Ras activates three mitogen-activated protein kinases (MAPKs) including ERK, JNK, and p38. Whereas the essential roles of ERK and JNK in Ras signaling has been established, the contribution of p38 remains unclear. Here we demonstrate that the p38 pathway functions as a negative regulator of Ras proliferative signaling via a feedback mechanism. Oncogenic Ras activated p38 and two p38-related activated protein kinases, MAPK-activated protein kinase 2 (MK2) and p38-related/activated protein kinase (PRAK). MK2 and PRAK in turn suppressed Ras-induced gene expression and cell proliferation, whereas two mutant PRAKs, unresponsive to Ras, had little effect. Moreover, the constitutive p38 activator MKK6 also suppressed Ras activity in a p38-dependent manner whereas arsenite, a potent chemical inducer of p38, inhibited proliferation only in a tumor cell line that required Ras activity. MEK was required for Ras stimulation of the p38 pathway. The p38 pathway inhibited Ras activity by blocking activation of JNK, without effect upon ERK, as evidenced by the fact that PRAK-mediated suppression of Ras-induced cell proliferation was reversed by coexpression of JNKK2 or JNK1. These studies thus establish a negative feedback mechanism by which Ras proliferative activity is regulated via signaling integrations of MAPK pathways.

Ras regulates multiple signaling pathways (1). Of these the best described are the mitogen-activated protein kinases (MAPKs),1 including extracellular mitogen-regulated kinase (ERK), Jun amino-terminal kinase (JNK), also called stress-activated protein kinase, SAPK, and p38 (2). Through interaction with Raf proteins, Ras activates MEK1 and MEK2, which in turn activate ERK (3–5). Whereas Raf-independent transduction of Ras signaling was reported (6–7), the Ras/Raf/MEK/ERK pathway provides a common route by which signals from different growth factor receptors converge to activate major transcription factors such as AP1. Ras also activates JNK, which also plays a role in regulating AP1 activity (8). The critical role of the JNK pathway in Ras signaling is suggested by the observation that Ras only poorly transforms c-jun null cells (9) and blocking of the JNK pathway can inhibit Ras-induced transformation (10). The signaling pathway from Ras to JNK, however, is less clear, although it has been shown that Ras can directly interact with c-Jun proteins, JNK (11), and its upstream activating kinase (MEK1) (12). Most Ras signaling is believed to be transmitted via these two pathways (8). Ras can also stimulate p38, albeit less efficiently (13, 14). The biological consequence of p38 activation in Ras signaling transduction, however, remains unclear. It is likely that even this moderate activation could have an important impact on Ras signaling.

p38 is most strongly activated by proinflammatory cytokines and environmental stresses (15, 16). It is activated by the upstream kinases MKK3, MKK6 (14, 17, 18), and MKK4 (also called SEK1 and JNKK1). Several protein kinases have been identified as p38 physiological substrates, including MAPKAP-K2 (MK2) (19), MAPKAP-K3 (20), and the recently cloned PRAK (p38-related/activated protein kinase) (21). PRAK is a 471 amino acid protein with 20–30% sequence identity to other MAPK-regulated protein kinases (21). In addition to phosphorylation and activation of transcriptional factors, some of the p38 pathway-mediated effects may be mediated via these downstream molecules.

Whereas the biological consequence of p38 activation may vary under different situations, the following evidence suggests that it may possess anti-mitogenic activity. 1) Although p38 responds to a variety of extracellular stimuli, it is activated most strongly by proinflammatory cytokines (22–24). Consequently, the p38 activation may play a role in controlling proliferation within the immune system. 2) Activation of the p38 pathway was shown to inhibit cyclin D1 expression (25), which functions downstream of Ras in the direct control of cell proliferation (26). 3) The activated p38 pathway is known to phosphorylate and activate Hsp27 (heat shock protein) via MK2 in vivo (27, 28), which by itself can inhibit cell proliferation (29, 30). The signaling mechanisms for this anti-mitogenic effect of p38, however, remain unknown.

It is believed that MAPKs regulate target genes by phosphorylation and activation of a group of transcriptional factors such as AP1 and SRE (8, 31). Recent evidence suggests, however, that signals from three MAPK pathways can be integrated before reaching the transcriptional factors. Activation of the ERK pathway, for example, suppresses p38/JNK-induced apoptosis in PC12 cells (32). Inhibition of p38, on the other hand, enhances interleukin 1β-induced expression of the low density lipoprotein receptor, which is inhibited by the MEK inhibitor PD98059 (33). Cross-talk also exists between JNK and p38 pathways. JNK opposes the stimulatory effect of p38 activity on induction of atrial natriuretic factor expression (34).
Forced expression of constitutively active p38-activating kinase MKK6, however, overcomes apoptosis induced by an active MEKK1, a JNK kinase kinase, in myocardial cells (35). The possibility thus exists that the activation of p38 by Ras may be able to determine the output of Ras signaling through interaction with the ERK/JNK pathways. The purpose of this study is to determine how the activation of the p38 pathway may affect Ras proliferative signaling.

