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Kinase-Deficient Pak1 Mutants Inhibit Ras Transformation of Rat-1 Fibroblasts

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Among the mechanisms by which the Ras oncogene induces cellular transformation, Ras activates the mitogen-activated protein kinase (MAPK or ERK) cascade and a related cascade leading to activation of Jun kinase (JNK or SAPK). JNK is additionally regulated by the Ras-related G proteins Rac and Cdc42. Ras also regulates the actin cytoskeleton through an incompletely elucidated Rac-dependent mechanism. A candidate for the physiological effector for both JNK and actin regulation by Rac and Cdc42 is the serine/threonine kinase Pak (p65^{pak}). We show here that expression of a catalytically inactive mutant Pak, Pak1^{R299}, inhibits Ras transformation of Rat-1 fibroblasts but not of NIH 3T3 cells. Typically, 90 to 95% fewer transformed colonies were observed in cotransfection assays with Rat-1 cells. Pak1^{R299} did not inhibit transformation by the Raf oncogene, indicating that inhibition was specific for Ras. Furthermore, Rat-1 cell lines expressing Pak1^{R299} were highly resistant to Ras transformation, while cells expressing wild-type Pak1 were efficiently transformed by Ras. Pak1^{L83,L86,R299}, a mutant that fails to bind either Rac or Cdc42, also inhibited Ras transformation. Rac and Ras activation of JNK was inhibited by Pak1^{R299} but not by Pak1^{L83,L86,R299}. Ras activation of ERK was inhibited by both Pak1^{R299} and Pak1^{L83,L86,R299}, while neither mutant inhibited Raf activation of ERK. These results suggest that Pak1 interacts with components essential for Ras transformation and that inhibition can be uncoupled from JNK but not ERK signaling.

Mutations in the small G protein Ras are found in about 20% of tumors, making the Ras oncogene one of the oncogenes most frequently associated with human cancers. Ras plays a key role in regulating cellular proliferation and differentiation. This is accomplished by mediating at least two different pathways. The first involves the stimulation of mitogen-activated protein kinase (MAPK) cascades which convey signals from the plasma membrane to the nucleus to regulate transcription (4, 18, 33, 37). The second pathway involves regulation of the actin cytoskeleton and results in membrane ruffling (5, 19).

Components of the MAPK cascade are well characterized. Growth factor receptors recruit to the membrane the Ras guanine nucleotide exchange factor, SOS, which then activates Ras via nucleotide exchange. Once activated, Ras binds and activates the Raf-1 protein kinase, which in turn phosphorylates and activates the MEK kinases, MEK1 and MEK2. The MEK kinases phosphorylate and activate the MAPK ERK1 and ERK2, which then phosphorylate and activate transcription factors leading to immediate-early gene expression (15, 22, 37). Oncogenic Ras differs from wild-type Ras by point mutations that reduce its intrinsic GTPase activity. This causes the mutant Ras to be predominantly GTP bound, hence activating it without growth factor stimulation of nucleotide exchange (6).

Ras regulation of the actin cytoskeleton does not require the interaction with Raf, since mutant Ras proteins that fail to interact with Raf still induce cytoskeletal changes (26). Although the cytoskeletal pathways have not been fully defined, they require the coordinated action of Ras-related small G proteins from the Rho family, i.e., Cdc42, Rac (Rac1 and Rac2), and Rho (RhoA, RhoB, and RhoC). Cdc42, Rac, and Rho each induce specific actin structures when microinjected into Swiss 3T3 fibroblasts. Cdc42 induces microspikes and filopodia, Rac causes membrane ruffling, and Rho induces stress fibers and focal adhesions. Microinjection of Ras protein induces membrane ruffling, and the ruffling is blocked by dominant-negative mutations in Rac, indicating that Ras regulation of the actin cytoskeleton is mediated by Rac. The actin cytoskeletal events can be further ordered into a cascade of Cdc42 activating Rac followed by Rac activating Rho (41).

