Activation and Nuclear Translocation of Mitogen-activated Protein Kinases by Polyomavirus Middle-T or Serum Depend on Phosphatidylinositol 3-Kinase*

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Several cellular signal transduction pathways activated by middle-T in polyomavirus-transformed cells are required for viral oncogenicity. Here we focus on the role of phosphatidylinositol 3-kinase (PI 3-kinase) and Ras and address the question how these signaling molecules cooperate during cell cycle activation. Ras activation is mediated through association with SHC-GRB2-SOS and leads to increased activity of several members of the mitogen-activated protein (MAP) kinase family, while activation of PI 3-kinase results in the generation of D3-phosphorylated phosphatidylinositols whose downstream targets remain elusive. PI 3-kinase activation might also ensue as a direct consequence of Ras activation. Oncogenicity of middle-T requires stimulation of both Ras- and PI 3-kinase-dependent pathways. Mutants of middle-T incapable to bind either SHC-GRB2-SOS or PI 3-kinase are not oncogenic. Sustained activation and nuclear localization of one of the MAP kinases, ERK1, was observed in wild type but not in mutant middle-T-expressing cells. Wortmannin, an inhibitor of PI 3-kinase, prevented MAP kinase activation and nuclear localization in middle-T-transformed cells. PI 3-kinase activation was also required for activation of the MAP kinase pathway in normal serum-stimulated cells, generalizing the concept that signaling through MAP kinases requires not only Ras but also PI 3-kinase-mediated signals.

Proteins expressed early in the virus life cycle of polyomavirus, the tumor antigens (T antigens), are responsible for tumor formation in virus-infected animals and virus-mediated transformation of cells in culture (1). Large tumor antigen (large-T) is a nuclear protein known to immortalize primary cells in culture (2) while middle tumor antigen (middle-T) causes phenotypic changes associated with malignant cell growth (3). The activity of middle-T results from its association with intracellular signal-transducing proteins like members of the Src family of tyrosine kinases (c-Src, Fyn, and c-Yes) (4), the 85- and 110-kDa subunits of a phosphatidylinositol 3-kinase (PI 3-kinase)1 (5), the catalytic and regulatory subunits of protein phosphatase 2A (PP2A) (6, 7), and the SH2 domain-containing protein SHC (8, 9) whose putative role is to activate the Ras signaling pathway (10, 11). More recently, middle-T immunoprecipitates have been found to contain a member of the 14-3-3 family of proteins, some of which are involved in stimulating ADP-ribosylation (12).

Middle-T activates intracellular signal transduction pathways mediated by PI 3-kinase and Ras, respectively. The latter becomes activated upon association of the SHC-GRB2-SOS complex with middle-T. Middle-T-transformed cells show an increase in the fraction of the GTP-bound form of Ras (13) and transfection with genes suppressing Ras activity results in reversion to a more normal phenotype (14). Activated Ras stimulates cell growth and differentiation through a kinase cascade involving Raf and MEK culminating in the activation and nuclear translocation of several members of the MAP kinase family (15–17). Middle-T has also been shown to activate transcription factors of the AP1 family like Jun and Fos (13, 18) or Myc (19), the former being direct targets of MAP kinases (20–24). The ability to activate transcription of cellular genes through MAP kinases is a prerequisite for cell transformation and delineating the underlying mechanisms is therefore of pivotal importance in understanding virus-mediated cell transformation.

In this study we investigate the signals activated by middle-T through SHC-GRB2-SOS and PI 3-kinase, respectively, and evaluate their importance for T antigen oncogenicity. Activation and translocation of ERK1 to the nucleus was observed in cells expressing wild type (WT) middle-T. Cells expressing T antigen mutants unable to bind SHC and PI 3-kinase, respectively, showed neither activation nor nuclear translocation of MAP kinases, suggesting that both pathways feed into the MAP kinase cascade. Similarly, we found that PI 3-kinase was also required for MAP kinase activation and translocation in untransformed cells stimulated with growth factors.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were purchased from Boehringer Mannheim. Pepstatin, apronin, myelin basic protein, and sodium vanadate were from Sigma. Wortmannin was purchased from Sigma. α-Glycerophosphate and protein A-Sepharose CL-4B were obtained from Pharmacia Biotech Inc., G418 (Geneticin) was from Life Technologies, Inc., and Micro BCA protein assay reagents were from Pierce. Luciferin was obtained from Chemie Brunschwig AG, and [γ-32P]ATP was from ICN.