**EXPERIMENTAL PROCEDURES**

cDNA Constructs and Expression Plasmids—The HA epitope-tagged wild-type PRAK, the mutant PRAK (PRAK(182D)), and the dominant-negative form of PRAK (PRAK/KM) in pcDNA3 have been previously described (21). The HA-tagged wild-type as well as dominant-negative MKK6 (MKK6/2A), with two phosphorylation residues replaced by alanine, have been described previously (17). These fusion proteins were purified with glutathione-agarose beads bought from Sigma. Anti-HA (clone 12CA5) and anti-c-Myc were from Sigma. Protein G-Sepharose 4B and protein A-Sepharose 4B were obtained from Celox (Hopkins, MN) and Sigma, respectively. MBP for the JNK assay was provided by Santa Cruz Biotechnology, Inc. DNA was prepared using Endofree kit from QIAGEN and DNA sequencing was performed at Celox (Hopkins, MN) and Sigma, respectively. DNA was prepared using Endofree kit from QIAGEN and DNA sequencing was performed at Celox (Hopkins, MN) and Sigma, respectively.

**Microinjections**—Microinjections were performed into cells within a circuitous area marked at the time of injection. For stimulation, the manufacturer’s protocol for calcium phosphate-mediated transfection (Promega) was followed. To compare the effect of 7 μg/ml of plasmid DNA for the proliferation assay and of 20 μg/ml for a 100-mm dish for the kinase assay and flow cytometry analysis. The next day, DNA-containing medium was removed, and cells were allowed to grow in 0.5% fetal calf serum for 48 h before cells were processed for the different assays.

**Cell Proliferation Assay**—For staining and autoradiography, cells were plated on coverslips and transfected or microinjected as above. At 24 h after transfection, cells were fixed in methanol, washed with 3% bovine serum albumin in phosphate-buffered saline for 45 min. (All of the staining procedures were performed at room temperature.) Cells were then incubated with mouse anti-β-Gal (1:30) for 1 h in a humidified chamber and then with anti-mouse Cy3 (1:1000) for 45 min. The coverslips were mounted with Permount solution, air-dried, and exposed to autoradiography emulsion for at least 24 h. After development and fixation, transfected or microinjected cells were identified under a fluorescence microscope as β-Gal-positive, and thymidine-positive cells among the total β-Gal-positive cells were counted. For microinjection, all β-Gal positive cells within the circle were counted, and for transfection, at least 200 β-Gal positive cells were scored for the labeling index.

** Luciferase Assay**—Cells were transfected as above although 1 μg of Topo IIα and 2 μg of p5E or SRE luciferase constructs instead of the transfection marker were used with different cDNAs of interest or proper vectors. By the end of the experiments (48 h after DNA removal), cells were washed with phosphate-buffered saline and collected in 200 μl of reporter lysis buffer (Roche Molecular Biochemicals) with scraper. The lysates were incubated with 30 μl of protein A-Sepharose beads (21), washed with 3% bovine serum albumin in phosphate-buffered saline for 45 min, and 10 min. The rest of the procedures for staining and autoradiography were the same as for the other proliferation assays.

**p38 Inhibits Ras Activity By Negative Feedback**

NIH3T3 cell line was obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and antibiotics at 37 °C with 5% CO2. The NIH3T3 line stably transfected by v-ras was provided by Dr. Lowy and has been described previously (47, 48). J82 and T24 human bladder carcinoma cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and have been previously characterized in this laboratory for the dependence (T24) and independence (J82) of Ras activity for their proliferation (48, 49). Cells were plated at 1 × 10^5/ml in 10% fetal calf serum, Dulbecco’s modified Eagle’s medium 1 day before in 6-well plates for the proliferation assay and in 100-mm dishes for the kinase assay. For microinjection, cells were switched to serum-starved medium (0.5% fetal calf serum) for 48 h before the injection. For transfection, the manufacturer’s protocol for calcium phosphate-mediated transfection (Promega) was followed. Two DNA concentrations were used, one at 150 μg/ml, and another at 450 μg/ml of total DNA (with each at 50 and 150 μg/ml, respectively). Following injection, 5 μCi of [3H]thymidine was added to each 2-ml plate, and cells were grown in 0.5% fetal calf serum for another 24 h. Cells were then washed twice with phosphate-buffered saline and fixed in 100% methanol at room temperature for 10 min. The rest of the procedures for staining and autoradiography were the same as for the other proliferation assays.

**Other Reagents**—Dulbecco’s modified Eagle’s medium and calf serum were obtained from Celox (Hopkins, MN) and Sigma (Sigma), respectively. All other materials for cell culture were supplied by Life Technologies, Inc. DNA was prepared using Endofree kit from QIAGEN and DNA sequencing was performed at Celox Laboratories Inc. (Hopkins, MN) and Sigma, respectively. Human DNA was prepared using Endofree kit from QIAGEN and DNA sequencing was performed at Celox Laboratories Inc. (Hopkins, MN) and Sigma, respectively. Human DNA was prepared using Endofree kit from QIAGEN and DNA sequencing was performed at Celox Laboratories Inc. (Hopkins, MN) and Sigma, respectively.

**Immunoprecipitation**—Cells were transfected and then serum-starved as described above. In some experiments, cells were treated with 50 μM PD98059 for the final 48 h, or with 20 μg SB203580 for the final 24 h. For ERK, JNK, and p38 kinase assay, cells were collected in HEPES lysis buffer (10 mM HEPES, pH 7.4, 50 mM NaF, 1% Triton X-100, 0.1 mM Na3VO4, and 20 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) as previously published (51) and used in this laboratory (38). 5 μl of protein A-Sepharose beads for GST-tagged ERK or -JNK, or 20 μl of anti-FLAG M2 affinity gel for FLAG-tagged p38 incubated for 4–20 h at 4 °C on a rotating plate. For immunoprecipitation of transfected HA-PRAK or Myc-MK2, cells were lysed in 25 mM HEPES, pH 7.6 buffer containing 137 mM NaCl, 3 mM EDTA, 3 mM β-glycerophosphate, 1% Triton X-100, 0.1 mM Na3VO4, and 1 mM phenylmethylsulfonyl fluoride (21). The lysates were then incubated with 2 μg of anti-HA or anti-c-Myc monoclonal antibody for 3 h and for another hour after adding 30 μl of protein A-Sepharose beads (HA-PRAK) or 20 μl of protein G-Sepharose (Myc-MK2).

