Activation of Rac1 and the p38 Mitogen-activated Protein Kinase Pathway in Response to All-*trans*-retinoic Acid*

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Several signaling pathways are activated by all-*trans*-retinoic acid (RA) to mediate induction of differentiation and apoptosis of malignant cells. In the present study we provide evidence that the p38 MAP kinase pathway is activated in a RA-dependent manner in the NB-4, acute promyelocytic leukemia, and the MCF-7, breast carcinoma, cell lines. RA treatment of cells induces a time- and dose-dependent phosphorylation of p38, and such phosphorylation results in activation of its catalytic domain. p38 activation is not inducible by RA in a variant NB-4 cell line, NB-4.007/6, which is resistant to the effects of RA, suggesting a role for this pathway in the induction of RA responses. Our data also demonstrate that the small G-protein Rac1 is activated by RA and functions as an upstream regulator of p38 activation, whereas the MAPKAPK-2 serine kinase is a downstream effector for the RA-activated p38. To obtain information on the functional role of the Rac1/p38/MAPKAPK-2 pathway in RA signaling, the effects of pharmacological inhibition of p38 on RA-induced gene transcription and cell differentiation were determined. Our results indicate that treatment of cells with the SB203580 inhibitor does not inhibit RA-dependent gene transcription via retinoic acid response elements or induction of Stat1 protein expression. However, treatment with SB203580 or SB202190 strongly enhances RA-dependent induction of cell differentiation and RA-regulated growth inhibitory responses. Altogether, our findings demonstrate that the Rac1/p38 MAP kinase pathway is activated in a RA-dependent manner and exhibits negative regulatory effects on the induction of differentiation.

All-*trans*-retinoic acid (RA)[1] is a potent inducer of cellular

differentiation and growth arrest of acute promyelocytic leukemia cells *in vitro* and *in vivo* and has significant clinical activity in the treatment of acute promyelocytic leukemia (1–4). RA and other retinoids also induce antitumor effects by inhibiting cell growth or promoting programmed cell death of neoplastic cells of diverse cellular origin (5–12). The important biological effects of RA have led to intense efforts to understand the mechanisms by which it generates its biological effects on malignant cells. It is well established that RA binds to the RAR and RXR nuclear receptors, resulting in the formation of RAR-RXR heterodimers or RXR homodimers (reviewed in Refs. 13 and 14). These complexes have the ability to induce gene transcription via binding to the retinoic acid response elements (RARE) in the promoters of RA-inducible genes (13, 14). Other mechanisms via which retinoids induce their biological effects in malignant cells include inhibition of activation of the AP-1 protein via a CBP-regulated mechanism (15, 16), inhibition of the c-Jun N-terminal kinase (17), modulation of histone acetylation (18), and up-regulation of transforming growth factor-β2 and insulin-like growth factor binding protein-3 expression (19). Recent evidence also indicates that the extracellular signal-regulated kinase (ERK2) mitogen-activated protein kinase is activated by all-*trans*-retinoic acid in HL-60 cells and mediates the induction of cell differentiation and growth arrest (20).

The family of the p38 mitogen-activated protein kinases includes four known members, all of which are mammalian homologues of the HOG-1 MAP kinase in *Saccharomyces cerevisiae* (21–23). All four homologues share significant identity with each other and include p38α (21–23), p38β (24–28), p38γ (29–31), and p38δ (32). The members of this family of kinases exhibit serine-kinase activities and upon their activation regulate phosphorylation/activation of other serine kinases, resulting in signals that mediate multiple biological responses. These include phosphorylation of transcription factors (33, 34), induction of cytokine production (33, 34), platelet aggregation (35), and regulation of apoptosis (36–40). The p38 pathway plays an important role in signaling for various extracellular stimuli and has been previously shown to be activated in response to cellular stress, as well as during treatment of cells with proinflammatory cytokines, thrombin, or hematopoietic growth factors (21–23, 41). In addition, this pathway is activated by the type I interferon receptor, and its function is essential for type I interferon-dependent transcriptional activation (42–44).

In the present study we determined if treatment of cells with

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[1] The abbreviations used are: RA, all-*trans*-retinoic acid, RARE, retinoic acid responsive element, MAP, mitogen activated protein kinase, SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, ERK, extracellular signal regulated kinase, JNK, c-Jun N-terminal kinase; GST, glutathione *S*-transferase; MTT, 3-(4,5-dimethylthiazol-2-

yl)-2,5-diphenyltetrazolium bromide; PAGE, polyacrylamide gel electrophoresis; RAR, retinoic acid receptor; RXR, retinoic X receptor.

RA induces activation of the p38 pathway in the NB-4, acute promyelocytic leukemia, and the MCF-7, breast carcinoma, cell lines. Our data demonstrate that p38 is phosphorylated and activated in an RA-dependent manner and that such activation is regulated by the small G-protein Rac1. We also demonstrate that the MAPKAPK-2 kinase is activated by RA, and its activation is inhibited by pretreatment of cells with the p38 inhibitor SB203580, indicating that it is a part of a signaling cascade downstream of p38. Finally, we demonstrate that inhibition of p38 activation using specific inhibitors has no significant effects on RA-dependent transcriptional regulation, but it strongly enhances RA-induced cell differentiation, suggesting a negative regulatory effect of this pathway on the induction of RA-dependent differentiation.