Members of the Rho family also regulate transcription through a another MAPK cascade similar to the ERK cascade (10, 39, 40). Rac and Cdc42 bind and activate a protein kinase called Pak, and Pak then activates a cascade that has not been completely defined but is likely to consist of MEK kinase, SEK, and then Jun kinase (JNK) or the related p38 kinase (2, 16, 35, 45, 56, 57). JNK phosphorylates transcription factors such as c-Jun. In many cell lines Ras also activates JNK (21). Ras activation of JNK is inhibited by dominant-negative mutations of Rac and Cdc42, suggesting that Rac and Cdc42 mediate Ras activation of JNK (39).

Several lines of research suggest that Rac and Rho are essential for Ras transformation. Dominant-negative mutations of Rac inhibit Ras transformation, and GTPase-deficient Rac and Rho can both weakly transform fibroblasts. Furthermore, activated Rac and Rho both can dramatically stimulate transformation by partially activated Raf mutants (46, 47). Several oncogenes, including the Ost, Dbl, and Tiam-1 oncogenes, are guanine nucleotide exchange factors specific for Rho family members (48). These observations demonstrate that although the Rho family members are not often found activated in tumors, they can transform cells and in many cases

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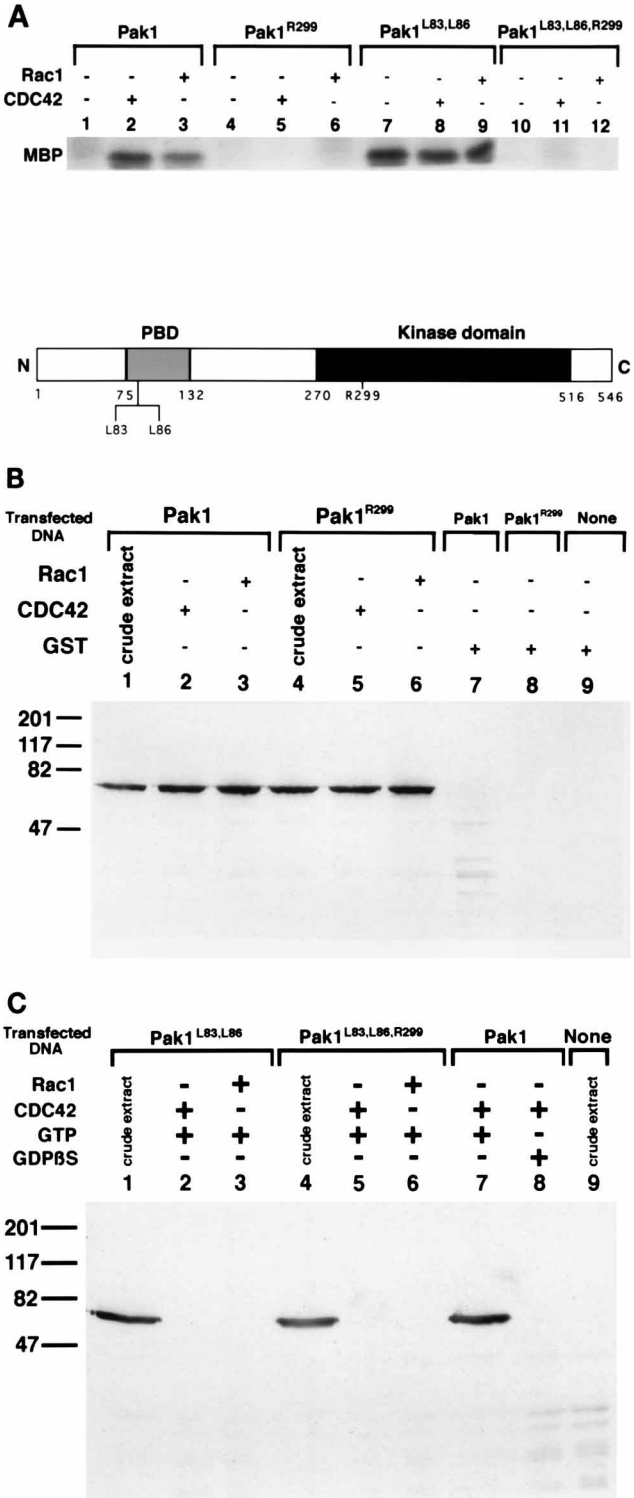