**Protein Kinase Assay**—The precipitates were then washed twice with respective lysis buffer and two times with kinase binding buffer (20 mM HEPES, pH 7.6, 50 mM NaCl, 0.05% Triton X-100, 0.1 mM Na3VO4, and 1 mM β-glycerophosphate, 20 mM MgCl2) and for 30 min at 37 °C with kinase reaction buffer (20 mM HEPES, pH 7.6, 20 mM MgCl2, 15 μM ATP, 20 mM β-glycerophosphate, 20 mM p-nitrophenylphosphate, 0.5 mM Na3VO4, 2 mM dithiothreitol) as described previously (38). 5 μCi of [3H]ATP and 1 μg of substrate protein were used for each sample. The substrate for the kinase reaction includes MBP for ERK, GST-Jun for JNK, ATP-2 for p38, HSP27 for PRAK and MK2. Following the reaction, an equal volume of 2X Laemmli buffer was added...
ERK, GST

GST bead or anti-FLAG precipitates by using MBP as a substrate for calculated based on phosphorimager quantitation.

GST-tagged p54SAPK(JNK) as described previously (36–38). As following transfection was determined by an antibody and the infected PRAK was then immunoprecipitated with anti-HA an-

genic Ras or a control vector into NIH 3T3 cells. The trans-

mimic activated PRAK (21), were cotransfected with the onco-

(PRAK/182D) in which Thr-182 was changed to aspartic acid to 
PRAK kinase activity, an HA epitope-tagged wild-type PRAK, 

regulated protein kinase PRAK was first analyzed. To assess 

on p38 downstream kinases was then investigated. The p38-

MAPKs in NIH 3T3 cells, although the effect on p38 is less than 

those reported from other laboratories (13–14).

Western blot analysis using corresponding specific antibodies. 

transfected eiptope-tagged plasmids in every case was confirmed by 

expression of the transfection and the determination of the protein kinase activity 

were repeated two to three times with similar results. Expression of the 

added to stop the reaction. The phosphorylated proteins were then 

separated in a 12.5% SDS-polyacrylamide gel. The gels were then dried, scanned, and quantitated in a phosphorimager. For each kinase assay, 

the transfection decreased Ras-induced proliferation by about 40%. If 

cotransfection with Myc-tagged MK2 with Ras, MKK6/2E, or a control vector, and the anti-FLAG immu

neproteins were used to determine the kinase activity with HSP27 as 

substrate.

RESULTS

Ras Activates the p38 Pathway—To study interactions be-

between Ras proliferative signaling and p38, effects of oncogenic 

Ras on p38 activity were first examined by transient transfection 

assay. For this purpose, p38 kinase activity was assessed by cotransfection of FLAG-tagged wild-type p38a together with oncogenic Ras (Ha-Ras, Leu-61) or a control vector. The trans-

fected p38 was then immunoprecipitated with an anti-FLAG 

(M2) antibody, and the in vitro kinase activity was determined 

using ATP2 as a substrate (18). The ERK and JNK activities 

were also assayed under similar experimental conditions in 

which Ras was cotransfected with GST-tagged p42MAPK(ERK) 

or GST-tagged p54SAPK(JNK) as described previously (36–38). As 

shown in Fig. 1, Ras was indeed able to activate all three 

MAPKs in NIH 3T3 cells, although the effect on p38 is less than 

with the other two kinases. These results are consistent with 

those reported from other laboratories (13–14).

To extend the observation with p38 kinase, the effect of Ras 

on p38 downstream kinases was then investigated. The p38-

regulated protein kinase PRAK was first analyzed. To assess 

PRAK kinase activity, an HA epiotpe-tagged wild-type PRAK, 

a dominant-negative form of PRAK (PRAK/KM) and a mutant 

(PRAK/182D) in which Thr–182 was changed to aspartic acid to 
mimic activated PRAK (21), were cotransfected with the oncogenic 

Ras or a control vector into NIH 3T3 cells. The trans-

fected PRAK was then immunoprecipitated with anti-HA 

antibody and the in vitro kinase activity was assayed by using 

HSP27 as a substrate (21). As shown in Fig. 2A, Ras activated 

PRAK by 19-fold, whereas it had no effect on the mutant 

PRAK/182D, even though this mutant shows some intrinsic 

kinase activity in vitro (21). Similarly, Ras failed to stimulate 

the dominant-negative PRAK (PRAK/KM, data not shown).