EXPERIMENTAL PROCEDURES

Cells and Reagents—The NB-4 human acute promyelocytic leukemia cell line was grown in RPMI 1640 supplemented with fetal bovine serum and antibiotics. The MCF-7 human breast carcinoma cell line was grown in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum and antibiotics. A polyclonal antibody against p38 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A polyclonal antibody against the phosphorylated/activated form of p38 was obtained from New England Biolabs and was used for immunoblotting. A polyclonal antibody against MAPKAP-2 kinase was obtained from Upstate Biotechnology, Inc., and was used for immunoprecipitations. A monoclonal antibody against Stat1 was obtained from Transduction Laboratories (Lexington, KY). An antibody against the phosphorylated form of Stat1 on serine 727 (anti-Ser-727Stat1) was obtained from Upstate Biotechnology, Inc. A polyclonal antibody that recognizes the phosphorylated/activated form of ATF-2 was obtained from New England Biolabs. The SB203580 and SB202190 inhibitors were obtained from Calbiochem.

Cell Lysis and Immunoblotting—Cells were treated with 1 μM RA for 48 h, unless otherwise indicated. The vehicle control was cells treated with Me$_2$SO (diluent for RA). The cells were subsequently lysed in phosphorylation lysis buffer as described previously (42). Immunoprecipitations and immunoblotting using an enhanced chemiluminescence (ECL) method were performed as in previous studies (45).

p38 MAP Kinase Assay—Cells were incubated in the presence or absence of the RA for the indicated times at 37 °C. The cells were subsequently lysed in phosphorylation lysis buffer (PLB) (42), and lysates were immunoprecipitated with an antibody against p38 using protein G-Sepharose. The immunocomplexes were subsequently washed 3 times with PLB containing 0.1% Triton X-100 and 2 times with kinase buffer (25 mM Hepes, 25 mM MgCl$_2$, 25 mM β-glycerophosphate, 2 mM dithiothreitol, and 0.1 mM Na$_3$VO$_4$, 20 μM ATP) and resuspended in 30 μl of kinase buffer containing 5 μg of GST ATF-2 fusion protein. The reaction was incubated for 30 min at room temperature and was terminated by the addition of SDS sample buffer. Proteins were analyzed by SDS-PAGE, and the phosphorylated form of ATF-2 was detected by immunoblotting with an antiphospho-ATF-2 antibody.

MAPKAP Kinase-2 Kinase Assay—Cells were treated with RA for the indicated times in the presence or absence of 10 μM SB203580. The cells were then lysed in phosphorylation lysis buffer (PLB), and lysates were immunoprecipitated with an antibody against MAPKAP kinase-2. Immunoprecipitated proteins were washed three times in PLB and two times in kinase buffer (25 mM Hepes, pH 7.4, 25 mM MgCl$_2$, 25 mM β-glycerophosphate 100 μM sodium orthovanadate, 2 mM dithiothreitol, 20 μM ATP). The immune complex kinase assays were initiated by the addition of 30 μl of kinase buffer containing 5 μg of Hsp-25 protein (StressGen Biotechnologies Corp.) as a substrate, and 10 μCi of [γ-^{32}P]ATP was added. The reaction was incubated for 30 min at room temperature and was terminated by the addition of SDS sample buffer. Proteins were subsequently analyzed by SDS-PAGE, and the phosphorylated form of Hsp-25 was detected by autoradiography.

Cell Proliferation Assays—Cell proliferation assays using the MTT method were performed as in previous studies (45).

Luciferase Reporter Assays—MCF-7 cells were transfected with a β-galactosidase expression vector and an RARE-luciferase plasmid (46) using the superfect transfection reagent as per the manufacturer's recommended procedure (Qiagen). Forty eight hours after transfection, triplicate cultures were either left untreated or treated with RA for 16 h in the presence or absence of 10 μM SB203580. The cells were washed twice with cold phosphate-buffered saline, and after cell lysis, luciferase activities were measured using the protocol of the manufacturer (Promega). The measured luciferase activities were normalized for β-galactosidase activity for each sample.

Flow Cytometric Analysis—Flow cytometric studies were performed as in previous studies (47). NB-4 cells were treated with Me$_2$SO or RA in the presence or absence of SB203580 (10 μM) or SB202190 (10 μM) or PD98059 (2 μM) for 5 days, and cell differentiation was determined by staining with the anti-CD11b monoclonal antibody. The anti-CD11b monoclonal antibody and a matched isotype control were purchased from Coulter Immunotech.

Rac1 Activation Assays—The activation of Rac1 by RA was determined using a recently described methodology (48), with minor modifications. Briefly, the pGEX-4T3 construct encoding for the GTPase binding domain of human PAK1 (48) was expressed in *Escherichia coli* as a GST fusion protein (GST-PBD). After treatment of cells with RA, cells were lysed in phosphorylation lysis buffer and were incubated with 5 μg of GST-PBD. Bound proteins were separated by SDS-PAGE and immunoblotted with a monoclonal antibody against Rac1 (Transduction Laboratories) to detect GTP-bound Rac1.

RESULTS

We determined whether treatment of cells with all-*trans*-retinoic acid induces activation of the p38 MAP kinase pathway in NB-4 and MCF-7 cells. Cells were incubated with RA for different times (24–48 h), and after cell lysis, total lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated/activated form of p38. RA treatment induced strong phosphorylation of p38 (Fig. 1*A*). Stripping and reprobing the same blot demonstrated that equal amounts of the p38 protein were detectable prior to and after RA treatment (Fig. 1*B*). To determine directly whether the phosphorylation of p38 results in induction of its kinase activity, cells were treated with RA; cell lysates were immunoprecipitated with an anti-p38 antibody, and *in vitro* kinase assays were performed using ATF-2 as an exogenous substrate. p38 from lysates of cells treated with RA induced strong phosphorylation of ATF-2 (Fig. 1*C*), indicating that the catalytic activity of the kinase is induced in a RA-dependent manner. Treatment with RA also induced phosphorylation/activation of the ERK2 kinase (Fig. 1, *D* and *E*), indicating that the ERK pathway is also activated by RA in these cells, consistent with previous reports using HL-60 cells (20).

