) whose putative role is to regulate GDP/GTP exchange factors involved in the activation of the Ras signalling pathway (Rozakis Adcock et al., 1992; Pronk et al., 1994; Sasaoka et al., 1994). More recently, middle-T immunoprecipitates have been found to contain an additional cellular component, a member of the 14-3-3 family of proteins, some of which seem to be involved in stimulating ADP ribosylation (Pallas et al., 1994).

Middle-T is associated with cellular membranes through a stretch of 22 hydrophobic amino acids localized close to the carboxy terminus (Carmichael et al., 1982; Kutay et al., 1993; Ito et al., 1977a). Cell fractionation studies have shown that middle-T is predominantly associated with the plasma membrane (Ito, 1979; Ito et al., 1977a, b), while a study using immunofluorescence light microscopy and electron microscopy points to association with internal membranes, most likely the endoplasmic reticulum (ER; Templeton et al., 1984; Dilworth et al., 1986).

A third T antigen, small-T (Ito et al., 1977b), whose biological function is not well defined, associates with PP2A through a sequence collinear with the amino terminus of middle-T (Pallas et al., 1990). Small-T, together with middle-T, is responsible for deregulation of PP2A (Scheidtmann et al., 1991; Cayla et al., 1993).

We investigated the localization of middle-T in mouse and rat fibroblasts using a series of monoclonal antibodies characterized recently (Dilworth & Horner, 1993). Studying various virus mutants we found that a middle-T lacking the putative membrane anchor domain (1387T middle-T; Carmichael et al., 1982) was localized in the

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* Author for correspondence. Fax +41 61 697 3976. e-mail ballmer@fmi.ch
† Present address: Royal Postgraduate Medical School, Hammersmith Hospital, London W12 0NN, UK.
Fig. 1. Detection of middle-T in NIH 3T3 and REF-52 cells by immunofluorescence. NIH 3T3 cells were infected with wt polyomavirus NG59RA (a) or with Py1387T mutant virus (c) and stained after 20 h with a mixture of mouse antibodies PAb750, PAb754, PAb758. REF-52 cells were microinjected with pcDNAmT (b) and pcDNA1387T (d) and stained 18 h later with mouse antibody PAb762. For visualization a Texas Red-coupled anti-mouse antibody was used. Cells were analysed on a Leica TCS4D confocal microscope using a 63× NA 1.4 oil immersion lens. Scale bar represents 25 μm.

nucleus, while the wild-type (wt) protein was concentrated in an asymmetrical perinuclear patch, most likely the ER, as shown previously (Templeton et al., 1984; Dilworth et al., 1986). We performed a detailed analysis of the localization of middle-T employing microinjection of cDNAs encoding wt or mutant T antigen into single cells. The results show that sequences present in the domain common to middle- and small-T directed these proteins to the nucleus. Since no classical nuclear targeting signal could be identified in the amino acid sequence of these proteins they might enter the nucleus via a mechanism distinct from the classical pathway, which requires a mono- or bipartite sequence of basic residues (Kalderon et al., 1984; Garcia-Bustos et al., 1991). Our data show that domains crucial for association with PP2A are also important for nuclear localization of these proteins and it could therefore be speculated that one of the subunits of this phosphatase mediates nuclear localization of T antigen. This would be in agreement with recently published data showing that a significant fraction of PP2A is nuclear (Turowski et al., 1995).

Methods

Cells and viruses. NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% newborn calf serum. Cells were infected either with wt polyomavirus (NG59RA) or with mutant virus Py1387T at a multiplicity of 10 p.f.u. per cell. After 20 h, cells were fixed and processed for immunofluorescence. Rat embryo fibroblasts (REF-52) were grown in DMEM supplemented
with 8% fetal calf serum (FCS). For microinjection studies cells were subcultured onto glass coverslips 2 days before use.