As a positive control, MKK6/2E, a constitutively active form of MKK6 (p38-activating kinase) (18), also increased PRAK activity (Fig. 2A, lower), as previously observed (18, 21). This activation was increased by coexpression of wild-type p38, indicating that endogenous p38 is limiting in concentration in these cells. The MKK6/2E-mediated PRAK activation was sen-

sitive to the pyridinyl imidazole derivative SB203580, a specific 
inhibitor of p38 (16, 21). Replacement of wild-type p38 kinase 

with a mutant p38 (p38/AGF) in which the dual phosphoryla-

tion motif Thr–Gly–Tyr was changed to the phosphorylation-

defective motif Ala–Gly–Phe (18) can also block MKK6-induced 
PRAK activation. These results further demonstrated that 

MKK6 activates PRAK via phosphorylation of p38 as described 

previously (21). Similar to Ras, MKK6/2E failed to activate 
PRAK/182D in vivo (Fig. 2A), indicating that residue Thr–182 is 
critical to activation as described previously (21).

Ras also activated MK2 (Fig. 2B), another p38 activated pro-

tein kinase, as demonstrated by cotransfection of Myc-tagged 

MK2 with Ras followed by in vitro kinase assay. MK2 was first 

identified as an ERK-regulated kinase (19) and was later found 
to respond most strongly to p38 (39, 52). As a positive control, 

MK2 is activated by MKK6/2E, which is enhanced by cotransfec-

tion with a wild-type p38 but completely inhibited by the mutant 
p38 (Fig. 2B). These results, therefore, confirm that MK2 func-

tions downstream of p38 in NIH 3T3 cells. Thus, our results 
demonstrate that Ras has the ability to stimulate the p38 path-

way in NIH 3T3 cells with its downstream kinases PRAK and 

MK2 stimulated more strongly than p38 itself.

Inhibition of Ras-induced Proliferation by the p38 Pathway—

Experiments were subsequently carried out to investigate the 
effect of p38 activity upon the biological consequences of Ras 

signaling. For this purpose, the ability of Ras to induce prolifer-

ation was assessed in the presence or absence of activated p38. A 

Marker plasmid, pSV-β-galactosidase (β-Gal) was included in 

transfections with Ras and p38 to identify positively transfected 

cells with a fluorescent antibody stain against β-Gal. Following 

transfection, serum was removed (0.5% fetal calf serum for 48 h), 

and cells were pulsed with [3H]thymidine for the final 24 h to 

identify proliferating cells (38). As shown in Fig. 3A, Ras led to a 

3-fold increase in the thymidine-labeling index over the vector 

control. The presence of a p38-expressing plasmid in the cotransfec-

tion decreased Ras-induced proliferation by about 40%. If 

cotransfected with p38, however, the ability of Ras to stimulate proliferation was essentially
p38 Inhibits Ras Activity By Negative Feedback

Fig. 3. Inhibition of Ras-induced proliferation by the p38 pathway. Cells were transfected (A and B) or microinjected (C) (total DNA concentration: 150 μg/ml) with a plasmid expressing β-galactosidase together with the indicated plasmids, and the serum removed for 48 h, the final 24 h of which were in the presence of [3H]thymidine. The transfected cells were identified by immunostaining against cotransfected β-Gal, and the thymidine labeling index from at least 200 β-Gal-positive cells was then determined by autoradiography. Results shown are mean of three experiments ± S.D.

Inhibition of Thymidine Incorporation in Ras-mutated Tumor Cells by the p38 Pathway—To assess the physiological relevance of inhibition of Ras-dependent proliferation and gene expression by the p38 pathway, experiments were carried out to assess effects of p38 activation on proliferation of tumor cells with or without a requirement for Ras activity. Human bladder carcinoma cell lines J82 and T24 (with Ha-Ras mutation) were used in this analysis. These have been previously shown to

Table 3. Inhibition of Ras-induced proliferation by the p38 pathway.

| Treatment     | Vector | Ras | p38 | p38/AGF | MKK6/2E | PRAK | PRAK/182D | PRAK/KM | MK2 |
|---------------|--------|-----|-----|---------|---------|------|-----------|---------|-----|
| Thymidine Labelling Index |          |     |     |         |         |      |           |         |     |
| Vector        | X      | X   | X   | X       | X       |      |           |         |     |
| Ras           | X      | X   | X   | X       | X       |      |           |         |     |
| p38           | X      | X   | X   | X       | X       |      |           |         |     |
| p38/AGF       |        |     |     |         |         |      |           |         |     |
| MKK6/2E       |        |     |     |         |         |      |           |         |     |
| PRAK          |        |     |     |         |         |      |           |         |     |
| PRAK/182D     |        |     |     |         |         |      |           |         |     |
| PRAK/KM       |        |     |     |         |         |      |           |         |     |
| MK2           |        |     |     |         |         |      |           |         |     |

blocked. Thus, the moderate activation of p38 by Ras (Fig. 1A) could apparently exhibit some inhibitory effect on Ras proliferative activity, and this suppression became highly efficient when the p38 pathway was exogenously activated. Moreover, the ability of MKK6/2E to inhibit proliferation was reversed in the presence of the kinase-inactive p38 (p38/AGF) (18), confirming the importance of signaling through p38 that leads to inhibition of Ras-induced proliferation (Fig. 3A).

The ability of the targets of p38 activity, PRAK and MK2, to block the proliferative activity of Ras was next determined. Both these p38-activated kinases almost completely inhibited Ras-induced proliferation (Fig. 3B), although these proteins had little effect upon the rate of proliferation in the absence of Ras induction (Fig. 9 and data not shown). Importantly, neither the mutant PRAK/182D, which was not activated by Ras, nor the dominant-negative PRAK/KM had inhibitory effect upon Ras-induced proliferation. These results were confirmed with flow cytometry. In this case pCMV-CD20 was cotransfected as a marker so that transfected cells could be identified by their expression of CD20. These cells were identified by flow cytometry, and their DNA content was measured. In this way PRAK was shown to reduce the percentage of S-phase cells following cotransfection with oncogenic ras by 80%, whereas the negative control, PRAK/182D had little effect (data not shown). These results further established the inhibitory role of members of the p38 pathway in Ras mitogenic signaling.