We subsequently determined whether RA-dependent phosphorylation/activation of p38 occurs in an NB-4 variant cell line, NB-4.007/6, which is refractory to RA-induced differentiation and inhibition of cell growth, due to constitutive degradation of PML-RAR (49). As shown in Fig. 2*A*, when NB-4 and NB-4.007/6 cells were analyzed in parallel, the phosphorylation of p38 was only inducible in NB-4 but not in NB-4.007/6 cells, despite the fact that similar amounts of the p38 protein were expressed in both cell lines. Thus, functional activation of a signaling cascade that requires PML-RAR expression is essential for downstream phosphorylation/activation of the p38 kinase in cells of acute promyelocytic leukemia origin.

We also determined whether RA-dependent activation of p38 occurs in the MCF-7 breast carcinoma cell line, which is sensitive to the growth inhibitory effects of RA (50–52). RA treatment of these cells resulted in a time-dependent phosphorylation of p38, with the signal reaching a maximum at ~24 h and diminishing by 120 h (Fig. 3, *A* and *B*), indicating that the RA-inducible activation of p38 is not restricted to cells of promyelocytic origin, but it also occurs in other RA-sensitive neoplastic cells.

Previous studies have demonstrated that the small G-protein Rac1 is an upstream regulator of the p38 MAP kinase in response to stress (53–55). We therefore determined if Rac1 is activated in RA-responsive manner and whether it regulates RA-inducible p38 activation. NB-4 or NB-4.007/6 cells were treated for different times with RA, and cell lysates were bound
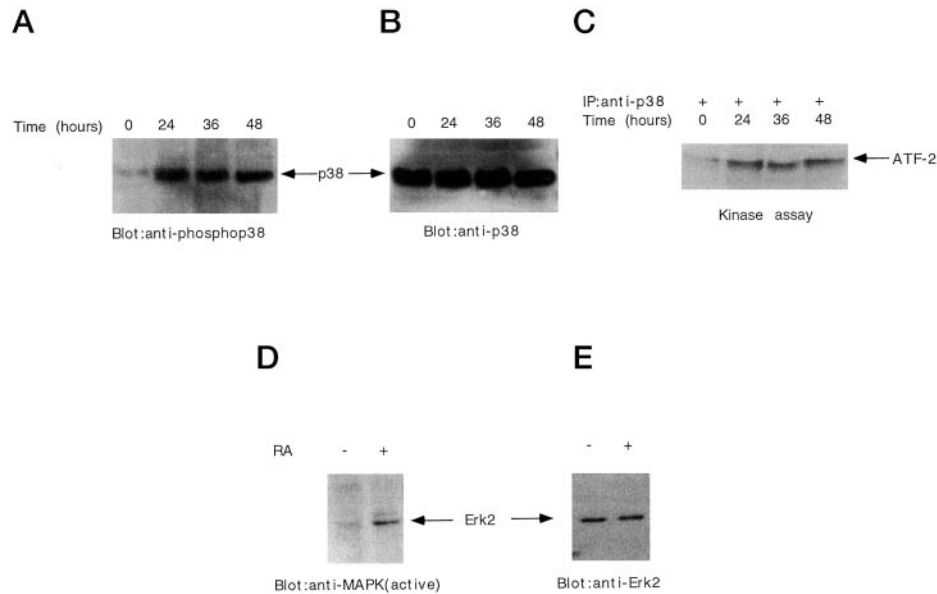
FIG. 1. **All-*trans*-retinoic acid induces phosphorylation and activation of the p38 MAP kinase in NB-4 cells.** *A,* cells were incubated with RA for the indicated times. Total cell lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated/activated form of p38. *B,* the blot shown in *A* was stripped and reprobed with an antibody against p38 to control for loading. *C,* NB-4 cells were incubated for the indicated times with RA at 37 °C as indicated. Cell lysates were immunoprecipitated (*IP*) with an antibody against p38, and an *in vitro* kinase assay, using ATF-2 as an exogenous substrate, was carried out on the immunoprecipitates. The phosphorylated form of ATF-2 is indicated. *D,* NB-4 cells were incubated with RA for 48 h as indicated. Total cell lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against the activated form of ERK. *E,* the blot shown in *D* was stripped and reprobed with an antibody against ERK2 to control for loading.
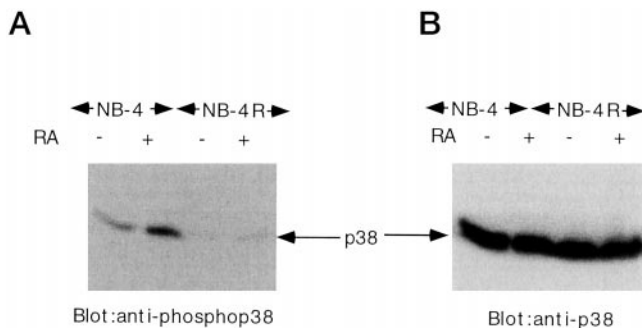