**Plasmid constructs.** pSVmT has been described before (Muser et al., 1989). pSV1387TmT is derived from pGM101 (Ballmer-Hofer et al., 1987). DC1 and DC2 mutations were cloned into pSVmT and pSV1387TmT by exchanging a BstXI–DraIII fragment with the corresponding sequence from pAS101DC1 or pAS101DC2 (Markland & Smith, 1987). pSVsTDC1 and pSVsTDC2 were created by transferring the BstXI–DraIII fragment of pSVmTDC1 and pSVmTDC2, respectively, to the BstXI- and DraIII-restricted pSVsT vector. pSVNG59 and pSV1387T.NG59 resulted from replacing the BstXI–DraIII fragment from pSVmT or pSV1387T with the BstXI- and DraIII-digested fragment from pAT53NG59 (Cheng et al., 1986). All genes were cloned into pcDNANeo (Invitrogen) at the HindIII and EcoRI sites. pcDNA1387T.C120W was cloned by transferring the HindIII–EcoRI fragment from pRSVMTC120W (Glenn & Eckhart, 1993) into pcDNA at HindIII and EcoRI sites and by exchanging the DraIII-digested fragment from pcDNA1387T for the corresponding fragment from pcDNAmt120W. pcDNAst.T.NG59 was constructed by swapping the DraIII fragment between pcDNA1387T.NG59 and pcDNAst.DC2.

**Microinjection.** REF-S2 cells were microinjected using an Eppendorf 5171 micromanipulator and an Eppendorf 5242 microinjection device. Microinjection buffer (48 mM-K$_2$HPO$_4$, 4.5 mM-KH$_2$PO$_4$, 14 mM-Na$_2$HPO$_4$) containing the expression vectors at a concentration of 0.3 gg/ml was centrifuged at 100000g for 1 h. Injected cells were studied in virus-infected NIH 3T3 or microinjected REF-52 cells at 20 and 18 h, respectively, after introduction of the corresponding DNA. For microinjection studies cells were incubated for 1 h either with the mouse monoclonal antibody PAb762, which is directed against the common amino-terminal region of large-T, middle-T and small-T, or with mouse antibodies (Southern Biotech) were added for 45 min. Cells were washed and permeabilized with 1% Triton X-100 for 5 min. After washing with PBS, cells were incubated for 1 h either with the mouse monoclonal antibody PA65177/194 directed against the p65 regulatory subunit of PP2A. After washing with Tween/Tris-buffered saline (TTBS; 0.05% Tween 20, 0.05% Tris–HCl pH 7.5, 150 mM-NaCl) the blot was washed and immunocomplexes detected by alkaline phosphatase linked to streptavidine using a chemiluminescence system (Tropix).

**Immunoprecipitation and Western blot analysis.** Immunoprecipitation was performed as described (Kech et al., 1991). For T antigen immunoprecipitation we used a polyclonal rat BNNasites. Immunoprecipitates were run on 10% SDS–polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked with 3% gelatine in TBS and incubated for 1 h at room temperature either with the T antigen-specific antibody PAb672 or with the rabbit serum Ab65177/194 (Turowski et al., 1995), which recognizes the p65 regulatory subunit of PP2A. After washing with Tween/Tris-buffered saline (TTBS; 0.05% Tween 20, 0.05% Tris–HCl pH 7.5, 150 mM-NaCl) the blot was incubated with biotinylated protein A in TTBS for 1 h. The membrane was washed and immunocomplexes detected by alkaline phosphatase linked to streptavidine using a chemiluminescence system (Tropix).

**Results**

In order to study the localization of wt and mutant middle-T antigen (1387Tmiddle-T), NIH 3T3 mouse fibroblasts were infected with polyomavirus and analysed by immunofluorescence 20 h after infection. Fig. 1(a) shows that wt middle-T was localized in a perinuclear patch, most likely the ER, while the truncated 1387Tmiddle-T lacking the putative membrane anchor sequence was predominantly nuclear (Fig. 1c). This was surprising since middle-T does not contain nuclear targeting sequences and was therefore expected to be cytoplasmic.

