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Polyomavirus middle-sized tumor antigen modulates c-Jun phosphorylation and transcriptional activity

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ABSTRACT **Polyomavirus middle-sized tumor antigen (MT) increases the expression of c-*jun* through a phorbol 12-*O*-tetradecanoate 13-acetate response element in the c-*jun* promoter. To investigate the cellular signaling pathways affected by MT, we studied the role of the c-Ras and Raf-1 proteins in MT-induced transactivation of c-*jun* and cell transformation. There was an increase in GTP complexed to Ras in MT-expressing cells, indicating an increase in Ras activity. Coexpression of dominant inhibitory mutants of Ha-*ras* and *raf-1* with MT inhibited MT-mediated transactivation and focus formation. Studies of the phosphorylation of c-Jun showed that MT expression increased the phosphorylation of Ser-63 and Ser-73 in the transactivation domain and decreased the phosphorylation of a peptide containing Ser-243, Ser-249, and Thr-231 in the DNA binding domain. MT increased the transcriptional activating ability of c-Jun but failed to increase the transcriptional activating ability of c-Jun mutants with Ser-63 and Ser-73 changed to nonphosphorylatable Ala, indicating that MT modulates c-Jun activity through phosphorylation. The dominant inhibitory mutants of Ha-*ras* and *raf-1* interfered with the ability of MT to activate c-Jun. The results indicate that MT induces a phosphorylation cascade through the activation of c-Ras and Raf-1 and that c-Jun is one of the downstream targets that may cause changes in gene expression leading to cell transformation.**

Polyomavirus middle-sized tumor antigen (MT) transforms established cell lines and renders them tumorigenic (1). MT is a membrane-associated protein that forms a complex with several cellular proteins potentially involved in growth control, including $pp60^{c\text{-}src}$ (2) and other members of the *src* family (3, 4), the 85-kDa and 110-kDa subunits of phosphatidylinositol (PI) 3-kinase (5, 6), and the A and C subunits of protein phosphatase 2A (7, 8). MT increases the activity of $pp60^{c\text{-}src}$ in the complex, at least in part by preventing the phosphorylation of an inhibitory site, Tyr-527 (9, 10). PI 3-kinase binds to phosphorylated tyrosine residues in a number of oncogene products and growth factor receptors, including Tyr-315 of MT (11, 12). Binding of MT to $pp60^{c\text{-}src}$ and PI 3-kinase appears to be necessary, but not sufficient, for transformation (13): mutants that fail to bind are defective for transformation, but a set of mutants affecting positions around position 248 in MT are defective for transformation, yet retain the ability to bind $pp60^{c\text{-}src}$ and PI 3-kinase (14). These mutants recently were shown to be defective in binding the SH2-containing adaptor molecule, SHC, which is phosphorylated on tyrosine in MT-transformed cells and binds to GRB2, providing a plausible link between MT and Ras activation (15). Polyoma-transformed cells show increases in the activities of protein serine/threonine kinases, including Raf-1 (16) and ribosomal protein S6 kinase (17), and an increase in the activities of transcriptional regulators, PEA1/AP-1 and PEA3/c-ets (18, 19). Therefore, it seems likely that association of MT with proteins in the cell membrane induces signaling pathways that activate serine/threonine kinases and transduce signals to transcriptional regulators in the nucleus.

The AP-1 transcription factors (Jun–Jun homodimers or Jun–Fos heterodimers) bind to phorbol 12-*O*-tetradecanoate 13-acetate response elements in the genes they regulate. Growth factors, oncoproteins, and tumor promoters induce AP-1 activity. Increased AP-1 activity can result from increased synthesis of Jun and Fos proteins or from posttranslational modifications (20–22). Five sites of phosphorylation have been identified in the Jun protein: two (Ser-63 and Ser-73) in the N-terminal region in the transactivation domain and three (Ser-243, Ser-249, and Thr-231) in the DNA binding domain. Dephosphorylation of the sites in the DNA binding domain increases the ability of c-Jun to bind DNA (21, 23, 24). The oncoproteins v-Src, v-Sis, Ha-Ras, and Raf-1 increase phosphorylation of Ser-63 and Ser-73 and increase c-Jun transcriptional activity (25–27). A protein kinase involved in phosphorylating these sites has been identified in Ha-Ras-transformed fibroblasts (28). Activation of Jun/AP-1 is necessary for transformation by activated *ras* (26, 29). MT enhances c-*jun* expression and AP-1 activity, and the degree of activation by MT mutants correlates with their transforming ability (19), suggesting that Jun/AP-1 activity may also play a role in transformation by MT.