To overcome low efficiency of transient transfection (typically about 12% in these cells), microinjection (50) was used to further verify these cotransfection data. In this case pCMV-β-Gal was co-microinjected as a marker. Cells were first stained with mouse-monoclonal antibody against β-Gal to identify successfully injected cells and then processed for autoradiography. Normally, about 30 to 40% of injected cells expressed β-Gal. The results (Fig. 3C) show that PRAK can efficiently suppress Ras-induced thymidine incorporation, whereas mutants PRAK/182D and PRAK/KM again showed little effect. These results, obtained independently of cotransfection, further confirm the inhibitory role of PRAK in Ras-induced proliferation.

Inhibition of Ras-dependent Gene Expression by the p38 Pathway—To confirm and extend the above results, the effects of p38 activity upon Ras-induced gene expression were next analyzed. The AP1 and SRE activity has been well characterized with regard to induction by Ras signaling (8, 53). Ras-dependent gene expression was therefore assessed with the luciferase gene driven by the minimal c-fos promoter (Δ56FosdE) containing an additional three AP1 binding sites (AP1) (43), or one containing five additional copies of the SRE sequence (SRE) (44, 45). As shown in Fig. 4, p38 alone inhibits Ras-induced AP1 and SRE activity by approximately one-half (Fig. 4, A and C). The SRE inhibition was increased dramatically when the p38-activating kinase MKK6/2E was added together with p38. As with cell proliferation, the effect of MKK6/2E was p38-dependent, because replacement of the p38 with p38/AGF reversed the suppression by MKK6/2E (Fig. 4, A and C). Moreover, MK2 and PRAK also have the ability to inhibit Ras-induced AP1- and SRE-dependent (B and D, respectively) gene expression, whereas PRAK/182D or PRAK/KM have little (AP1) or moderate (SRE) effects (Fig. 4, B and D). These results thus clearly demonstrate that the p38 pathway is suppressive for Ras-induced AP1- and SRE-dependent gene expression and indicate that the inhibitory activity of the p38 pathway may occur prior to the activation of AP1 or SRE.

The promoter for DNA Topo IIα was next utilized to determine the effects of p38 signaling upon the ability of Ras to stimulate a native promoter element. Topo IIα is considered a Ras-responsive gene because Ras oncogenic transformation increases Topo IIα RNA and protein (54), and because our previous work demonstrated that Ras stimulates the Topo IIα promoter independently of cell cycle progression (38). As with AP1 and SRE, p38 plus the constitutively active p38 activator MKK6/2E completely blocked the Ras-induced Topo IIα gene expression, which was reversed after replacing p38 with the mutant counterpart (p38/AGF) (Fig. 5, left). The inhibition of the Topo IIα promoter was also apparent when PRAK and MK2 were used in the cotransfection, whereas PRAK/KM or PRAK/182D again showed little effect (Fig. 5, right). These results thus establish an inhibitory role of the p38 pathway in Ras signaling to a target gene.

Inhibition of Thymidine Incorporation in Ras-mutated Tumor Cells by the p38 Pathway—To assess the physiological relevance of inhibition of Ras-dependent proliferation and gene expression by the p38 pathway, experiments were carried out to assess effects of p38 activation on proliferation of tumor cells with or without a requirement for Ras activity. Human bladder carcinoma cell lines J82 and T24 (with Ha-Ras mutation) were used in this analysis. These have been previously shown to
proliferate in either a Ras-dependent (T24) or in a Ras-inde-
pendent (J82) manner (48, 49). The stress-inducer arsenite was
used here to activate endogenous p38 because it is one of most
potent p38 stimuli (51, 52) and has been demonstrated to
activate several kinases in a MKK6/p38-dependent manner
(55, 56). Following a 30-min exposure to arsenite, the endoge-
nous p38 activity was increased by 112- and 44-fold in J82 and
T24 cells, respectively (Fig. 6). Interestingly, this treatment
inhibited thymidine incorporation in T24 cells ($p < 0.02$) but
not in J82 cells (Fig. 6). Despite the fact that arsenite may
activate more than one MAPK pathway, its effect on p38 ap-
pears stronger (55, 56), and consequently the p38 induction
might be more relevant to biological responses. Whereas in-
volution of additional signaling pathways in this process
cannot be ruled out, the fact that the selective inhibition of
thymidine incorporation was seen only in the Ras-dependent
T24 cells, even though p38 was activated only to about 40% of
the level of that seen in J82 cells, strongly suggests the sup-
pressive role of the p38 pathway in endogenous Ras activity.
This observation was confirmed by transfection experiments.
The p38 pathway was activated in tumor cells by transfection
with the constitutively active MKK6 (MKK6/2E) together with
p38, and a β-Gal expression plasmid as a marker. Once again,
positively transfected cells were identified by expression of
β-Gal as described in Fig. 3. Twenty-four hours following trans-
fecition into the Ras-independent J82 cells, the thymidine la-
beling pattern was essentially identical to that seen in cells
transfected with a control plasmid. Activation of the p38 path-
way thus had no effect upon proliferation in these cells. On the
other hand, the activation of the p38 pathway in the Ras-de-
pendent T24 cells reduced thymidine incorporation from 50 to
80% in different experiments. These transfections to activate
the p38 pathway were also inhibitory to the proliferation of
NIH3T3 cells stably transformed by oncogenic HaRas (85% in-
hibition). These results, therefore, confirm that the p38 path-
way is only inhibitory in malignant cells that require Ras
activity for proliferation.