To investigate further this phenomenon, a variety of mutant middle-T antigens that could not be expressed...
Fig. 3. Immunofluorescence of full-length wt and mutant middle-T. At 18 h after microinjecting REF-52 cells with pcDNAmT (a), pcDNAmT.DC1 (b), pcDNAmT.NG59 (c) and pcDNAmT.DC2 (d) cells were processed as above with PAb762. Scale bar represents 25 μm.

from an entire viral genome due to interference with the large-T reading frame (Tooze, 1980) were cloned into a eukaryotic expression vector. Wt and mutant T antigen-encoding constructs were introduced into REF-52 rat fibroblasts by microinjection. This approach further excluded interference with other viral proteins expressed in virus-infected cells. Fig. 1(b) shows that wt middle-T cDNA microinjected into REF-52 cells resulted in the expression of T antigen in perinuclear structures similar to those observed in virus-infected cells, while truncated middle-T (Fig. 1d) was almost exclusively nuclear. Quantification of the data using the Imaris image processing software package is shown in Fig. 6 (see below). Approximately 60% of the truncated mutant proteins were nuclear while more than 80% were found in perinuclear structures in wt protein-expressing cells. Thus we conclude that middle-T localization is not dictated by ancillary viral proteins but rather by its ability to interact with membranes through a carboxy-terminal hydrophobic domain. Truncated forms of middle-T are not cytoplasmic as expected but accumulate in the nucleus.

The truncated form of middle-T is defective in binding Src tyrosine kinases, PI 3-kinase and SHC yet is still capable of interaction with PP2A (Fig. 2). We therefore concentrated our efforts on T antigen mutants unable to bind PP2A. A series of amino-terminal mutations [NG59 (Hattori et al., 1979); DC1 and DC2 (Markland & Smith, 1987); C120W (Glenn & Eckhart, 1993)] are known to prevent association of full-length middle-T...
Fig. 4. Subcellular localization of various forms of truncated middle-T. REF-52 cells were microinjected with pcDNA1387T (a), pcDNA1387T.DC1 (b), pcDNA1387T.DC2 (c), pcDNA1387T.C120W (d) and pcDNA1387T.NG59 (e) and fixed after 18 h. Middle-T was detected with PAb762 and Texas Red-coupled anti-mouse antibody. Pictures were taken on a Leica TCS4D confocal microscope using a 63 × NA 1.4 oil immersion lens. Scale bar represents 25 μm.
with PP2A. These mutations, mapping to a region encompassing approximately 60 amino acids, were introduced into wt or truncated middle-T. To verify that these mutants were disabled in PP2A-binding, immunoprecipitates made with T antigen-specific antibodies were analysed on Western blots using an antiserum raised against the 65 kDa subunit of PP2A (Fig. 2). Similar data were obtained for an antibody specific for the catalytic subunit of PP2A (data not shown).

Plasmids encoding mutant middle-T were microinjected into REF-52 cells and the localization of the expressed proteins studied by immunofluorescence. Fig. 3 shows that full length middle-T (Fig. 3a) as well as NG59 middle-T (Fig. 3c) were localized in perinuclear structures whereas DC1 and DC2 middle-T were predominantly cytoplasmic (Fig. 3b, d). The same mutations introduced into the truncated nuclear form of middle-T (1387Tmiddle-T) prevented nuclear accumulation of the corresponding proteins (Fig. 4; compare a with b–e). The DC1 and DC2 mutant proteins were confined to the cytoplasm while C120W and NG59 were partially nuclear, with the bulk of the protein accumulating in the cytoplasm. The change in cellular distribution of the various middle-T mutants was quantified (see Fig. 6). The results are consistent with the pictures shown in Fig. 4. The quantification of mutant C120W was somewhat ambiguous as indicated by the large sd. This might be a consequence of the varying amounts of protein made in individual cells dictating the specificity of the association with cellular structures.