Recently there has been rapid progress in identifying elements of signaling pathways that link external stimuli to intracellular targets. Receptor protein tyrosine kinases are linked to Ras proteins through adaptor proteins, like GRB2 and SHC, and guanine nucleotide exchange factors (30–33). Activated Ras binds Raf-1 and triggers activation of a cascade of serine/threonine kinases, culminating in changes in gene expression (34–38). The signaling pathway(s) by which MT activates transcription factors and causes cell transformation are still not clear. Transformation by MT is inhibited by expression of *src* antisense RNA (39, 40) and by expression of *ras* antisense RNA (41) or a dominant inhibitory mutant of *ras* (42), suggesting that both Src and Ras are required for MT-mediated transformation. Raf-1 and Rsk kinase activities are elevated in MT-transformed cells, but their involvement in MT-mediated alterations in gene expression and transformation has not been defined (16, 17).

After our observations (19) that MT induces c-*jun* expression and AP-1 activity, we studied the mechanism of c-Jun activation in MT-expressing cells. We present evidence that MT expression activates Ras and Raf and alters the phosphorylation pattern of c-Jun, resulting in enhanced transcriptional activity and changes in gene expression that may contribute to cell transformation.

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Abbreviations: MT, middle-sized tumor antigen; PI, phosphatidylinositol; CAT, chloramphenicol acetyltransferase.

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MATERIALS AND METHODS

Cell Culture. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) calf serum. NIH 3T3 cells and NIH 3T3 cells expressing MT or the vector PSLXCMV (43) were kindly provided by Gary Glenn (The Salk Institute, San Diego).

Immunoprecipitation and Phosphopeptide Mapping. Cells were seeded at a density of 2×10^5 cells per 6-cm dish. After 24 h, the cultures were radiolabeled with [^{32}P]orthophosphate at 1 mCi/ml (1 Ci = 37 GBq) for 6 h in phosphate-free DMEM. Cells were washed twice with ice-cold Tris-buffered saline and lysed in modified RIPA buffer (10 mM sodium phosphate, pH 7.0/0.15 M NaCl/1% SDS/1% Nonidet P-40/1% sodium deoxycholate/1% aprotinin/2 mM EDTA/50 mM sodium fluoride) containing 100 μM sodium orthovanadate as the phosphatase inhibitor. The c-Jun protein was immunoprecipitated using polyclonal antibodies raised against the C-terminal region of c-Jun, kindly provided by Michael Karin (University of California San Diego, La Jolla, CA) as described (25). The immunoprecipitated c-Jun protein was electrophoresed on an SDS/polyacrylamide gel, oxidized, and digested to completion with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin. The peptides thus generated were separated on cellulose thin layer chromatography plates by electrophoresis at pH 1.9 for 30 min at 1 kV in the first dimension, followed by ascending chromatography in phospho chromo buffer [1-butanol/glacial acetic acid/pyridine/H_2O, 75:15:50:60 (vol/vol)] in the second dimension (21). Phosphopeptides were detected by exposure to preflashed x-ray film using intensifying screens.

Ras–GTP Association. Measurements of GTP and GDP associated with Ras were performed as described by Gibbs *et al.* (44), with minor modifications. Cells were plated at a density of 2×10^5 per 6-cm dish. The following day the medium was changed and the cultures were incubated for 24 h in medium containing 0.1% or 0.5% calf serum. Subsequently, the cells were radiolabeled with [^{32}P]orthophosphate at 1 mCi/ml for 15 h in phosphate-free DMEM supplemented with the same concentrations of dialyzed calf serum. The cells were lysed for 10 min in 600 μl of ice-cold lysis buffer [50 mM Tris·HCl, pH 7.5/20 mM $MgCl_2$/150 mM NaCl/0.5% Nonidet P-40/0.5 mM phenylmethylsulfonyl fluoride/aprotinin (10 μg/ml)]. After clarification of the lysates, the supernatants were immunoprecipitated with the anti-Ras monoclonal antibody Y13-259 kindly provided by Bart Sefton (The Salk Institute, San Diego). Immune complexes were collected with protein A-Sepharose (Repligen) coupled to rabbit anti-rat IgG. The complexes were washed six times with lysis buffer and once with phosphate-buffered saline. Guanine nucleotides bound to the Ras protein were eluted in 20 μl of 2 mM EDTA/2 mM dithiothreitol/0.2% SDS/0.5 mM GTP/0.5 mM GDP and incubated at 68°C for 20 min. After centrifugation to remove the protein A-Sepharose beads, supernatants were spotted on a polyethylenimine-cellulose thin layer chromatography plate, which was developed in 1 M KH_2PO_4 (pH 3.5) and exposed to x-ray film for 12–24 h. The radioactivity associated with GDP and GTP was quantified using a PhosphorImager.