Cell Lines—NIH 3T3 and Fisher rat F111 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum at 37 °C in a humidified CO2 incubator. 3T3 cell lines expressing wild type (mT4 and mT8) or mutant forms of middle-T were generated by stable transfection as described earlier (25). The cell line mT7 was obtained by infection of NIH 3T3 cells with the middle-T carrying retrovirus N-TKmT (26).

Plasmid Constructs—All plasmid construction steps were carried out using standard molecular cloning techniques. The plasmids

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1 To whom correspondence should be addressed. Tel.: 61-697-6689; Fax: 61-697-3976.
2 The abbreviations used are: PI 3-kinase, phosphatidylinositol 3-kinase; WT, wild type; MAP, mitogen-activated protein; DMEM, Dulbecco’s modified Eagle’s medium; uPA, urokinase-type plasminogen activator.
Polyomavirus Middle-T Activates MAP Kinases—Middle-T forms complexes with cellular proteins mediating signal transduction like the SHC-GRB2-SOS complex stimulating Ras activity. This initiates a cascade of kinase reactions culminating in the activation of ERK1 and ERK2. The activation of these kinases in response to Middle-T expression is demonstrated in Fig. 1. Western analysis confirmed the expression of Middle-T and its mutant forms in NIH 3T3, REF-52, and F111 cells. Immunofluorescence localization studies showed an increase in the cellular distribution of ERK1 and T antigens in cells expressing Middle-T compared to control cells.

Microinjection of Ref-52 cells with recombinant Middle-T constructs demonstrated the ability of Middle-T to activate MAP kinases in vivo. The transformation assay revealed an increase in focus formation in NIH 3T3 mouse fibroblasts and F111 rat fibroblasts in cells expressing Middle-T compared to control cells. These results support the hypothesis that Polyomavirus Middle-T can act as a transforming agent by activating MAP kinases, leading to cellular transformation.

**RESULTS**

Polyomavirus Middle-T Activates MAP Kinases—Middle-T forms complexes with cellular proteins mediating signal transduction like the SHC-GRB2-SOS complex stimulating Ras activity. This initiates a cascade of kinase reactions culminating in the activation of ERK1 and ERK2. The activation of these kinases in response to Middle-T expression is demonstrated in Fig. 1. Western analysis confirmed the expression of Middle-T and its mutant forms in NIH 3T3, REF-52, and F111 cells. Immunofluorescence localization studies showed an increase in the cellular distribution of ERK1 and T antigens in cells expressing Middle-T compared to control cells.

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Middle-T Stimulates Transcription from Various Promoters—MAP kinases phosphorylate several transcription factors such as J un and F os resulting in the activation of a variety of promoters. Activation of MAP kinases was therefore measured as the increase in transcription of a series of luciferase reporter gene constructs carrying the uPA promoter, the F os promoter, and an artificial promoter containing three AP1 sites, respectively. The effect of middle-T on these promoters was assessed in NIH 3T3 cells transiently transfected with a reporter plasmid together with a middle-T expression vector. Fig. 3A shows that middle-T expression resulted in approximately 30-fold activation of the uPA and 20-fold activation of the F os and AP1 promoter while the SV40 promoter was only slightly induced. Similar activation of the uPA promoter was observed when the reporter construct was expressed in middle-T-transformed cell lines (mT7, Fig. 3A). These data establish that the uPA promoter and the PEA3/AP1 sites present in the F os and AP1 promoter are regulated by signaling pathways activated by middle-T. The most likely candidates mediating this activation are members of the MAP kinase family.

Middle-T Activates ERK1 and ERK2 via the Ras, Raf, MEK Cascade—In order to identify signaling intermediates responsible for activation of MAP kinases in middle-T-transformed cells, we introduced dominant negative SOS or Raf together with the luciferase reporter plasmid into 3T3 fibroblasts. Fig. 3B shows that dominant negative Raf or SOS block T antigen-mediated activation of the uPA promoter and suggests that Ras acts as signaling intermediate. MKP-1 (33), an ERK-specific dual specificity phosphatase required for down-regulation of MAP kinases, also blocked the response to middle-T in reporter gene-expressing cells, further demonstrating that middle-T activates the MAP kinase cascade (Fig. 3B).