Truncated forms of middle-T might be considered to be functional homologues of small-T, the third protein expressed by polyomavirus in the early part of the life-cycle. This protein also associates with PP2A (Pillas et

Fig. 5. Subcellular localization of small-T by immunofluorescence. REF-52 cells were microinjected with pcDNAsT(a), pcDNAsT2.DC1(b), pcDNAsT2.DC2(c) and pcDNAsT2.NG59(d). Cells were labelled for small-T with PA76. Scale bar represents 25 μm.
Polyomavirus T antigen localization

Fig. 6. Quantification of the subcellular distribution of various T antigens. (a) NIH 3T3 cells were infected with polyomavirus NG59RA or Py1387T and stained for middle-T 20 h later with a mixture of mouse antibodies PAb750, PAb754 and PAb738. REF-52 cells microinjected with expression vectors encoding wt or mutant T antigen were stained with PAb762. Visualization was performed with Texas Red-coupled anti-mouse antibody. Pictures were recorded on a Leica TCS4D confocal microscope and quantified with image processing software as described in Methods. Quantification of subcellular distribution of the different T antigens is based on an evaluation of 10 cells in each experiment. Average percentage fluorescence intensity in the nucleus relative to that in the whole cell with sd is shown for wt and mutant T antigens. (b) Map of the various T antigen mutants used in this study.

al., 1990; Cayla et al., 1993) and has been shown to be predominantly nuclear using immunofluorescence microscopy (Noda et al., 1986). The same mutations introduced into 1387T middle-T described above were investigated in the context of small-T and found to block PP2A binding (Fig. 2). Fig. 5 shows that wt small-T expressed from microinjected vector DNA is found both in the cytoplasm and the nucleus. When the same mutations described above for middle-T were introduced into small-T, no significant alteration in the subcellular distribution was detected. The amount of protein localized in the nucleus or the cytoplasm was quantified in a series of wt or mutant small-T-expressing cells as shown in Fig. 6. In contrast to the corresponding truncated middle-T mutants, small-T mutants unable to bind PP2A were still partially nuclear, like the wt protein. This is most likely due to the fact that small-T is able to cross the nuclear pore complex by passive diffusion and is retained in the nucleoplasm by binding to nuclear components whereas middle-T, due to its higher molecular mass, depends on active transport facilitated through association with PP2A (Dingwall & Laskey, 1991; Paine, 1993).

To corroborate the data presented, wt and mutant T antigen cDNA were also introduced into NIH 3T3 cells by LipofectAMINE-mediated transient transfection. Localization of the expressed proteins was similar to that in virus-infected NIH 3T3 or microinjected REF-52 cells.
cells. Middle-T localization was confined to a perinuclear middle-T consisting of only 304 amino acids had the (data not shown). In addition, further truncated forms of middle-T lacking a hydrophobic membrane-targeting sequence located close to the carboxy terminus were specifically localized in the nucleoplasm and excluded from nucleoli. Passive diffusion of middle-T through the nuclear pore complex can be excluded since proteins with a molecular mass above approximately 40 kDa are unable to cross the nuclear envelope. Active transport of proteins across the nuclear envelope requires a mono- or bipartite targeting signal consisting of basic amino acids (Dingwall & Laskey, 1991; Paine, 1993). We could not find such a sequence in middle-T and suggest that this protein reaches the nucleoplasm as a passenger bound to cellular proteins carrying nuclear targeting sequences. Based on our studies with T antigen mutants we propose that amino-terminal sequences that have been shown to be essential for PP2A-binding are also required for nuclear targeting of truncated middle-T. Interestingly, all middle-T mutants unable to associate with PP2A were cytoplasmic suggesting a role of this phosphatase in nuclear targeting. These findings are in good agreement with recently published data showing that the regulatory and catalytic subunits of PP2A are also found in the nucleus (Turowski et al., 1995).

The fact that the membrane anchor at the carboxy terminus is dominant over amino-terminal signals mediating nuclear localization is exemplified by the finding that all full-length middle-T mutants were localized either in perinuclear structures, most likely membranes, or in the cytoplasm. Since middle-T seems to be integrated into membranes post-translationally (Kutay et al., 1993), the preferentially cytoplasmic distribution of some of the full-length middle-T mutants may result from impaired targeting to intracellular membranes.