Transfections and Chloramphenicol Acetyltransferase (CAT) Assays. NIH 3T3 cells were transfected and analyzed for CAT activity as described (19). Cells were seeded at a density of 3×10^5 cells per 6-cm dish 24 h prior to transfection. Cells were cotransfected with 10 μg of plasmid DNAs using 2-[bis(2-hydroxyethyl)amino]ethane sulfonic acid (Bes) and calcium phosphate precipitation methods as described (45). After incubation for 16–20 h at 37°C in an atmosphere containing 3% CO_2/97% air, the cells were washed and incubated in DMEM supplemented with 0.5% calf serum for 24 h. Cells were harvested, and lysates were normalized for transfection efficiency prior to CAT assays, as follows: 2 μg of a Rous sarcoma virus β-galactosidase expression plasmid was included with the reporter plasmid, and the total amount of DNA in the transfection mixtures was adjusted to 10 μg with pGEM-4Z before transfection. β-Galactosidase activity was used to adjust the CAT assays to contain equivalent amounts of expressed protein.

Plasmids. Plasmids RSVJUN/GHF-1 and RSV(A63, 73)-Jun/GHF-1 and the Raf-1 expression vectors encoding either activated Raf(BXB) or dominant negative Raf(301), and dominant negative Ras(N17) have been described elsewhere (27) and were kindly provided by Tod Smeal (University of California San Diego, La Jolla, CA). RSVMT and −132/+170junCAT constructs were as described (19, 43).

RESULTS

Activated Ras in MT-Expressing Cells. Ras proteins exist in an activated state, bound to GTP, and an inactive state, bound to GDP. To determine whether Ras is activated in MT-expressing cells, guanine nucleotides complexed with Ras were measured in normal and MT-expressing NIH 3T3 cells. MT-expressing cells showed an increase in Ras bound to GTP, compared to vector-expressing control cells: the percentage of Ras bound to GTP increased from 7.9 ± 0.4% to 11.7 ± 1.7% and from 8.3 ± 1.6% to 13.8 ± 1.2% for cells incubated in medium containing 0.1 and 0.5% serum, respectively (average of two experiments). The increases are consistent with increases reported (44) for v-*src*- or v-*abl*-transformed NIH 3T3 cells (from 7 to 22%), indicating that Ras activity is elevated in these MT-expressing cells.

Involvement of Ras and Raf-1 in MT Signaling. Previous studies using dominant inhibitory mutants of Ha-*ras* and *raf-1*, Ras(N17) and Raf(301), showed that Ras functions downstream of various growth factor receptor tyrosine kinases and nonreceptor tyrosine kinases (46, 47) and that Raf-1 functions downstream of Ras to activate transcription in the nucleus (48). The Ras(N17) mutant blocks the activity of Ha-, N-, and K-Ras and blocks cell growth induced by growth factors and cell transformation by v-*src* (46, 47). The Raf(301) mutant interferes with endogenous Raf-1 function and blocks cell growth induced by growth factors and cell transformation by Ha-*ras* (49). To determine whether Ras and Raf-1 are involved in MT-mediated transactivation of the c-*jun* promoter, we studied the effects of Ras(N17) and Raf(301) on MT-mediated transactivation. Transient transfection of NIH 3T3 cells was carried out with a MT expression vector and a reporter construct (−132/+170junCAT) containing the c-*jun* promoter (nt −132 to +170) linked to the CAT gene. CAT activity was used as a measure of the response of the c-*jun* promoter. Fig. 1 shows that MT enhanced the expression from the c-*jun* promoter 7-fold, confirming our previous results (19). Expression of either of the dominant inhibitory mutants, Ras(N17) and Raf(301), inhibited MT transactivation of c-*jun*. Coexpression of Ras(N17) with an activated Raf-1 construct, Raf(BXB), did not inhibit transactivation of c-*jun* by Raf(BXB), which acts downstream of Ras. Coexpression of Raf(301) with Raf(BXB) inhibited transactivation of c-*jun* only slightly, as observed (27). These results indicate that the activities of Ras and Raf-1 are required for activation of the c-*jun* promoter by MT and that Ras and Raf-1 function downstream of MT in the signaling pathway.