FIG. 2. **RA induces phosphorylation/activation of p38 in NB-4 cells but not the RA-resistant NB-4.007/6 (NB-4R) cell line.** *A,* cells were incubated with RA for 48 h at 37 °C as indicated. Cell lysates were analyzed by SDS-PAGE and immunoblotted with an antiphospho-p38 antibody. *B,* the blot shown in *A* was stripped and reprobed with an antibody against p38 to control for loading.
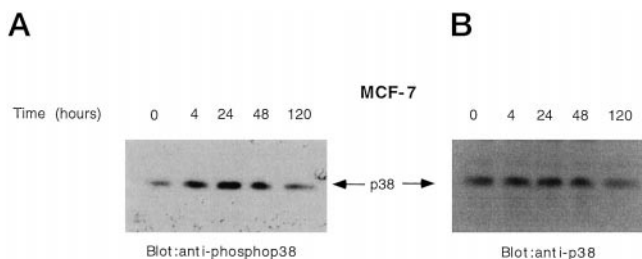

FIG. 3. **Time course of the RA-dependent activation of the p38 MAP kinase in MCF-7 breast carcinoma cells.** *A,* MCF-7 cells were incubated with RA for the indicated times at 37 °C. Total cell lysates were analyzed by SDS-PAGE and immunoblotted with an antiphospho-p38 antibody. *B,* the same blot was subsequently stripped and reprobed with an antibody against p38 to control for loading.

to a GST fusion protein for the binding domain of the Pak1 kinase, which is the downstream effector for Rac1. The bound proteins were subsequently analyzed by SDS-PAGE and immunoblotted with an anti-Rac1 antibody to identify the activated/GTP-bound form of Rac1. RA treatment induced strong activation of Rac1 in NB-4 but not in NB-4.007/6 cells (Fig. 4*A*). Such activation was dose-dependent, with maximum amounts of GTP-bound Rac1 detectable at RA concentrations of 10^{-5} to 10^{-6} M (Fig. 4*B*). Rac1 activation was also detected in response to RA treatment in the MCF-7 cell line, consistent with the RA-inducible p38 activation in these cells (Fig. 4*C*).

To determine directly whether Rac1 activation regulates downstream engagement of p38, MCF-7 cells were transiently transfected with either wild-type Rac1 or a dominant-negative form of Rac1 (Rac1T17N) (53). The phosphorylation/activation of the p38 MAP kinase was subsequently determined in the transfected cells. Overexpression of wild-type Rac1 slightly increased the RA-dependent activation of p38, as compared with cells transfected with control empty vector (Fig. 5*A*). On the other hand, overexpression of the dominant-negative form of Rac1 abrogated p38 activation (Fig. 5*A*). There were no changes in the amounts of p38 protein in response to overexpression of wild-type Rac1 or the dominant-negative Rac1 mutant (Fig. 5*B*). Thus, activation of the Rac1 GTPase by RA ultimately regulates RA-dependent activation of p38.

Previous work has demonstrated that MAPKAPK-2 is an *in vivo* substrate for the kinase activity of p38 in response to stress (41, 42, 56). To determine whether this serine-threonine kinase is also activated downstream of p38 after RA treatment of NB-4 or MCF-7 cells, lysates from RA-treated cells were immunoprecipitated with a specific antibody against MAP-KAPK-2, and *in vitro* kinase assays were carried out on the immunoprecipitates. As shown in Fig. 6, *A* and *B*, MAPKAPK-2 was strongly activated by RA, as evidenced by the strong phosphorylation of heat shock protein 25 (Hsp25) used as an exogenous substrate. Such an activation was blocked by treatment of cells with the specific p38 inhibitor SB203580 (Fig. 6*C*), indicating that MAPKAK-2 is a downstream effector for the RA-activated p38 kinase.