FIG. 1. Characterization of the Pak mutants used in this study. Myc-tagged Pak1 and the indicated mutants were transfected into COS cells and then assayed for kinase activity and Rac and Cdc42 binding. (A) Top, kinase activities of different Pak mutants. Activity was measured by immunoprecipitating with the anti-Myc tag antibody 9E10 and incubating precipitates with, where indicated, GST-Cdc42 or GST-Rac1 with myelin basic protein (MBP) as a substrate. Reactions were started by addition of labeled ATP, and then the mixtures were incubated for 10 min at 22°C as described in Materials and Methods. Bottom, map of Pak1 showing the PBD, kinase domain, and mutations used in this study. (B) Cdc42 and Rac binding. Cdc42 and Rac binding to Pak1 and Pak1^{R299} was measured by mixing ~50 μg of extract from the transfected cells as indicated with ~50 μg of purified GST-Cdc42 or GST-Rac bound to either GTP or GDP-βS and glutathione beads. (C) Cdc42 and Rac binding to PBD mutants. Similar results were obtained in three independent experiments. The same extracts were used in panels A, B, and C. The numbers on the left in panels B and C are molecular masses in kilodaltons.

cooperate with the Ras/Raf signaling pathway to transform cells.

The Pak family of protein kinases are regulated by GTP-bound Rac and Cdc42 and are candidates for effectors that mediate both actin and JNK signaling (32, 35, 51). Three Pak kinases, Pak1, Pak2, and Pak3, have been found in mammals. All are related to the STE20 gene of the yeast *Saccharomyces cerevisiae*, which is regulated by a Cdc42 homolog (3, 30, 36, 43, 49). GTP-bound Rac and Cdc42 both stimulate kinase activity through direct binding to a conserved region near the N terminus of Pak called the p21 binding domain (PBD). Regions homologous to the PBD are found in other proteins that bind Rac and Cdc42 in vitro, such as Ste20 (8). We report here that expression of a catalytically inactive Pak1 kinase inhibits Ras transformation in Rat-1 fibroblast cells. Surprisingly, a functional PBD was not required for Ras inhibition, indicating that this inhibition was not due to sequestration of Rac and Cdc42.

MATERIALS AND METHODS

Plasmids. cDNA expression plasmids utilizing the cytomegalovirus (CMV) promoter to express Myc-tagged Pak1 and Pak1^{R299}, based on the plasmid pCMV6M (a modified version of pCMV5), have been described elsewhere (52). Pak1^{L83,L86} and Pak1^{L83,L86,R299} were constructed by using a unique-site-elimination mutagenesis protocol to introduce the desired mutations (12). Human H-*ras* and K-*ras4B* (K-*ras*) expression systems that utilize pZIP-NeoSV(x)1, a retrovirus vector (neomycin resistant), were a gift from C. Der (42). Expression of the inserted gene is regulated from the Moloney long terminal repeat promoter. v-*raf* expression plasmids were described elsewhere (28). Glutathione *S*-transferase–Rac1 (GST-Rac1) and GST-Cdc42 bacterial expression vectors based on the plasmid pGEX-2T were generous gifts from R. Cerione and S. Bagrodia.