Similar cotransfection experiments were carried out to analyze the role of Ras and Raf-1 in cell transformation by MT. NIH 3T3 cells were transfected with a MT expression vector, with or without each of the dominant inhibitory mutants, Ras(N17) and Raf(301), and focus formation by MT was measured. The results shown in Table 1 indicate that expression of the dominant inhibitory mutants reduced trans-

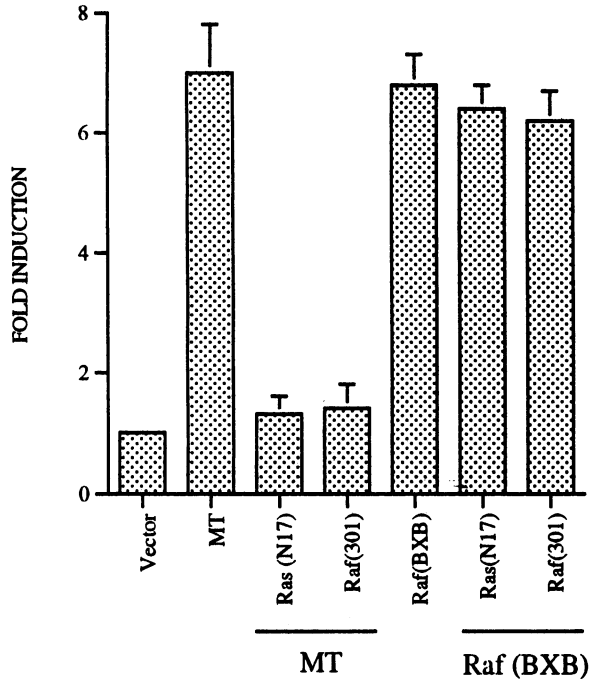

FIG. 1. Dominant inhibitory mutants of c-Ha-Ras and Raf-1 interfere with activation of c-Jun by MT. NIH 3T3 cells were cotransfected with the −132/+270c-JunCAT reporter plasmid and vectors expressing MT or activated Raf-1(BXB). Vectors expressing the dominant inhibitory mutants Ras(N17) and Raf(301) were included where indicated. Fold induction is the CAT activity in cells transfected with the MT or Raf expression vectors divided by the CAT activity in cells transfected with the reporter plasmid and the noncoding expression vector only, normalized to β-galactosidase activity. Values are averages and SD from three experiments.

formation by MT 90–100%. Transformation by the activated Ha-*ras* construct was not affected by expression of the Ras(N17) mutant. These results indicate that Ras and Raf-1 are required for initiation and/or maintenance of cell transformation by MT.

Modulation of c-Jun Phosphorylation by MT. As noted above, the transcriptional activity of c-Jun is modulated by phosphorylation. Hyperphosphorylation of sites in the transactivation domain increases the transcriptional activity of the protein, and dephosphorylation of sites in the DNA binding domain increases DNA binding activity (21, 25, 26). We studied the effects of MT expression on c-Jun phosphorylation, using a clone of NIH 3T3 cells stably expressing MT. Fig. 2 shows two-dimensional tryptic phosphopeptide maps of c-Jun isolated from actively growing control cells (expressing the vector alone) and MT-expressing cells. Spots x and y

Table 1. Dominant inhibitory mutants of Ras and Raf-1 inhibit transformation by MT

Transfection	Foci, no.
Vector	0, 0
MT	46, 36
MT + Ras(N17)	0, 1
MT + Raf(301)	0, 0
Raf(BXB)	35, 29
Raf(BXB) + Ras(N17)	32, 28
Raf(BXB) + Raf(301)	23, 21

Approximately 2 × 10^5 NIH 3T3 cells were transfected with 1 μg of plasmids encoding MT or activated Raf(BXB), either alone or with 5 μg of plasmids encoding the dominant inhibitory mutants Ras(N17) or Raf(301). The cultures were subsequently grown in medium supplemented with 5% calf serum. After 15 days, when transformed foci were visible, the cells were fixed and stained with crystal violet.