Altogether, these studies demonstrated that a cellular cascade involving Rac1, the p38 MAP kinase, and the MAP-KAPK-2 kinase is activated by RA. In subsequent studies we sought to obtain information on the functional relevance of this
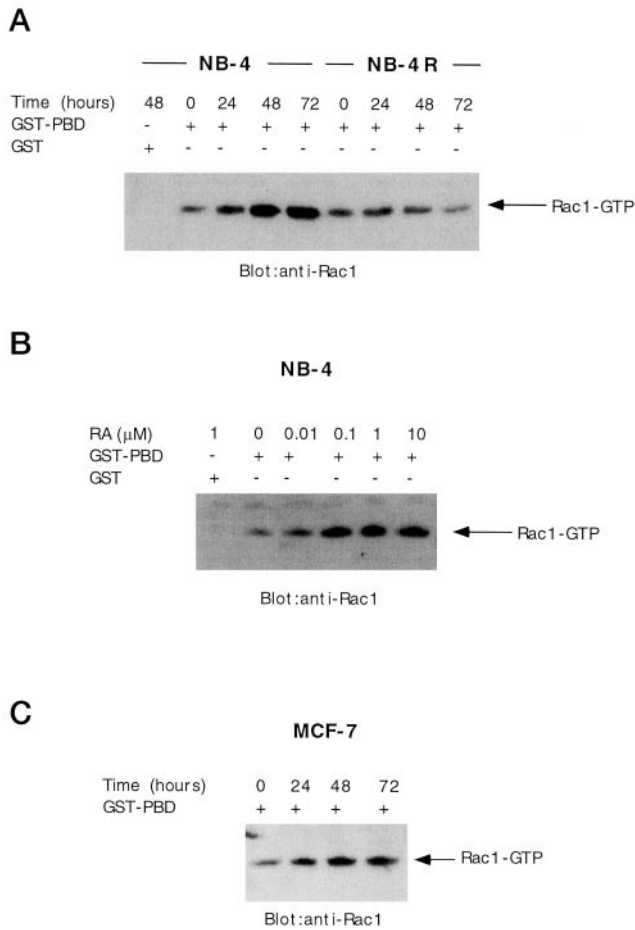
FIG. 4. **RA-dependent activation of the small G-protein Rac1.** *A,* NB-4 cells or NB-4.007/6 cells (NB-4R) were treated with RA for the indicated times. The cells were lysed, and lysates were bound to either GST alone (control) or the GST-PBD fusion protein. Bound proteins were analyzed by SDS-PAGE and immunoblotted with an antibody against Rac1. The GTP-bound form of Rac1 is indicated. *B,* NB-4 cells were treated for 48 h with the indicated amounts of RA. Cell lysates were bound to either GST alone (control) or the GST-PBD fusion protein. Bound proteins were analyzed by SDS-PAGE and immunoblotted with an antibody against Rac1. The GTP-bound form of Rac1 is indicated. *C,* MCF-7 cells were treated with RA for the indicated times. The cells were lysed, and the cell lysates were bound to either GST alone (control) or the GST-PBD fusion protein. Bound proteins were analyzed by SDS-PAGE and immunoblotted with an antibody against Rac1.
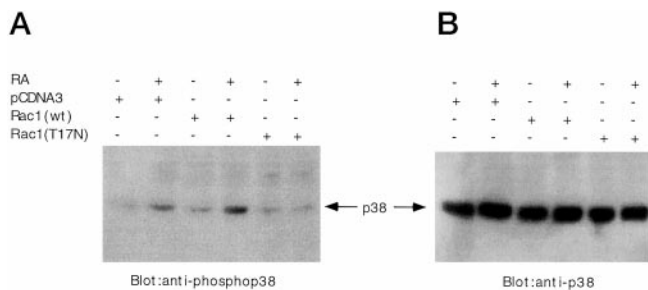

FIG. 5. **Rac1 regulates the RA-dependent activation of p38.** *A,* MCF-7 cells were transiently transfected with control empty vector (pCDNA3) or the pCDNA3-Rac1WT, or the pCDNA3-Rac1T17N constructs as indicated. The cells were subsequently treated with RA for 48 h and lysed in phosphorylation lysis buffer. Total cell lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against phospho-p38. *B,* the blot shown in *A* was subsequently stripped and reprobed with an antibody against p38.

pathway in the induction of RA responses. Previous studies have demonstrated that, in other systems, the function of the p38 pathway is required for transcriptional regulation (42–44,
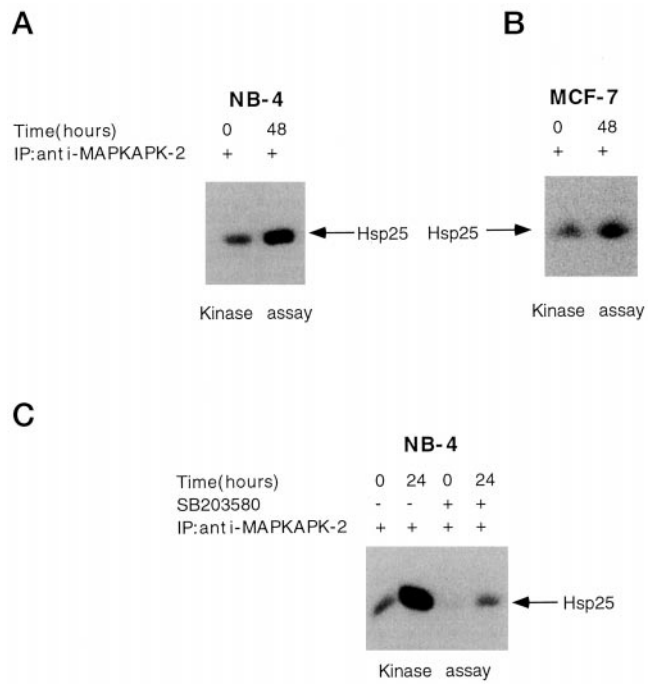

FIG. 6. **The MAPKAPK-2 kinase is activated by RA and is a downstream effector of p38.** *A,* NB-4 cells were treated in the presence or absence of RA for 48 h as indicated. Cell lysates were immunoprecipitated (*IP*) with an anti-MAPKAPK-2 antibody, and the immunoprecipitated proteins were subjected to an *in vitro* kinase assay using Hsp-25 as an exogenous substrate. Proteins were analyzed by SDS-PAGE, and phosphorylated Hsp-25 was detected by autoradiography. *B,* MCF-7 cells were treated with RA for 48 h as indicated. Cell lysates were immunoprecipitated with an anti-MAPKAPK-2 antibody, and immunoprecipitated proteins were subjected to an *in vitro* kinase assay using Hsp-25 as an exogenous substrate. Proteins were analyzed by SDS-PAGE,, and phosphorylated Hsp-25 was detected by autoradiography. *C,* NB-4 cells were treated with RA for 24 h, in the presence or absence of 10 μM SB203580 as indicated. Cell lysates were immunoprecipitated with an anti-MAPKAPK-2 antibody, and immunoprecipitated proteins were subjected to an *in vitro* kinase assay using Hsp-25 as an exogenous substrate. Proteins were analyzed by SDS-PAGE, and phosphorylated Hsp-25 was detected by autoradiography.