### Requirement of MEK and MKK6 for Ras Signaling to the p38 Pathway—

The results above demonstrated that molecules of the p38 pathway (p38, MK2, and PRAK) are activated by oncogenic Ras and have the ability to suppress Ras signaling. Experiments were next designed to investigate how Ras signals to the p38 pathway. Ras signaling is to a large extent trans-
duced downstream through MEK (1, 8). MKK6, on the other
hand, activates for all known p38 isoforms selectively (17, 18).
p38 Inhibits Ras Activity By Negative Feedback

We, therefore, examined whether Ras requires these two molecules to activate p38. A dominant-negative form of MEK1 (MEK/2A) and of MKK6 (MKK/6/2A) were used in the cotransfections with Ras, together with FLAG-tagged p38. Interestingly, both MEK/2A and MKK/6/2A completely blocked p38 activation by Ras (Fig. 7A). The inhibitory effect of MEK/2A was further confirmed by the MEK-specific inhibitor PD98059 (57). No inhibitory effect upon Ras-induced p38 activation, however, was found when a dominant-negative SEK (SEK/AL) was used to block JNK activation (data not shown). The selective inhibition of Ras-activated ERK by MEK/2A (but not by SEK/AL), and that of Ras-stimulated JNK by SEK/AL (but not by MEK/2A) has been demonstrated previously in these cells (38) and is illustrated again in Fig. 8. These experiments thus demonstrate the requirement of both MEK and MKK6 for Ras activation of p38.

To determine whether MEK or MKK6 alone is sufficient to activate p38 in this cell line, effects of the constitutively active MEK1 (MEK/2E) or MKK6 (MKK/6/2E) on all three MAPKs were assessed. MEK/2E selectively activates ERK but not JNK, whereas MKK/6/2E has no effect on either ERK or JNK (data not shown). With regard to p38 stimulation, only MKK/6/2E but not MEK/2E was active (Fig. 7A). These results thus demonstrate that MEK was only necessary for Ras stimulation of p38, whereas MKK6 was both necessary and sufficient for p38 activation.

The next experiment was carried out to examine the relationship between MEK and MKK6 during Ras signaling. A FLAG-tagged MKK6 was cotransfected with Ras in the presence or absence of dominant inhibitory MEK/2A, and MKK6 activity was assessed in immunoprecipitates using GST/p38/KM as a substrate (17). Ras was shown to stimulate MKK6, which was blocked by the dominant-negative MEK (Fig. 7B). These results indicate that the signal from Ras to MKK6 requires MEK.

Because Ras requires MEK to stimulate MKK6 (Fig. 7B) and p38 (Fig. 7A), and because both PRAK and MK2 function downstream of p38 (Fig. 2), interruption of Ras signaling at either MEK or p38 would be expected to interfere with Ras signaling to these two p38-activated protein kinases. To test this possibility, dominant-negative MEK (MEK/2A) and a kinase-inactive form of p38 (p38/AGF) were examined for their effects on Ras activation of PRAK or MK2. These inhibitory plasmids were cotransfected with Ras, together with HA-tagged PRAK, or Myc-tagged MK2. Immunoprecipitates from the resulting cells were then used to assess the kinase activity of PRAK or MK2 as described in Fig. 2. As expected, either MEK/2A or p38/AGF abolished Ras-induced PRAK activation, and the inhibitory effect of MEK/2A was further confirmed with the MEK inhibitor PD98059 (Fig. 7C). A similar effect was also seen with MK2 activation (Fig. 7D). Taken together, these results demonstrate that Ras requires MEK to stimulate each member of the MKK6/p38/PRAK/MK2 pathway.

Involvement of the JNK Pathway in the Inhibition of Ras Proliferative Signaling by the p38 Pathway—The above data demonstrate that Ras activity stimulates the p38 pathway, and that the resulting activity of p38 and its targets provide a negative feedback to the biological effects of Ras signaling. A final analysis was made to determine how p38 signaling was able to block the biological consequences of Ras activity. Studies focused on the ERK and JNK pathways because both are known to be essential for Ras mitogenic signaling (3, 10), and because antagonism between p38 and JNK/ERK has been previously described (25, 32). As shown in Fig. 8, both PRAK and MK2 displayed only a slight effect on Ras-stimulated ERK activity. The inhibition was similar to that seen with negative controls SEK/AL and PRAK/182D (50–60% inhibition). In contrast, the positive control MEK inhibitor (MEK/2A) (38) completely blocked Ras-induced ERK activation.

The next experiment was carried out to examine the relationship between MEK and MKK6 during Ras signaling. A FLAG-tagged MKK6 was cotransfected with Ras in the presence or absence of dominant inhibitory MEK/2A, and MKK6 activity was assessed in immunoprecipitates using GST-p38/KM as a substrate (17). Ras was shown to stimulate MKK6, which was blocked by the dominant-negative MEK (Fig. 7B). These results indicate that the signal from Ras to MKK6 requires MEK.