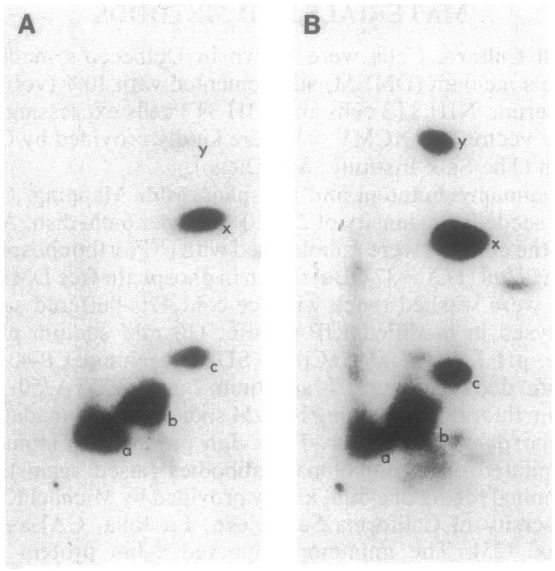

FIG. 2. Alterations in c-Jun phosphorylation in MT-expressing cells. c-Jun from NIH 3T3 cells stably expressing the vector alone (*A*) or the vector encoding MT (*B*) was analyzed by two-dimensional tryptic phosphopeptide mapping. The anode is on the left, and the cathode is on the right. Spots x and y are peptides containing Ser-73 and Ser-63, respectively. Spots a–c represent tri-, di-, and monophosphorylated forms of a tryptic peptide containing Ser-243, Ser-249, and Thr-231.

are phosphopeptides containing Ser-73 and Ser-63, respectively, in the transactivation domain. Spots a–c are tri-, di-, and monophosphorylated forms of the peptide containing Ser-243 and Ser-249 and Thr-231 in the DNA binding domain (50). Expression of MT stimulated phosphorylation of Ser-63 and Ser-73. There also was a modest increase in phosphorylation of spot c and a corresponding decrease in phosphorylation of spots a and b, suggesting that MT expression may lead to partial dephosphorylation of sites in the DNA binding region of c-Jun.

Stimulation of c-Jun Transcriptional Activity by MT. To confirm that phosphorylation of Ser-63 and Ser-73 in MT-expressing cells is required for activation of the transcriptional activity of c-Jun, we used a c-Jun/GHF-1 chimeric construct encoding the transactivation domain of c-Jun linked to the DNA binding domain of transcription factor GHF-1 (25–27). The chimeric protein lacks the c-Jun DNA binding domain, including the C-terminal phosphorylation sites, and the leucine zipper, making it insensitive to changes in levels of Fos proteins and phosphorylation of C-terminal sites that may affect DNA binding. The chimeric protein activates transcription from the growth hormone promoter through binding sites for GHF-1 (25). This chimeric protein and a corresponding protein, (A63,73)Jun/GHF-1, in which Ser-63 and Ser-73 are changed to nonphosphorylatable Ala, have been characterized (28); they are expressed equally well, but the mutant protein is not phosphorylated. The two proteins have similar binding affinities to GHF-1 binding sites (27). Fig. 3 shows that expression of MT enhances the ability of Jun/GHF-1, but not of (A63,73)Jun/GHF-1, to stimulate transcription from the growth hormone promoter in transient assays, confirming that phosphorylation of Ser-63 and Ser-73 in the transactivation domain, induced by MT, increases the transcriptional activity of the Jun protein.

To test whether activation of the transcriptional activity of the Jun/GHF-1 chimera by MT is dependent on Ras and Raf-1 activities, we tested the ability of MT to stimulate transcription from the growth hormone promoter by Jun/GHF-1 in the presence and absence of the dominant inhibi-

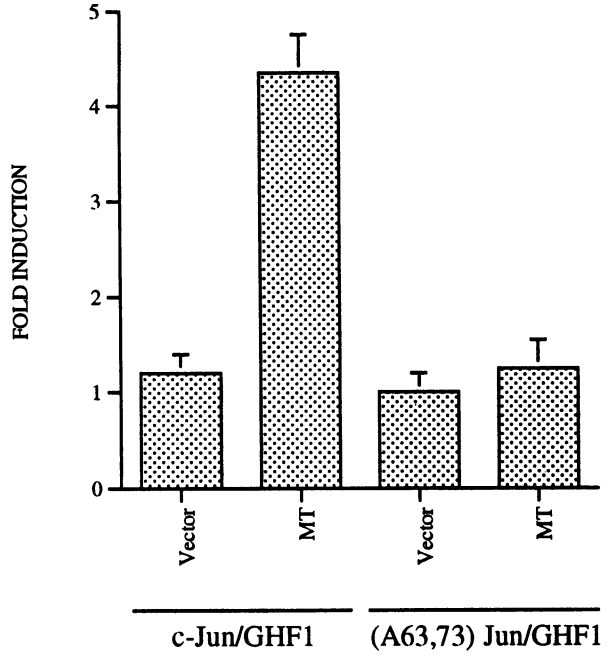

FIG. 3. MT stimulates c-Jun transcriptional activity through phosphorylation of Ser-63 and Ser-73. NIH 3T3 cells were transfected with the GH–CAT reporter plasmid and either RSVJUN/GHF-1 or RSV(A63,73)JUN/GHF-1, in the presence or absence of RSVMT. Fold induction is as described for Fig. 1. MT failed to stimulate transcriptional activity of the mutant (A63,73)Jun/GHF-1 protein. Values are the averages and SD from three experiments.