57). It is also well established that one of the mechanisms of action of RA involves RA-dependent up-regulation of the transcriptional activator Stat1 (58–62). Such Stat1 up-regulation may be particularly important for the induction of the synergistic effects of RA and type I interferons (59–61), which also activate the p38 MAP kinase pathway (42–44). In addition, there is recent evidence that p38 may be required for serine phosphorylation of Stat-1 in response to various stimuli (63–65). We therefore sought to determine whether inhibition of RA-dependent p38 activation abrogates RA-dependent gene transcription, as well as serine phosphorylation and/or up-regulation of Stat1 protein expression.

We first performed studies to determine whether SB203580, which selectively blocks p38 activation, abrogates gene transcription via RARE elements. MCF-7 cells were transfected with a plasmid containing an RARE-luciferase construct and treated with RA in the presence or absence of the SB203580 inhibitor. The RA-dependent induction of luciferase activity was subsequently determined. As expected, RA treatment of cells resulted in a significant increase in luciferase activity (Fig. 7*A*). Treatment of cells with SB203580 did not abrogate such RA-dependent induction of luciferase activity (Fig. 7*A*), indicating that the function of the p38 pathway is not required for RA-induced transcriptional regulation. We also determined whether p38 inhibition blocks up-regulation of Stat1 protein expression by RA. NB-4 cells were incubated with RA for 48 h,
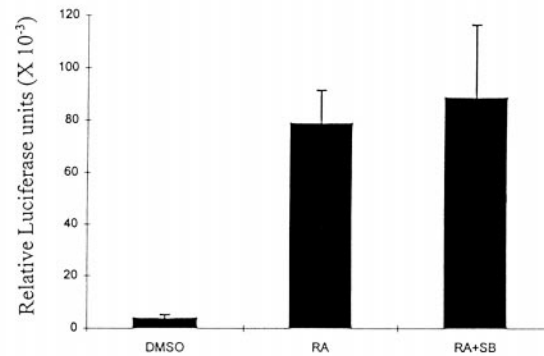
RA Activates the p38 MAP Kinase

A

B

C

FIG. 7. **The p38 MAP kinase is not required for RA-dependent gene transcription via RARE elements or up-regulation of Stat1 protein expression.** *A,* MCF-7 cells were transfected with an RARE-luciferase construct. 48 h after transfection the cells were incubated for 12 h with either Me_2SO (*DMSO*, control) or with RA (1 μM), in the presence or absence of SB203580 (10 μM), as indicated. The cells were then harvested and assayed for luciferase activity. The data are expressed as relative luciferase units, normalized for β-galactosidase activity. Means ± S.E. of three independent experiments are shown. *B,* NB-4 cells were incubated for the indicated times at 37 °C. Equal amounts of total cell lysates (100 μg/lane) were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of Stat1 on serine 727 (anti-Ser-727Stat1). *C,* the blot shown in *B* was stripped and reprobed with an antibody against Stat1.
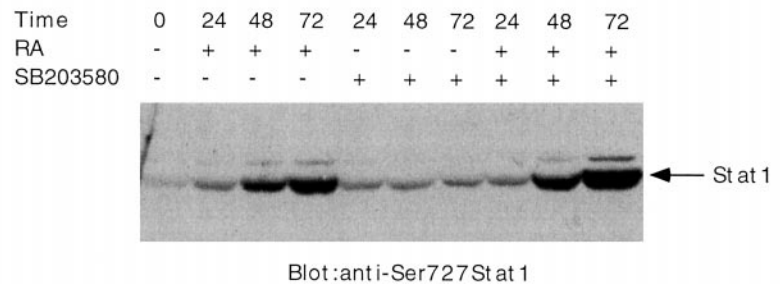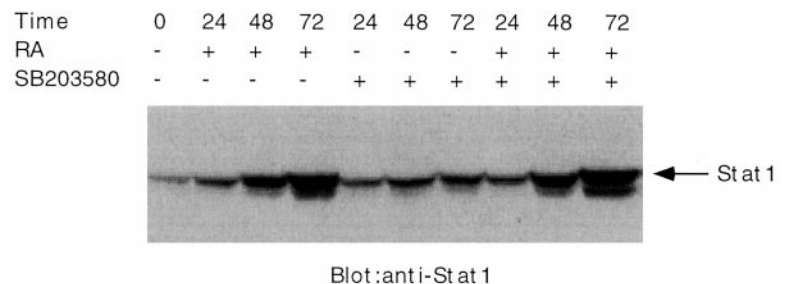
and the cells were lysed, and total cell lysates were analyzed by SDS-PAGE and immunoblotted with a monoclonal antibody against the serine-phosphorylated form of Stat1 on Ser-727 (Fig. 7*B*) or against Stat1 (Fig. 7*C*). Consistent with previous reports (59–62), significantly higher levels of Stat1 were detectable in the RA-treated samples (Fig. 7*C*). Also, there was an increase in the level of Stat1 phosphorylated on Ser-727 after treatment of cells with RA (Fig. 7*B*). However, treatment of the cells with SB203580 did not affect Stat1 protein expression (Fig. 7*C*) or Stat1 serine phosphorylation (Fig. 7*B*), indicating that the p38 pathway does not exhibit regulatory effects on RA-induced up-regulation of Stat1.

