Involvement of Stress-Activated MAP Kinase p38 in p53-Independent Induction of *p21/WAF1/Cip1* Gene Expression

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A p53-independent increase in *p21/WAF1/Cip1* gene expression is induced by anti-cancer agents such as trichostatin, actinomycin D and butyrate, and the signaling cascade of this induction was examined in cells stably transformed with a luciferase reporter under transcriptional control of the *p21/WAF1/Cip1* gene. An inhibitor of stress-activated protein kinase p38 (SB203580) efficiently inhibited the induction, while PD98059, an inhibitor of MEK1, showed weaker inhibition. The dominant negative form of MKK6 inhibited the transcriptional activation of *p21/WAF1/Cip1* gene in transient assay, whereas the dominant negative form of MKK4 did not affect it. Western blotting using antibodies against activated MAP kinases showed that butyrate, trichostatin and actinomycin D activated p38 in p53-defective human osteoblastic cells, but ERK1/2 and JNK activities were not increased significantly by these treatments. These results indicate that p38 kinase is the principal signaling molecule involved in the induction of *p21/WAF1/Cip1* gene expression by butyrate, trichostatin and actinomycin D.

Key words——p21/WAF1, MAP kinase, p38 kinase, antitumor agent

INTRODUCTION

The *p21/WAF1/Cip1(p21)* gene was identified as a target of p53 and has also been shown to encode a protein that inhibits the activity of cyclin–Cdk complexes, thereby, negatively controlling cell cycle progression.[1–3] Expression of the *p21* gene is, however, controlled by diverse mechanisms in a p53-dependent and -independent manner. Induction of *p21* by γ-irradiation is a result of direct transcriptional activation by p53,[4,5] whereas various signals have been reported to induce *p21* independently of p53, including serum, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), okadaic acid, 2-*O*-tetradecanoylphorbol 13-acetate (TPA), granulocyte colony stimulating factor (G-CSF), interleukin (IL)-6, interferon (IFN)-γ and transforming growth factor (TGF)-β.[6–12]

A number of chemotherapeutic and DNA-damaging agents, such as alkylating agents, metal complexes, intercalating agents, vinca alkaloids, nucleosides and folate analogs, have been shown to enhance wild-type p53 accumulation in the cell nucleus.[13] There is evidence that enhanced p53 levels are required for growth arrest in response to DNA damage, since cells lacking wild-type p53 do not exhibit increased p53 levels in response to DNA damage and do not exhibit growth arrest.[14] Although the hypothesis that the DNA damage-induced p53-dependent G_1 arrest is mediated by p21 remains to be investigated, the *p21* gene product is known to inhibit cyclin-dependent kinase activity and blocks the cellular transition from G_1 to S.[1,15] In our previous study, we identified several anti-tumor agents such as actinomycin D and trichostatin from screening using *p21* reporter assay, and determined transcriptional response elements involved in the induction by these drugs.[16]

The signals that elicit transcription of the *p21* gene are a matter of controversy. MAPK activity was reported to be required for growth factor-induced p21 induction and the dominant negative form of ras inhibited this induction in

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HeLa cells.[17] Induction by ethylmaleimide was blocked by dominant negative MEK in HeLa cells,[18] whereas ERK1/2 seems to be required for induction by butyrate and trichostatin in neuroepithelioma cells.[19] MAP kinase cascades are essential for transduction of extracellular signals to nuclei, and these cascades play a role both in growth stimulation and inhibition, depending on the cell-type and cellular context.[20–23] Here, we report evidence that the stress-activated MAP kinase p38 is involved in the induction of *p21* gene transcription in human osteosarcoma Saos-2 and mouse fibroblastic cells.

MATERIALS AND METHODS

Cells—Mouse embryonic fibroblasts defective in p53, 10(1) cells, described by Harvey and Levine,[24] and the human osteosarcoma cell line Saos-2 were cultured in DMEM and αMEM supplemented with heat-inactivated fetal bovine serum, respectively. Construction of WAF1/Pst/luciferase containing the transcriptional element from about nt -200 of the *p21* gene deprived of p53-responsive elements (WAF1/Pst/Luc), and a stable cell line of Saos-2 transfected with the plasmid were as described previously.[16]

Transfection of Plasmids—Plasmids were transfected into 10(1) cells by the conventional calcium phosphate-precipitation method. Flag-tagged expression vectors for the dominant negative forms of MKK4 [MKK4(Ala)], and MKK6 [MKK6(K/A)] were kind gifts from Dr. R. Davis.[25]