Because Ras requires MEK to stimulate MKK6 (Fig. 7B) and p38 (Fig. 7A), and because both PRAK and MK2 function downstream of p38 (Fig. 2), interruption of Ras signaling at either MEK or p38 would be expected to interfere with Ras signaling to these two p38-activated protein kinases. To test this possibility, dominant-negative MEK (MEK/2A) and a kinase-inactive form of p38 (p38/AGF) were examined for their effects on Ras activation of PRAK or MK2. These inhibitory plasmids were cotransfected with Ras, together with HA-tagged PRAK, or Myc-tagged MK2. Immunoprecipitates from the resulting cells were then used to assess the kinase activity of PRAK or MK2 as described in Fig. 2. As expected, either MEK/2A or p38/AGF abolished Ras-induced PRAK activation, and the inhibitory effect of MEK/2A was further confirmed with the MEK inhibitor PD98059 (Fig. 7C). A similar effect was also seen with MK2 activation (Fig. 7D). Taken together, these results demonstrate that Ras requires MEK to stimulate each member of the MKK6/p38/PRAK/MK2 pathway.

Involvement of the JNK Pathway in the Inhibition of Ras Proliferative Signaling by the p38 Pathway—The above data demonstrate that Ras activity stimulates the p38 pathway, and that the resulting activity of p38 and its targets provide a negative feedback to the biological effects of Ras signaling. A final analysis was made to determine how p38 signaling was able to block the biological consequences of Ras activity. Studies focused on the ERK and JNK pathways because both are known to be essential for Ras mitogenic signaling (3, 10), and because antagonism between p38 and JNK/ERK has been previously described (25, 32). As shown in Fig. 8, both PRAK and MK2 displayed only a slight effect on Ras-stimulated ERK activity. The inhibition was similar to that seen with negative controls SEK/AL and PRAK/182D (50–60% inhibition). In contrast, the positive control MEK inhibitor (MEK/2A) (38) completely blocked Ras-induced ERK activation. The in vitro kinase assays were performed as described previously with MBP as the substrate for ERK and GST-Jun for JNK. PRAK/KM was shown in independent experiments to have little effect on either ERK or JNK activity (data not shown).

FIG. 7. Signaling components required for Ras stimulation of the p38 pathway. A, Ras requires MEK and/or MKK6 to stimulate p38. Cells were cotransfected with various plasmids as indicated, together with FLAG-tagged p38. The kinase activity of the transfected p38 was determined 48 h after the transfection in immunoprecipitates as described above. 50 μg PD98059 was added in one set of determinations to confirm the results with MEK/2A. B, Ras activates MKK6 in a MEK-dependent manner. Cells were transfected with various combinations of plasmids of interest, together with FLAG-MKK6 for MKK6 assay, with the FLAG-tagged constitutively active MKK6/2E included as a positive control of MKK6 activity. C, Ras requires MEK and p38 to stimulate PRAK. D, Ras requires MEK and p38 for stimulation of MK2.

FIG. 8. Inhibition of Ras-induced JNK but not ERK by PRAK and MK2. Cells were transfected with various combinations of plasmids as indicated, together with GST-tagged p42MAPK for ERK assay, or GST-tagged p54SAPK for JNK assay. The dominant-negative SEK (SEK/AL) was included as a control for the JNK suppression, whereas the inhibitory MEK (MEK/2A) served as a control for the ERK inhibition. The in vitro kinase assays were performed as described previously with MBP as the substrate for ERK and GST-Jun for JNK. PRAK/KM was shown in independent experiments to have little effect on either ERK or JNK activity (data not shown).
p38 Inhibits Ras Activity By Negative Feedback

DISCUSSION

Previous studies have shown that both the ERK and JNK pathways are essential for Ras-induced proliferation and transformation (9, 10, 58), while the role of the p38 MAP kinase pathway is less clearly understood (59). Our results here demonstrate that the p38 pathway functions as a negative regulator in Ras proliferative signaling by a feedback mechanism. This conclusion is based on the observation that Ras activates each member of the p38 pathway beginning with its upstream activating kinase MKK6 and including two targets of p38 activity, PRAK and MK2. Once activated, each of these molecules was shown to inhibit Ras-dependent gene expression and Ras-induced proliferation. The inhibitory activity of the p38 pathway upon proliferation was then demonstrated in tumor cells containing endogenous Ras mutations. This was the case whether the p38 activation was achieved chemically or by transfection of appropriate plasmids. No such inhibition was observed in tumor cells that do not require Ras activity for proliferation. The intact p38 pathway is required for this inhibitory activity, as the kinase inactive p38 (p38/AGF) or a chemical p38 inhibitor blocked activation of downstream targets and blocked the inhibitory effects described above.

Our results further demonstrated that the p38 pathway may suppress Ras proliferative signaling by inhibition of JNK activation. This is because PRAK or MK2, which completely block Ras proliferative signaling, also completely inhibit Ras-induced JNK activation, and because coexpression of the JNK pathway can block this inhibition. This result is consistent with the essential property of the JNK pathway in Ras signaling (9, 10) and is in agreement with the published reports about the antagonistic nature between the p38 and the JNK pathways (34, 35). Thus, Ras has ability to stimulate three MAPK pathways, with the ERK and JNK pathways essential for its proliferative activity; whereas the p38 pathway serves as a negative regulator to restrain Ras signaling (Fig. 10). This interaction between Ras-induced MAPK pathways might have important implications in the development of signal transduction pathways-oriented therapeutics against human malignancies.