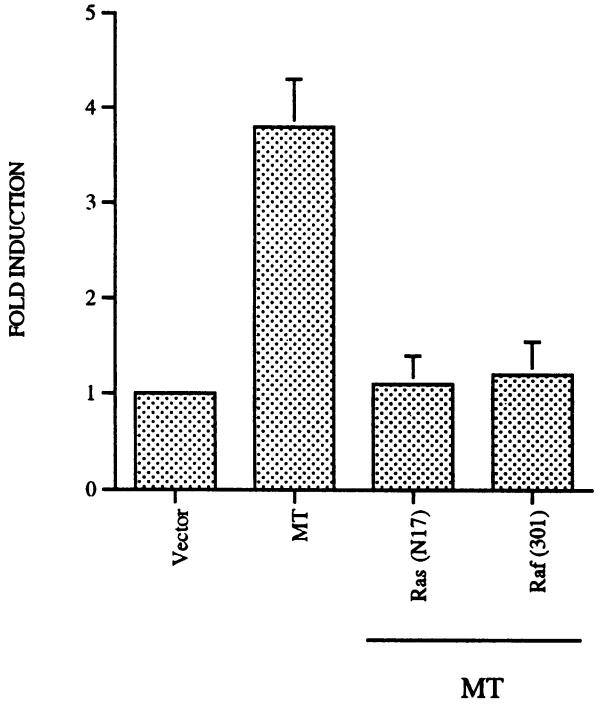

FIG. 4. MT-mediated stimulation of the c-Jun activation domain requires Ras and Raf-1 activities. NIH 3T3 cells were transfected with the GH–CAT reporter plasmid, the expression vectors, RSV-Jun/GHF-1, RSVMT, and the dominant inhibitory mutants, Ras(N17) and Raf(301) as indicated. Fold induction is the CAT activity in cells transfected with MT divided by the CAT activity in cells transfected with the GH–CAT reporter plasmid and the Jun/GHF-1 expression vector only. Values are averages and SD from three experiments. The dominant inhibitory mutants interfered with MT-mediated stimulation of c-Jun transcriptional activity.

tory mutants, Ras(N17) and Raf(301). Fig. 4 shows that the dominant inhibitory mutants interfered with MT-mediated activation of Jun/GHF-1, further confirming that Ras and Raf-1 activities are required for activation of c-Jun by MT.

DISCUSSION

The results described above support the conclusion that MT enhances the transcriptional activity of c-Jun by stimulating phosphorylation of sites in the transactivation domain of c-Jun and that the signaling pathway from MT to c-Jun involves Ras and Raf-1. Thus the pathway used by MT is similar to the signaling pathways used by other oncoproteins that stimulate c-Jun, such as v-Sis and v-Src (27). Previous studies showed that Ras activity is required for transformation by MT (41, 42). Our results confirm that conclusion and show that Ras and Raf-1 are common elements in the pathways used by MT for activation of c-Jun and for cell transformation. These observations and previous observations that the ability of MT mutants to activate c-Jun correlates with their transforming ability (19) suggest that alterations in AP-1-induced gene expression may contribute to transformation by MT.

Several oncoproteins stimulate c-Jun transcriptional activity by increasing the phosphorylation of Ser-63 and Ser-73 in the transactivation domain of c-Jun (27). Our results showed that expression of MT also causes a marked increase in phosphorylation of Ser-63 and Ser-73 and an increase in transcriptional activity. The increase in transcriptional activity was shown to depend on phosphorylation of Ser-63 and Ser-73. For this purpose we used chimeric constructs of c-Jun/GHF-1, which contain only the transactivation domain of c-Jun and activate transcription from the growth hormone promoter. MT failed to enhance transcriptional activity of c-Jun/GHF-1 when Ser-63 and Ser-73 were changed to nonphosphorylatable Ala.

A Jun kinase, JNK, distantly related to mitogen-activated protein kinase (MAP kinase), has been isolated from Ha-Ras-transformed cells (28). The kinase phosphorylates Ser-63 and Ser-73, and the activities of Ha-Ras and Raf-1 are required for its activation by oncoproteins or UV irradiation. The N-terminal region of c-Jun is required for interaction with JNK and phosphorylation of Ser-63 and Ser-73. It seems likely that JNK participates in the activation of c-Jun in MT-expressing cells. MAP kinases appear to act downstream of Ras and Raf-1 as receptor tyrosine kinase signal transduction pathways (for review, see ref. 37). It will be of interest to determine whether MT expression activates MAP kinases.