So far we have shown that middle-T activates MAP kinases. Earlier reports suggest that constitutively activated MEK, the kinase acting upstream of ERK1 and ERK2, is sufficient for cell transformation (34–36). This implies that Ras-mediated signaling is sufficient for ERK activation. An analysis of various middle-T mutants, on the other hand, suggests that both SHC- and PI 3-kinase-initiated pathways are required for T antigen oncogenicity (4, 37). To address this discrepancy, we investigated a series of non-oncogenic middle-T mutants (Table I). 1178T, a truncated mutant protein lacking the membrane anchor sequence) only binds PP2A (39, 60), and dl1015 associates with all cellular enzymes described so far, but is unable to activate PI 3-kinase (40, 41). Mutant genes were introduced into NIH 3T3 cells and activation of MAP kinase measured in the luciferase reporter assay. Fig. 3B shows that only WT middle-T fully stimulated the reporter gene. NG59, 1387T, and dl1015 were almost completely inactive, while 1178T and Y250F were about 50% as effective as WT in inducing the uPA promoter.
Residual stimulation of the uPA promoter by mutant middle-Ts was totally blocked by dominant negative SOS and Raf or by MKP-1. These findings further support the view that both SHC- and PI 3-kinase-initiated signaling pathways are required for efficient stimulation of the MAP kinase cascade. In Middle-T-transformed cells, ERK1 is localized in the nucleus—it is well established that in order to accomplish mitogenic stimulation, MAP kinases have to translocate to the nucleus upon growth factor stimulation (15, 42–44). We therefore studied the localization of a representative member of this family of kinases, ERK1, in a variety of normal and middle-T-transformed cell lines. Serum stimulation of resting 3T3 cells resulted in accumulation of ERK1 in the nucleus as expected (Fig. 4, A–C). Panels D–F show that most of ERK1 was nuclear in middle-T-expressing cells and, most importantly, that nuclear localization of ERK1 was only slightly influenced by the growth conditions of the cells. Similarly, translocation of ERK1 to the nucleus was observed in REF-52 cells microinjected with plasmids carrying a WT middle-T-specific cDNA (Fig. 4, G and H). None of the non-oncogenic mutant T antigens stimulated translocation of ERK1 to the nucleus. Y250F middle-T, the mutant impaired in SHC binding, was completely defective (Fig. 4, I and K), while 1178T and dl1015, mutants unable to activate PI 3-kinase, had dramatically reduced potential to relocalize ERK1. A quantification of the data obtained with several hundred microinjected cells is shown in Fig. 5. Our data show that both PI 3-kinase and Ras activation are required for stimulation of the MAP kinase cascade by middle-T.

Wortmannin, an Inhibitor of PI 3-Kinase, Prevents Accumulation of MAP Kinases in the Nucleus and Blocks Entry into S Phase—We have shown so far that in WT middle-T-expressing cells, basal activity of MAP kinases is increased concomitantly with relocalization of ERK1 to the nucleus. This activity requires both SHC and PI 3-kinase-dependent pathways. Middle-T-expressing cells were treated with wortmannin, a specific inhibitor of phosphatidylinositol 3-kinases (45), as shown in Fig. 5. The number of cells expressing nuclear ERK1 dropped to less than 10% in drug-treated WT or mutant middle-T-injected cells, indicating that PI 3-kinase activation was essential for initiation of the MAP kinase cascade by polyomavirus.

To test the relevance of these findings for growth factor-mediated signaling in normal cells, we studied the localization of ERK1 in serum-stimulated fibroblasts in the absence and presence of wortmannin (Fig. 4, L and M). Nuclear accumula-
and ERK2 was abolished when G0-arrested cells were stimulated. Similarly, the expression of ERK1 was completely blocked by the drug, in agreement with the data shown for microinjected cells expressing middle-T. The gray bars indicate the percentage of middle-T-expressing cells that show nuclear localization of ERK1. As a control the same number of un.injected control cells was counted. The same experiment was performed in the presence of 100 nM wortmannin (black bars). In each experiment several hundred cells were counted.

**TABLE II**

Inhibition of DNA synthesis by wortmannin in serum-stimulated cells

| Arrested cells | Stimulated cells |
|---------------|-----------------|
| Serum-arrested NIH 3T3 and F111 cells were stimulated for 8 hours with calf serum. For the next 16 h, the serum concentration was reduced to 0.1% and bromodeoxyuridine was added to a final concentration of 10 μM. The same experiment was performed in the presence of 100 nM wortmannin added 3 h before serum stimulation. The numbers represent percentage of bromodeoxyuridine-positive cells from two independent experiments. |
| -Wortmannin | +Wortmannin |
| F111 | 10.89/5 | 2.1/1.7 |
| NIH 3T3 | 16/13 | 1.0/1.5 |

Fig. 5. Translocation of ERK1 to the nucleus in middle-T-expressing cells. Starved REF-52 cells were microinjected with pcDNAmT, pcDNA1178TmT, pcDNA250FmT, or pcDNA11015mT expression plasmids. After injection the cells were kept for 8 h in low serum and immunostained for both middle-T and ERK1. The gray bars indicate the percentage of middle-T-expressing cells that show nuclear localization of ERK1. As a control the same number of un.injected control cells was counted. The same experiment was performed in the presence of 100 nM wortmannin (black bars). In each experiment several hundred cells were counted.