Cell culture and transformation assays. Rat-1 cells were from the Merck collection of strains and have been described elsewhere (28). NIH 3T3 cells were obtained from the American Type Culture Collection, Rockville, Md. Rat-1 cells and NIH 3T3 cells were grown in high-glucose (4.5 g/liter) Mediatech Dulbecco's modified Eagle medium (DMEM) (Fisher Scientific, Pittsburgh, Pa.) supplemented with 10% fetal bovine serum (Sigma, St. Louis, Mo.), penicillin (100 U/ml), and streptomycin (100 mg/ml) and kept at 37°C in 5% CO_2–95% air. DNA transfections were performed by the calcium phosphate precipitation technique. Twenty micrograms of total DNA (10 μg of each test DNA and, when single plasmids were tested, 10 μg of plasmid pUC19) were briefly mixed with 0.5 ml of 0.25 M $CaCl_2$ and 0.5 ml of 2× *N*,*N*-bis (2-hydroxethyl)-2-aminoethanesulfonic acid (BES)-buffered saline and incubated for 10 to 20 min at room temperature. The mixture was then added dropwise to a 25 to 50% confluent, freshly fed 100-mm-diameter dish of cells, swirled gently, and incubated for 18 to 24 h at 37°C in 5% CO_2. Cells were washed twice with growth medium, refed, incubated for 24 to 48 h, and then split 1:5 into 100-mm-diameter dishes. Posttransfection cultures were fed twice a week with fresh growth medium. Cell foci were scored 14 to 18 days posttransfection by fixing the cells in a 10% acetic acid–10% methanol solution and staining the dishes with 0.4% crystal violet in 10% ethanol. Soft-agar assays were performed as described previously (11). Posttransfection cells (10^3) were plated on 60-mm-diameter dishes. After 12 to 15 days, colonies were examined under a Nikon DIAPhot microscope with phase contrast.

To establish stable Rat-1 cell lines expressing Pak1, Pak1^{R299}, Pak1^{L83,L86,R299}, and Pak1^{L83,L86}, each construct was cotransfected with pCDNA3 into Rat-1 cells. The transfected cells were selected in growth medium containing 400 μg of G418 (Geneticin; GIBCO/BRL, Grand Island, N.Y.) per ml. Protein expression levels were determined by Western blot (immunoblot) analysis of G418-selected cell lysates by using the anti-Myc tag monoclonal antibody 9E10 (Calbiochem, Cambridge, Mass.) with the procedure outlined in the enhanced chemiluminescence kit (Amersham, Arlington Heights, Ill.).

Transfection efficiency assays were performed by transfecting Rat-1 cells with the Pak and Ras plasmids to be tested along with pRSV-β-gal (a generous gift from Randy Pittman). At 48 h after transfection, cultured cells were rinsed with phosphate-buffered saline (PBS) and then fixed in PBS containing 2% formaldehyde and 0.2% glutaraldehyde for 5 min at 4°C. The cells were then washed with PBS and overlaid with a histochemical reaction mixture containing 1 mg of