The fact that Ras stimulates all three MAP kinase pathways, with the net effect of the p38 pathway serving as a negative regulator of Ras signaling, indicates that the biological effects of Ras action can be regulated by the integration of the three MAP kinase pathways. This fact might help explain the many potential biological effects of Ras action. The net result of a Ras signal will depend upon the interactions of the various signaling pathways which it effects. Depending upon the interactions between these pathways the Ras signal might potentially lead to proliferation, differentiation or apoptosis. Importantly, it might be possible to alter the biological outcome of the Ras signal by altering the balance between the multiple signaling pathways which it effects. For example, whereas under certain experimental conditions Ras can induce differentiation or apoptosis (1), its proliferative/oncogenic activity is most clinically relevant, as its mutation/activation occurs in about 30% of human malignancies (60). It is possible that inhibition of Ras proliferative signaling by the p38 pathway may constitute a novel strategy against Ras-related cancer. Moreover, p38 is mostly responsive to proinflammatory cytokines (22, 61). Consequently, the negative regulation of Ras proliferative signaling by the p38 pathway may constitute a possible mechanism.
for the action of cytokines used in cancer therapy. If this observation can be shown to be a general phenomenon, the p38 pathway may be considered as a novel Ras suppressor.

One recent study demonstrated that MEK3 (MEK kinase 3), a MEKK1 analog, which stimulates all three MAPK pathways, inhibits cell cycle progression and Ras-induced transformation (62). Because MEKK3-induced anti-mitogenic effect is reversed upon inhibition of the p38 pathway, it was concluded that this effect is mediated by the p38 pathway (62). The mechanism of the inhibition in that report, however, was not determined. Our results presented here have demonstrated that the inhibition is a negative feedback. Moreover, evidence is also presented that this inhibition is exercised through the p38 downstream kinases PRAK and MK2. It would be of interest to explore further which downstream targets of PRAK or MK2 are involved in inhibition of Ras proliferative signaling.

MEK, a downstream kinase target of Ras, is an essential molecule for Ras signaling to the p38 pathway, because Ras-induced p38 activation was blocked by the MEK inhibitors. Of interest, a constitutively active MEK (MEK/2) failed to activate p38 on its own. This suggest that the MEK/ERK pathway is essential but not sufficient for p38 activation. Similar results have also been described for the role of MEK/ERK in induction of differentiation by Ral guanine exchange factor RaGEF (63). The MKK6 kinase, which functions upstream of p38, was likewise demonstrated to be required for the Ras-dependent induction of p38 activity, because the dominant negative MKK6 was efficiently inhibitory to p38 activation. This conclusion is further strengthened by the observation that Ras, in a MEK-dependent manner, activates MKK6. These results thus outline a signaling pathway from Ras via MEK/ERK to MKK6/p38. A similar pathway was described recently in which cellular Ras molecules. These results may explain why MK2 has been previously reported to function downstream of both the ERK and p38 pathways (19, 39, 52). Thus, the MEK/ERK pathway is a previously reported to function downstream of both the ERK and p38 pathways (19, 39, 52). Thus, the MEK/ERK pathway is a pathway that is critical in Ras signaling. It stimulates the activity of the AP1 and SRE transcription factors, as well as other Ras dependent genes (38) which are essential for cell proliferation. On the other hand, it also transmits the signal to the p38 pathway to trigger the negative feedback to restrain the proliferative response (Fig. 10).

Acknowledgments—We thank Drs. Dennis Templeton, Roger J. Davis, Matthias Gaestel, Craig Hauser, Christopher B. Newgard, Anning Lin, and Michael Karin for providing reagents, which made this work possible.

REFERENCES

1. Campbell, S. L., Khooravari-Far, R., Rossman, K. L., Clark, G. J., and Der, C. J. (1999) Oncogene 17, 1395–1413
2. Marshall, C. J. (1996) Curr. Opin. Cell Biol. 8, 197–204
3. Westwick, J. K., Cox, A. D., Der, C. J., Cobb, M. H., Iibi, M., Karin, M., and Brenner, D. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6030–6034
4. Khooravari-Far, R., Solski, P. A., Clark, G. J., Kineh, M. S., Burridge, K., and Der, C. J. (1998) Mol. Cell. Biol. 18, 15443–15452
5. Downward, J. (1996) Curr. Opin. Gen. Dev. 8, 49–54
6. Ganganaro, L. M., Sizemore, N., Graves-Deal, R., Oldham, S. M., Der, C. J., and Coffey, A. J. (1997) J. Biol. Chem. 272, 18926–18931
7. Klebes, L. M., Frey, K. A., Marshall, C. J., and Parada, L. F. (1999) Oncogene 18, 2055–2068
8. Karin, M. (1995) J. Biol. Chem. 270, 16483–16486
9. Johnson, R., Spiegelman, B., Hanahan, D., and Wisdom, R. (1996) Mol. Cell. Biol. 16, 4504–4511
10. Clark, G. J., Westwick, J. K., and Der, C. J. (1997) J. Biol. Chem. 272, 1677–1681
11. Adler, V., Pincus, M. R., Brandt-Rauf, P. W., and Ronai, Z. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10585–10589
12. Russell, M., Lange-Carter, C., and Johnson, G. L. (1995) J. Biol. Chem. 270, 11737–11760