Changes in phosphorylation of c-Jun in the DNA binding domain were also observed. These are potential sites of phosphorylation by glycogen synthase kinase 3 (21, 24) or casein kinase II (23). A phosphatase that dephosphorylates these sites may be activated by phorbol 12-myristate 13-acetate and protein kinase C, or a kinase that phosphorylates the sites may be inhibited (21). Protein kinase C activity is increased in MT-expressing cells (51), and this may lead to dephosphorylation of the sites in the DNA binding domain. Dephosphorylation of these sites increases the binding of c-Jun to DNA and increases its transactivating activity (21). Protein phosphatase 2A dephosphorylates negative regulatory sites of Jun *in vitro* and activates promoters containing AP-1 binding elements (52). The changes in phosphorylation we observed in the DNA binding domain were less striking than the changes in the transactivation domain, and we did not attempt to assess the possible contribution of increased DNA binding to the increase in c-Jun transcriptional activity, for example, by performing gel-shift assays.

MT appears to function, at least in part, by assembling a signaling complex at the cell membrane that activates a

pathway involving SHC, Ras, Raf-1, and downstream protein kinases, resulting in phosphorylation of c-Jun and increases in AP-1 transcriptional activity. Because the pathways leading to c-Jun activation and to transformation are so similar, it seems likely that increases in AP-1 activity contribute to transformation. MT probably affects growth control in additional ways, and it will be important to continue to seek a better understanding of the effects of the cellular proteins that interact with MT.