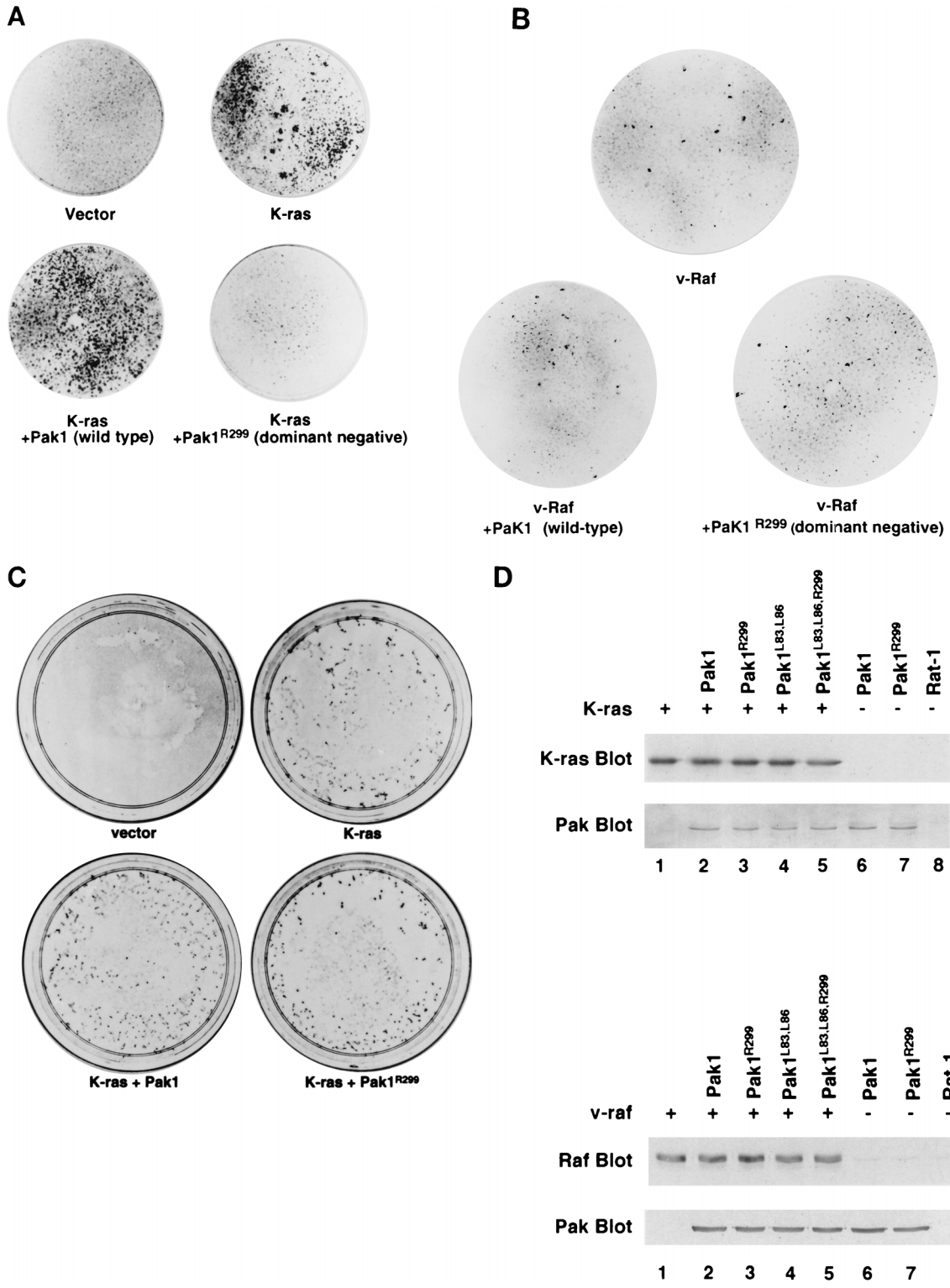

FIG. 2. (A) Effect of Pak1 and Pak1^{R299} on K-*ras* transformation in focus assays. Rat-1 fibroblasts were transfected with 10 μg of the indicated plasmids and stained with crystal violet to visualize foci as described in Materials and Methods. Similar results were obtained in more than 10 independent experiments. (B) Effect of Pak1 and Pak1^{R299} on Raf transformation. Similar results were obtained in more than three independent experiments. (C) Effect of Pak^{R299} on Ras EJ transformation of NIH 3T3 cells. Cells were transfected with, where indicated, 10 μg of Ras and 10 μg of Pak^{R299}. The total amount of DNA was brought to 20 μg with carrier DNA. (D) Western blots of Pak, Raf, and Ras expression. Rat-1 cells were cotransfected with K-*ras* or v-*raf* and the various Pak1 expression plasmids. Extracts were prepared, and 50 μg of each was run on a 12% gel and tested on a Western blot probed with anti-K-*ras* (antibody F234; Santa Cruz Biotechnology Inc., Santa Cruz, Calif.), anti-Pak (the anti-Myc epitope antibody 9E10), or anti-Raf-1 (antibody C12; Santa Cruz Biotechnology). Detection was performed by enhanced chemiluminescence. Similar results were obtained in two independent experiments.