We subsequently determined the effects of inhibition of p38 activation on the induction of RA-dependent cell differentiation of NB-4 cells. Cells were treated with RA in the presence or absence of SB203580, and the induction of differentiation was determined by staining the cells with the CD11b antibody, whose expression is a marker for RA-induced myeloid differen-

tiation. As shown in Fig. 8, RA treatment induced up-regulation of CD11b expression. Surprisingly, concomitant treatment with SB203580 strongly enhanced RA-dependent CD11b expression (Fig. 8). To confirm that the p38 pathway plays a negative regulatory role on the induction of cell differentiation, another specific p38 inhibitor, SB202190, was studied. Consistent with the effects obtained using SB203580, treatment of cells with SB202190 strongly enhanced the induction myeloid differentiation of NB-4 cells in response to RA (Fig. 8). On the other hand, inhibition of ERK kinase activation using the PD98059 inhibitor abrogated RA-dependent up-regulation of CD11b (Fig. 8), consistent with previous reports (20). Thus, in contrast to the function of the ERK pathway that exhibits positive regulatory effects on RA-mediated myeloid cell differentiation (20), the p38 pathway antagonizes such effects.

In further studies, we sought to identify the functional role of the p38 MAP kinase pathway in the generation of the growth inhibitory effects of RA on NB-4 cells. Cells were treated with
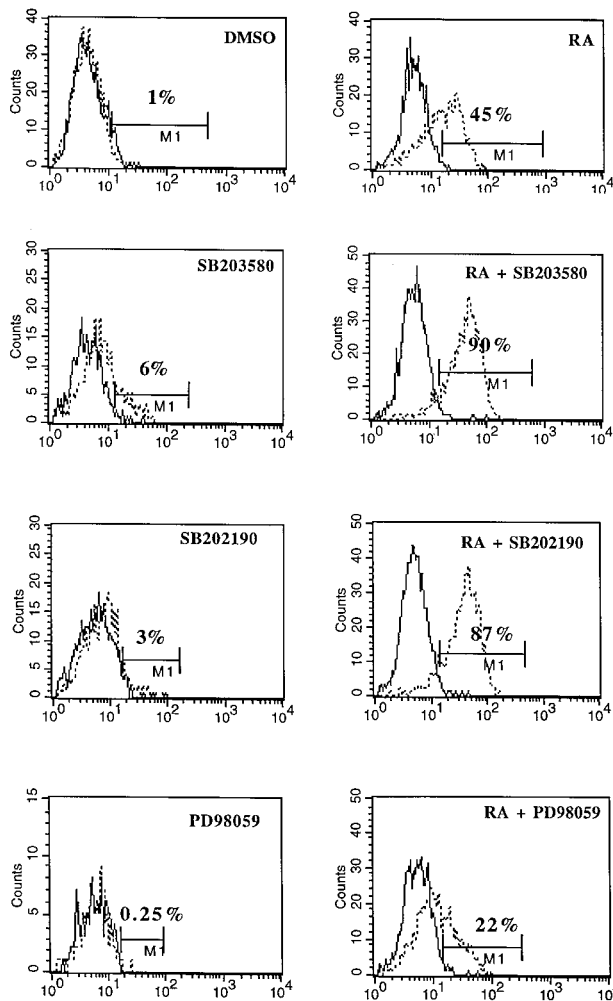
FIG. 8. **Inhibition of p38 MAP kinase activation enhances RA-induced differentiation.** NB-4 cells were incubated with RA for 5 days in the presence or absence of SB203580 or SB202190 or PD98059, as indicated. The cells were subsequently stained with a fluorescein isothiocyanate-conjugated anti-CD11b antibody and analyzed by flow cytometry. *Solid lines* indicate cells labeled with isotype control. *Dotted lines* indicate cells labeled with anti-CD11b. The percentage of cells positive for CD11b is indicated for each condition. *DMSO*, Me₂SO.

FIG. 9. **Inhibition of the p38 MAP kinase pathway enhances RA-dependent inhibition of cell proliferation.** NB-4 cells were incubated with or without RA for 5 days, in the presence or absence of SB203580 at concentrations of either 5 (*SB 5*) or 10 μM (*SB 10*), and cell proliferation was assessed using an MTT assay. Means ± S.E. of two independent experiments are shown.

RA in the absence or presence of SB203580, and cell proliferation was assessed using an MTT assay. As expected, RA diminished cell proliferation of NB-4 cells (Fig. 9). Concomitant treatment of cells with SB203580 at doses of 5 or 10 μM further enhanced the growth inhibitory effects of RA on these cells, whereas treatment with SB203580 alone had no significant effect on cell growth (Fig. 9). Thus, in addition to regulating RA-dependent cell differentiation, the p38 pathway appears to play a negative regulatory role in the generation of growth inhibitory responses by RA on target cells.

DISCUSSION

The family of MAP kinases includes the ERK, JNK, and p38 kinases (reviewed in Ref. 66). These kinases are activated in response to a variety of stimuli and mediate signals important for the generation of various biological responses (66). Two members of this family, ERK2 and JNK, have been previously shown to be involved in the generation of retinoid responses. ERK2 is activated in response to all-*trans*-retinoic acid treatment in the HL-60 acute myelogenous leukemia cell line (20), and such an activation is apparently required for the induction of RA-dependent cell differentiation and growth arrest. This has been established by studies demonstrating that treatment
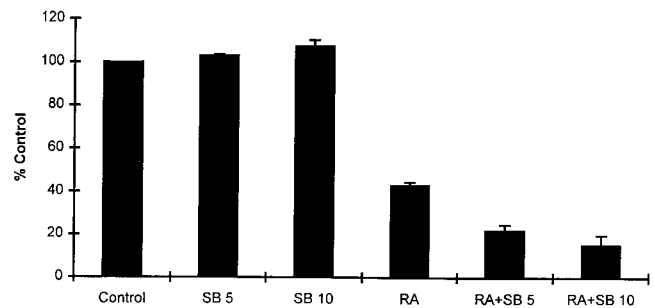
of cells with a specific inhibitor of ERK2 activation, PD98059, reverses the effects of RA on these cells (20). Other studies have established that RA inhibits JNK kinase-dependent signaling pathways (19) via activation of the MAP kinase phosphatase-1 (MKP-1) and abrogation of MKK4 activity (67). Although the precise functional role of inhibition of JNK kinase activity by RA is unknown, it has been proposed that this inhibition may mediate the suppression of c-Fos expression by RA to facilitate growth inhibition (19).