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1. Treisman, R., Novak, U., Favaloro, J. & Kamen, R. (1981) *Nature (London)* **292,** 595–600.
2. Courtneidge, S. A. & Smith, A. E. (1983) *Nature (London)* **303,** 435–439.
3. Kornbluth, S., Sudol, M. & Hanafusa, H. (1987) *Nature (London)* **325,** 171–173.
4. Cheng, S. H., Harvey, R., Espino, P., Semba, K., Yamamoto, T., Toyoshima, K. & Smith, A. E. (1988) *EMBO J.* **7,** 3845–3855.
5. Whitman, M., Kaplan, D. R., Schaffhausen, B., Cantley, L. & Roberts, T. M. (1985) *Nature (London)* **315,** 239–242.
6. Otsu, M., Hiles, I., Gout, I., Fry, M. J., Ruiz-Larrea, F., Panayotou, G., Thompson, A., Dhand, R., Hsuan, J., Totty, N., Smith, A. D., Morgan, S. J., Courtneidge, S. A., Parker, P. J. & Waterfield, M. D. (1991) *Cell* **65,** 91–104.
7. Pallas, D. C., Shahrik, L. K., Martin, B. L., Jaspers, S., Miller, T. B., Brautigan, D. L. & Roberts, T. M. (1990) *Cell* **60,** 167–176.
8. Walter, G., Ruediger, R., Slaughter, C. & Mumby, M. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 2521–2525.
9. Courtneidge, S. A. (1985) *EMBO J.* **4,** 1471–1477.
10. Cartwright, C. A., Kaplan, P. L., Cooper, J. A., Hunter, T. & Eckhart, W. (1986) *Mol. Cell. Biol.* **6,** 1562–1570.
11. Courtneidge, S. A. & Heber, A. (1987) *Cell* **50,** 1031–1037.
12. Kaplan, D. R., Whitman, M., Schaffhausen, B., Pallas, D. C., White, M., Cantley, L. & Roberts, T. M. (1987) *Cell* **50,** 1021–1029.
13. Markland, W. & Smith, A. E. (1987) *Biochim. Biophys. Acta* **907,** 299–321.
14. Druker, B. J., Ling, L. E., Cohen, B., Roberts, T. M. & Schaffhausen, B. S. (1990) *J. Virol.* **64,** 4454–4461.
15. Dilworth, S. M., Brewster, C. E. P., Jones, M. D., Lanfrancone, L., Pelicci, G. & Pelicci, P. G. (1994) *Nature (London)* **367,** 87–90.
16. Morrison, D. K., Kaplan, D. R., Rapp, U. & Roberts, T. M. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8855–8859.
17. Talmage, D. A., Blenis, T. & Benjamin, T. J. (1988) *Mol. Cell. Biol.* **8,** 2309–2315.
18. Gutman, A. & Wasylyk, B. (1991) *Trends Genet.* **7,** 49–54.
19. Schönthal, A., Srinivas, S. & Eckhart, W. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 4972–4976.
20. Angel, P. & Karin, M. (1991) *Biochim. Biophys. Acta* **1072,** 129–157.
21. Boyle, W. J., Smeal, T., Defize, L. H. K., Angel, P., Woodgett, J. R., Karin, M. & Hunter, T. (1991) *Cell* **64,** 573–584.
22. Karin, M. & Smeal, T. (1992) *Trend. Biochem. Sci.* **17,** 418–422.
23. Lin, A., Frost, J., Deng, T., al-Alawi, N., Smeal, T., Kikkawa, U., Hunter, T., Brenner, D. & Karin, M. (1992) *Cell* **70,** 777–789.
24. deGroot, R. P., Auwrex, J., Bourouis, M. & Sassone-Corsi, P. (1993) *Oncogene* **3,** 841–847.
25. Binetruy, B., Smeal, T. & Karin, M. (1991) *Nature (London)* **351,** 122–127.
26. Smeal, T., Binetruy, B., Mercola, D., Birrer, M. & Karin, M. (1991) *Nature (London)* **354,** 494–496.
27. Smeal, T., Binetruy, B., Mercola, D., Grover-Bradwick, A., Heidecker, G., Rapp, U. R. & Karin, M. (1992) *Mol. Cell. Biol.* **12,** 3507–3513.
28. Hibi, M., Lin, A., Smeal, T., Minden, A. & Karin, M. (1993) *Genes Dev.* **7,** 2135–2148.
29. Lloyd, A., Yancheva, N. & Wasylyk, B. (1991) *Nature (London)* **352,** 635–638.
30. Pelicci, G., Lanfrancone, I., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Pawson, T. & Pelicci, P. G. (1992) *Cell* **70,** 93–104.
31. Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T. & Botwell, D. (1993) *Nature (London)* **363,** 83–85.
32. Rozakis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J. & Pelicci, P. G. (1992) *Nature (London)* **360,** 689–692.
33. Egan, S. E., Giddings, B. W., Brooks, M. W., Buday, L., Sizeland, A. M. & Weinberg, R. A. (1993) *Nature (London)* **363,** 45–51.
34. Zhang, X.-F., Settleman, J., Kyriakis, J. M., Takeuchi-Suzuki, E., Elledge, S. T., Marshall, M. S., Bruder, J. T., Rapp, U. R. & Avruch, J. (1993) *Nature (London)* **364,** 308–313.
35. Warne, P. H., Viciana, P. R. & Downward, J. (1993) *Nature (London)* **364,** 352–355.
36. Davis, R. J. (1993) *J. Biol. Chem.* **268,** 14553–14556.
37. Blenis, J. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 5889–5892.
38. Vojtek, A. B., Hollenberg, S. M. & Cooper, J. A. (1993) *Cell* **74,** 205–214.
39. Talmage, D. A., Riney, C. & Benjamin, T. L. (1991) *Cell Growth Differ.* **2,** 51–58.
40. Bolen, J. B., Amini, S., DeSeau, V., Reddy, S. & Shalloway, D. (1987) *J. Virol.* **61,** 1079–1085.
41. Raptis, L., Marcellus, R., Corbley, M. J., Krook, A., Whitfield, J., Anderson, S. K. & Haliotis, T. (1991) *J. Virol.* **65,** 5203–5210.
42. Jelinek, M. A. & Hassell, J. A. (1992) *Oncogene* **7,** 1687–1698.
43. Glenn, G. M. & Eckhart, W. (1993) *J. Virol.* **67,** 1945–1952.
44. Gibbs, J. B., Marshall, M. S., Scolnick, E. M., Dixon, R. A. F. & Vogel, U. S. (1990) *J. Biol. Chem.* **265,** 20437–20442.
45. Chen, C. & Okayama, H. (1987) *Mol. Cell. Biol.* **7,** 2745–2752.
46. Fieg, L. A. & Cooper, G. M. (1988) *Mol. Cell. Biol.* **8,** 3235–3243.
47. Cai, H., Szeberen, Y. I. & Cooper, G. M. (1990) *Mol. Cell. Biol.* **10,** 5314–5323.
48. Rapp, U. (1991) *Oncogene* **6,** 495–500.
49. Kolch, W., Heidecker, G., Lloyd, P. & Rapp, U. (1991) *Nature (London)* **349,** 426–428.
50. Chou, S.-Y., Baichwal, V. & Ferrell, J. E. J. (1992) *Mol. Biol. Cell* **3,** 1117–1130.
51. Marcellus, R. C., Whitfield, J. F. & Raptis, L. (1991) *Oncogene* **6,** 1037–1040.
52. Alberts, A. S., Deng, T., Lin, A., Meinkoth, J. L., Schönthal, A., Mumby, M. C., Karin, M. & Feramisco, J. R. (1993) *Mol. Cell. Biol.* **13,** 2104–2112.