**FIG. 6.** Focus formation on F111 fibroblasts by various middle-T mutants. F111 rat fibroblasts were transfected with 20 μg of the middle-T-encoding expression plasmids pcDNAmT, pcDNA250FmT, and pcDNA1178TmT. For double transfections, 10 μg of each expression plasmid was used. Focus assays were performed as described under “Experimental Procedures.” The data shown represent the average of four independent experiments.

**DISCUSSION**

Middle-T transforms cells through association with a variety of proteins involved in cell signaling (4, 37) and activates intracellular signal transduction pathways mediated by the PI 3-lipid kinase and Ras, respectively. It is well established that Ras stimulates the MAP kinase pathway (15–17). Owing to its ability to induce the phosphorylation of cellular transcription factors like J un and Fos, middle-T has been suggested to activate the MAP kinase cascade (13, 18, 46). It has also been shown that middle-T activates genes coding for various transcription factors (19, 47) like Fos (18) and jun (46). Investigating the effect of middle-T on the MAP kinase pathway we studied several parameters. (i) We measured the activity of MAP kinases in vitro using myelin basic protein as substrate; (ii) we determined the shift in the apparent Mr of MAP kinases upon cell stimulation indicative of activation upon phosphorylation by MEK; (iii) we measured the activity of MAP kinase-regulated promoters in reporter plasmid-transfected cells using a luciferase reporter gene; and (iv) we studied the intracellular localization of a representative member of the MAP kinase family, ERK1, by immunofluorescence microscopy.

Expression of WT middle-T, but not of transformation-defective mutant proteins, resulted in high basal MAP kinase activity in asynchronously growing or serum-starved cells. WT middle-T-expressing cells showed increased ERK1 and ERK2 activity, as determined in the myelin basic protein phosphorylation assay but showed no corresponding shift in the apparent Mr of these kinases typical for MAP kinase activation in growth factor-stimulated cells. This might be explained by the fact that middle-T-transformed cells do not accumulate in G0 upon serum starvation and are refractory to further stimulation by growth factors. The Mr shift in MAP kinases might only arise in cells entering the cell cycle from G0 but not in cycling cells. Alternatively, the Mr shift of ERK1 and ERK2 might be transient preceding translocation to the nucleus. Since MAP kinases are constitutively localized in the nucleus of middle-T-transformed cells, transient phosphorylation by MEK might escape detection on Western blots. Earlier data obtained with cells overexpressing mutant forms of ERK1 and ERK2 suggest that the shift in Mr resulting from phosphorylation by MEK as well as enzymatic activity of ERK1 and ERK2 are not required for translocation to the nucleus (15, 17, 43). A change in activity and/or specificity of PP2A upon association with T antigens...
might further contribute to altered phosphorylation and activity of ERK1 and ERK2 in polyomavirus-transformed cells. In agreement with this idea, recently published papers show that SV40 small-T activates the MAP kinase pathway by blocking PP2A-mediated down-regulation (48, 49).

A detailed analysis of the pathways targeted by middle-T was performed in cells co-transfected with middle-T and a reporter gene consisting of the coding region derived from the firefly luciferase gene under the control of the uPA, Fos, or AP1 promoters, respectively. Emphasis was on the uPA promoter, since it has been shown previously that expression of uPA is increased in middle-T-induced endotheliomas. This protease has been shown to be one of the major determinants in T antigen-induced morphological transformation (26). Signaling through the MAP kinase pathway was dramatically reduced in cells expressing transformation-defective middle-T mutants. Dominant negative Raf, dominant negative SOS, and overexpression of MKP-1, a phosphatase involved in down-regulation of MAP kinases, blocked T antigen-mediated activation of the uPA promoter reminiscent of experiments performed earlier with tyrosine kinase growth factor receptors (33, 50).