Wt polyomavirus encodes an additional T antigen lacking a membrane targeting sequence, small-T, that is capable of association with PP2A (Pallas et al., 1990). Small-T has a molecular mass of 22 kDa and is therefore expected to enter the nucleus by passive diffusion. This might explain why small-T mutants defective in PP2A-binding due to amino-terminal mutations still enter the nucleus. Once in the nucleus both wt and mutant small-T might associate with nucleoplasmic components resulting in nuclear accumulation. We suggest that truncated middle-T and authentic small-T might be functionally equivalent: in virus mutants lacking middle-T, small-T might at least partially compensate for this defect, presumably by provoking the necessary modifications of PP2A activity or specificity required for efficient virus replication (Liang et al., 1984; Garcea et al., 1989). The 1387T virus expressing truncated middle-T and normal small-T grows almost like wt (Carmichael et al., 1982; Templeton et al., 1986), hinting at the possibility that the truncated mutant protein cooperates with small-T in the final steps of virus encapsidation as suggested before (Garcea et al., 1989). Hr-t mutant viruses, lacking functional small- and middle-T, grow very poorly and require special permissive cells for efficient replication (Garcea & Benjamin, 1983; Benjamin, 1982). In these cells the function of small- and middle-T antigens might be supplied in trans by cellular factors.

A likely function of small- and middle-T is their ability to promote increased coat protein phosphorylation late in the replicative cycle as shown before (Garcea & Benjamin, 1983; Garcea et al., 1985). Nuclear PP2A has the capacity to dephosphorylate VP1, resulting in poor encapsidation efficiency of the virus. Upon binding to T antigen the specificity of PP2A might change as suggested before (Cayla et al., 1993) allowing increased steady state phosphorylation of cellular and viral phosphoproteins. Since virus encapsidation occurs in the nucleus (Delos et al., 1993; Forstova et al., 1993; Montross et al., 1991) a nuclear form of T antigen might be required to maintain high levels of VP1 phosphorylation required during the final steps of the replicative cycle. In agreement with this concept it has been shown recently that phosphorylation of VP1 at Thr-156 is required for encapsidation of the virus (Li & Garcea, 1994).

Addressing the relevance of our findings for the life-cycle of polyomavirus we looked for nuclear middle-T in virus-infected cells. With a new set of highly specific middle-T monoclonal antibodies (Dilworth & Horner, 1993) and the high sensitivity of a confocal microscope we measured the amount of middle-T in the nucleus of infected cells. These cells showed a 10-100-fold increase in average fluorescence intensity in the nucleus as compared with uninfected control cells. This accounts for approximately 10% of the signal observed in an infected cell using a large-T-specific antibody, suggesting
that a small fraction of middle-T might be present in the nucleus of virus-infected cells. Since full length middle-T does not enter the nucleus as shown in this study we suspect that truncated forms are made during the replicative cycle of the virus. They might be derived from the intact protein by proteolytic degradation. Alternatively, cryptic splice sites upstream from the sequence might enable the virus to form a fourth T antigen by alternative splicing of the primary transcript. Finally, truncated middle-T lacking the hydrophobic carboxy terminus might arise by frameshifting as shown earlier (Wilson et al., 1986). These possibilities are currently being investigated.

A.M. and C.D. contributed equally to this work. We would like to thank Drs W. Eckhart, T. Benjamin and A. Smith for supplying the various polyomavirus T antigen mutants and virus stocks. We also thank Dr B. Hemmings for PP2A-specific antibodies. A.M. was generously supported by grant 2.93.39 from Daimler-Benz Stiftung. We also thank our colleagues Drs B. Hemmings, S. Kaech and P. Turowski for critical reading of the manuscript.

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(Received 6 July 1995; Accepted 17 August 1995)