Western Bloting Analysis—Cells were solubilized in lysis buffer (50 mM HEPES, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1% Triton X-100, 0.5% deoxycholate (DOC), 0.1% SDS, 2 mM $MgCl_2$, 1 mM phynylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin) or disrupted using a Dounce homogenizer and fractionated into Triton X-100-soluble and -resistant fractions in a buffer consisting of 10 mM Tris, pH 8.0, 10 mM NaCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 1mM PMSF and 10 μmg/ml aprotinin. The extracts were subjected to SDS-PAGE, and resolved proteins were electrophoretically transferred onto nitrocellulose membranes (Amersham Life Science). The membranes were then probed with a primary, followed by a secondary, antibody. Antibodies against activated kinases, anti-phospho-p38 MAP kinase (Thr180/Tyr182), anti-phospho-SAPK/JNK (Thr183/Tyr185) and anti-phospho-p42/p44 MAP kinase (Thr202/Tyr204) were obtained from New England Biolabs. A monoclonal antibody against ERK2 (pan-ERK) was obtained from Transduction Laboratory. The immune complexes were detected using enhanced chemiluminescence, according to the manufacturer's protocol (NEN™ Life Science Products).

RESULTS AND DISCUSSION

Effects of MAP Kinase Inhibitors on the Expression of *p21*/Luciferase

The effects of inhibitors of the MAP kinase cascade on *p21* gene expression were first examined using a stable Saos-2 transformant harboring the WAF1/Pst/Luc reporter, the activity of which reflects p53-independent activation. The p53-independent induction of *p21* by butyrate, trichostatin and actinomycin D was strongly inhibited by SB203580, a specific inhibitor of p38,[27] while PD98059, a specific inhibitor of ERK activation,[28] inhibited the induction much more weakly than SB203580 (Fig. 1). However, the induction by actinomycin D was more sensitive to PD98059 than that by butyrate and trichostatin.

Effects of Dominant Negative Forms of MKK (MAP Kinase Kinase)

To obtain insight into the involvement of

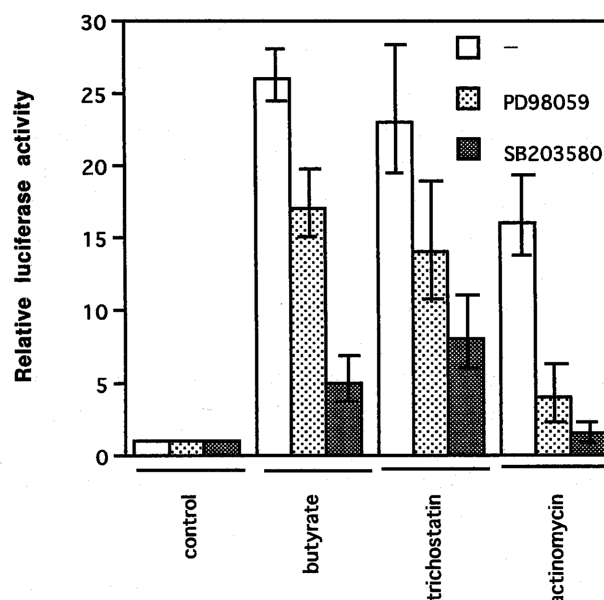

Fig. 1. Effects of MAP Kinase Inhibitors on the Induction of WAF1/Pst/Luc

Saos-2 cells stably transfected with WAF1/Pst/Luc (clone #22) were treated with 2 mM butyrate, 3 μM trichostatin A or 30 ng/ml actinomycin D in the absence or presence of PD98059 (50 μM) or SB203580 (10 μM) for 24 h, and the luciferase activity was measured. The results are means of three independent experiments±S.D.

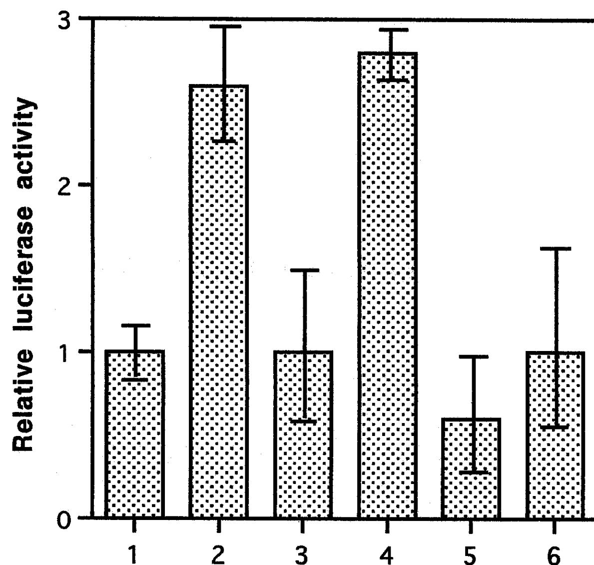

Fig. 2. Dominant Negative MKK6 Inhibited the Induction of WAF1/Pst/Luc

Mouse fibroblasts 10(1) cells that are deficient in the *p53* gene were transiently transfected with WAF1/Pst/Luc (1—6), with or without dominant negative (dn) forms of MKK4 (3, 4) or MKK6 (5, 6). After transfection, cells were either untreated (1, 3, 5) or treated with 2 mM butyrate for 24 h (2, 4, 6), and luciferase activity was measured. The results are means of three independent experiments±S.D.