While short term treatment of cells with growth factors is sufficient to transiently activate MAP kinases, sustained activation accompanied by nuclear translocation of ERK1 and ERK2 are required for mitogenic stimulation of cells through this pathway (17, 51, 52). A variety of T antigen mutants was used to address the question which of the pathways initiated by middle-T were required for sustained activation and nuclear translocation of MAP kinases. Our data show that only WT middle-T efficiently stimulates nuclear translocation of ERK1 establishing that SHC-mediated activation of Ras was not sufficient for activation of the MAP kinase cascade. Thus PI 3-kinase activation seems to be an important factor in T antigen-mediated MAP kinase activation and mitogenic signaling. To corroborate these findings, we treated middle-T-expressing cells with wortmannin, an inhibitor of PI 3-kinase. Relocalization of ERK1 upon middle-T expression was completely blocked by the drug, confirming the results obtained with middle-T mutants unable to activate PI 3-kinase. Nuclear translocation of MAP kinase observed in a small fraction of 1178T middle-T-expressing cells is therefore most likely the consequence of residual PI 3-kinase activity and not due to a PI 3-kinase-independent pathway (5, 31, 53). This explanation is consistent with earlier studies showing that mutation of tyrosine 315, the major binding site for the 85-kDa subunit of PI 3-kinase, to phenylalanine in the 1178T mutant, reduced but did not completely abolish oncogenicity and PI 3-kinase activity (5, 31).

Remains activity might be the consequence of residual binding of p85 to this mutant protein. Alternatively, elevated D3-phosphorylated PIP3 levels might arise from SHC-mediated Ras activation, resulting in stimulation of PI 3-kinase (54). Our interpretation of these data was confirmed with another mutant, d1101S, still able to associate with this enzyme yet unable to activate PI 3-kinase activity (41).

The fact that Y250F middle-T was totally defective in causing nuclear localization of ERK1 and ERK2 yet only 2-fold reduced in inducing the uPA gene can be explained in three ways. (i) Detection of nuclear localization of ERK1 by immunostaining depends on accumulation of a significant fraction of the enzyme in the nucleus while only a small amount of nuclear ERK1 might be sufficient to activate the uPA promoter; (ii) Transient transfections of Y250F middle-T together with the reporter plasmid had to be performed in the presence of serum that might compensate for the defect of this mutant in initiating the Ras pathway; (iii) activation of the uPA promoter might also ensue after phosphorylation of transcription factors in the cytoplasm followed by their translocation to the nucleus. Oncogenicity of WT middle-T is most likely the result of the induction of a complex set of cellular genes upon phosphorylation of various transcription factors by MAP kinases. Our data suggest that activation and translocation of MAP kinases to the nucleus upon expression of middle-T best correlates with mitogenicity and oncogenicity of this protein. Partially defective mutants unable to cause relocalization of MAP kinases do not initiate the cell cycle, suggesting that some of the crucial substrates of MAP kinases are nuclear.

Wortmannin blocked nuclear translocation of MAP kinases in middle-T-expressing cells and efficiently prevented phosphorylation and relocalization of these kinases to the nucleus in serum-stimulated control cells, establishing our findings as a general phenomenon during mitogenic stimulation of cells. Support for our observation also comes from the fact that wortmannin has been shown by others to reduce the efficiency of signaling through the MAP kinase pathway upon insulin or serum treatment (55, 56).

To test the hypothesis that SHC-GRB2-SOS- and PI 3-kinase-initiated pathways operate independently on MAP kinases, we performed focus assays with cells transfected with two plasmids encoding 1178T and Y250F middle-T, respectively. While each mutant alone was unable to induce foci, a combination of both efficiently transformed cells indicating that the two pathways can be initiated from separate middle-T complexes and efficiently cooperate to activate the MAP kinase cascade.

In summary, we have shown here that both SHC-GRB2-SOS as well as PI 3-kinase-induced signaling pathways are required for full stimulation of the MAP kinase cascade by polyomavirus middle-T or serum growth factors. It remains the goal of further studies to identify the level at which these pathways cross-talk. Recently published data demonstrate that a constitutively activated PI 3-kinase activates Ras, Raf, and MAP kinases and stimulates transcription of Fos, suggesting a role for this enzyme upstream of Ras (57). Studies with mutant growth factor receptors point to a role of PI 3-kinase in mitogenesis, cell migration, and receptor internalization (58). Whether localization of MAP kinases is affected by mutations preventing binding of PI 3-kinase to activated growth factor receptors remains to be determined. Other signaling molecules such as S6 kinase and the proto-oncogene akt1 have been identified as putative downstream targets of PI 3-kinase (59). It will be interesting to investigate whether these kinases are necessary for middle-T-mediated transformation and nuclear translocation of MAP kinases.

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