In the present study we provide the first evidence that the p38 MAP kinase is activated by all-*trans*-retinoic acid treatment of target cells. Our data clearly establish that RA treatment induces phosphorylation of p38 and induction of its kinase activity in two RA-sensitive cell lines, NB-4 and MCF-7. Furthermore, they demonstrate that the small G-protein Rac1 is activated by RA and that its function is essential for RA-induced p38 activation. The activation of Rac1 and p38 eventually leads to engagement of the MAPKAPK-2 kinase, which functions as a downstream effector for this pathway.

The engagement of the p38 signaling cascade by all-*trans*-retinoic acid is of considerable interest, as the function of this pathway is critical for the generation of signals required for important biological activities in response to stress and/or engagement of certain cytokine receptors. These include activation of transcription factors (33, 34), transcriptional regulation (42–44, 54), induction of cytokine production (33, 34), platelet aggregation (35), and induction of erythroid cell differentiation (68, 69). In addition, recent evidence has suggested that p38 may function as serine kinase for Stat proteins in certain systems (62–64). Therefore, our finding that the p38 pathway is activated during all-*trans*-retinoic acid treatment of cells led us to further studies to determine the functional role of this kinase in the induction of RA responses. Our results indicate that pharmacologic blockade of p38 activation does not inhibit RARE-dependent gene transcription and, on the contrary, exerts a slight stimulatory effect. Consistent with this, p38 inhibition does not abrogate RA-induced up-regulation of Stat1 protein expression or serine phosphorylation of Stat-1 on Ser-727. Thus, our data clearly establish that the RA-dependent activation of the p38 MAP kinase pathway is not a positive regulator of gene transcription.

Surprisingly, we found that treatment of cells with the p38 inhibitors SB203580 or SB202190 strongly enhances RA-induced differentiation, whereas inhibition of ERK kinase activity using the PD98059 inhibitor partially reverses such differentiation, confirming that the ERK kinase pathway mediates such responses. The pyridinyl imidazole compounds SB203580 and SB202190 specifically inhibit p38 activation by binding to the ATP site and inhibiting its kinase activity (71). Both of

these inhibitors exhibit the same specificity and block activation of the p38 (p38α) and p38β isoforms but not p38γ and p38δ (25, 27, 32, 71). Thus, it is possible that, in addition to p38α, p38β is also activated by RA and exhibits negative regulatory effects on differentiation, although this needs to be determined in future studies. The specificity of our findings is also established by the fact that another MAP kinase inhibitor, PD98059, which inhibits activation of ERK kinases, but not p38, blocks RA-induced differentiation. Our data also implicate p38 as a negative regulator on the RA-induced growth inhibitory activities and such a finding is consistent with its effects on RA-induced cell differentiation, as differentiated cells exhibit lower proliferation potential.

Altogether, our data implicate the activation of the p38 pathway by RA treatment in the negative regulation of induction of differentiation and growth inhibition. Such a function for p38 may represent a novel mechanism to control the rate of RA-dependent differentiation and apoptosis of normal cells, also conserved in malignant cells. However, the precise signals downstream of p38 that mediate such negative regulatory effects remain to be determined. It has been previously shown that the ERK and p38 pathways cross-talk and compete with each other for the induction of certain biological responses and that inhibition of ERK leads to p38 activation (72). Thus, the negative regulatory effects of p38 on RA-induced differentiation and growth inhibitory responses may be mediated via an antagonistic effect on ERK2, but this remains to be established in future studies. Recent studies have also shown that retinoic acid induces the up-regulation of mRNA for *blr*1, a chemokine receptor that promotes activation of ERK2 and RA-dependent cell differentiation (73). Thus, the negative regulatory effects of p38 activation on RA-induced responses may be mediated by down-regulation of *blr*1 expression. If this hypothesis proves to be correct, it would provide a mechanism by which the opposing effects of the ERK and p38 MAP kinases on the induction or RA-dependent cell differentiation occur.

Independent of the precise mechanisms involved, the finding that pharmacological inhibition of the p38 MAP kinase pathway enhances RA-dependent differentiation and growth arrest may prove of clinical value in the future. RA is used successfully in the treatment of acute promyelocytic leukemia and several other malignancies (1–4) but resistance to its effects eventually develops in nearly all cases. It is conceivable that combined use of RA with pharmacologic agents that inhibit p38 may enhance its differentiating and growth inhibitory effects *in vivo*. Further studies along these lines may provide important insights on the mechanisms by which all-*trans*-retinoic acid and other retinoids generate their biological effects, and provide the basis for the rational development of novel therapeutic approaches in RA-responsive malignancies.

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Activation of Rac1 and the p38 Mitogen-activated Protein Kinase Pathway in Response to All- *trans*-retinoic Acid

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