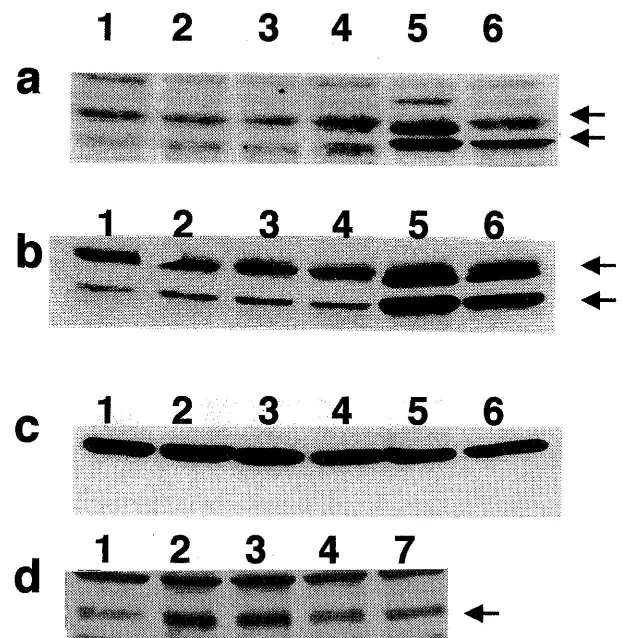

Fig. 3. Activation of MAP Kinases by Butyrate, Trichostatin and Actinomycin D

Saos-2 cells stably transfected with WAF1/Pst/Luc reporter (clone # 22) were untreated (1), or treated with 2 mM butyrate (2), 3 μM trichostatin (3), 30 ng/ml actinomycin D (4), 0.3 M sorbitol (5), 100 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate (6) or 200 nM anisomycin (7) for 30 min. Cell lysates were prepared, and active forms of JNK (a), ERK1/2 (b), and p38 (d), and total levels of ERK (c) were determined using specific antibodies. Arrows indicate activated forms of JNK (a), ERK1/2 (b), and p38 (d), respectively.

MAP kinases in the activation of *p21* gene transcription by butyrate, trichostatin and actinomycin D, the effects of dominant negative forms of MKK (dnMKK) were examined using mouse embryonic fibroblast 10(1) cells that are defective in the *p53* gene. Cells were transiently transfected with the reporter WAF1/Pst/Luc, together with the expression vectors of the dominant forms of MKK4 or MKK6. Co-transfection of dnMKK6, an upstream regulator of p38 kinase, inhibited the reporter activity induced by butyrate but dnMKK4, which regulates the JNK family kinases, had essentially no effect (Fig. 2). Similar effects were observed with induction by trichostatin and actinomycin D (data not shown). These results, together with those shown in Fig. 1, indicate that p38 is a key regulator in the induction of WAF1/Pst/Luc caused by butyrate, trichostatin and actinomycin D.

Activation of p38 by Trichostatin, Butylate and Actinomycin D

The above results prompted us to examine the activation of members of the MAP kinase family by western blotting using antibodies specific for the activated forms of these kinases. The results in Fig. 3 indicated that the activity of ERK1/2 was not affected by butyrate, trichostatin or actinomycin D at doses that induced WAF1/Pst/Luc activity, while the level of the active form of p38 was increased by these treatments. Activation of p38 by actinomycin D was weaker than by other inducers, and actinomycin D induced about a two-fold activation of JNK (Fig. 1a).

MAP kinases are activated by various types of extracellular stimuli, and play essential roles in the regulation of specific gene expression.[29] Although the drugs used in the present study were structurally unrelated, our previous results indicated that they all activated WAF1/Pst/Luc promoter through the same Sp1 element.[16] The DNA-binding activity of Sp1 was, however, unaffected. The Sp1 factor associates with the transcriptional coactivator p300/CBP[30,31] to form transcriptionally active complexes. The p38 activity may regulate some components of these complexes and activate *p21* gene transcription. Although ERK kinases, rather than p38, seem to be involved in p53-independent induction of the

p21 gene by growth factors,[17,19,23] treatment of cells with drugs used in the present study may induce non-specific cellular damage, such as membrane pertrubation and inhibition of RNA synthesis, that cause stressful conditions. These stress pathways may activate p38 kinase.

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