5-bromo-4-chloro-3-indolyl-β-galactosidase (X-Gal) per ml, 5 mM potassium ferricyanide, and 2 mM $MgCl_2$ in PBS. After being incubated for 18 to 24 h, the cells were then rinsed with PBS and fixed in PBS containing 2% formaldehyde and 0.2% glutaraldehyde for 15 min. Cells were examined under a microscope and scored positive for LacZ if they turned blue. Transfection efficiencies ranged from 5 to 10% and were not affected by any of the plasmids used in this study.

Rac and Cdc42 binding and kinase assays. Biochemical assays for Pak1 were performed with extracts from COS cells transfected with Pak plasmids. Lipofectamine-mediated transient transfections of COS-7 cells were performed according to the manufacturer's protocol (Life Technologies, Inc., Gaithersburg, Md.). At 18 to 24 h prior to transfection 2×10^5 to 3×10^5 COS cells were plated on 35-mm-diameter dishes. A total of 1.5 μg of DNA and 10 μl of Lipofectamine reagent were added to the plates containing 1 ml of DMEM (in the absence of serum). After 5 h, 1 ml of DMEM containing 20% fetal bovine serum was added. After 18 to 24 h, the medium was replaced with fresh DMEM containing 10% fetal bovine serum. At 48 to 72 h after addition of DNA, transfected COS cells were washed with cold PBS and lysed in 40 mM HEPES (pH 7.4)–1% Nonidet P-40–100 mM NaCl–1 mM EDTA–25 mM NaF–1 mM sodium orthovanadate–10 μg of leupeptin per ml–10 μg of aprotinin per ml and centrifuged at 12,000 × *g* for 25 min at 4°C (3). Protein concentrations ranged from 5.3 to 7.2 mg/ml.

Pak kinase assays were performed with immunoprecipitates from COS cells as follows. Extracts were incubated with antibody 9E10 and protein A beads for 2 h at 4°C. Precipitates were washed three times with lysis buffer. Immunoprecipitates were washed twice in 2× phosphorylation buffer (10 mM $MgCl_2$, 40 mM HEPES, pH 7.4) and, where indicated, incubated with soluble GTP-bound GST-Cdc42, GST-Rac1 (~5 μg of protein), and 5 μg of myelin basic protein (Sigma) for 5 min on ice. Kinase assays were initiated by the addition of 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) and 20 μM ATP (final concentration) followed by incubation for 10 min at 22°C (3). Reactions were stopped by the addition of 2× sodium dodecyl sulfate (SDS) sample buffer, the mixtures were heated to 95°C, and the products were resolved by SDS-polyacrylamide gel electrophoresis (12% gel) and visualized by autoradiography.

To perform Rac and Cdc42 binding assays, 50 μg of purified GST-Cdc42 or GST-Rac1 was incubated with lysis buffer for 15 min at room temperature to release any nucleotide, washed with lysis buffer, incubated with 1 mM GDP-βS [guanosine-5′-*O*-(2-thiodiphosphate)] or GTP in lysis buffer with 10 mM $MgCl_2$ for 30 min at room temperature, and then washed with lysis buffer with 10 mM $MgCl_2$ to remove unbound nucleotides. Next, the proteins were incubated with 10 μl of COS cell lysates and glutathione beads supplemented with 10 mM $MgCl_2$ for 1.5 h at 4°C (3). Precipitates were washed three times with lysis buffer containing 10 mM $MgCl_2$. Bound proteins were eluted in SDS sample buffer, subjected to SDS–12% polyacrylamide gel electrophoresis, Western blotted, and probed for Pak1 with anti-Myc epitope monoclonal antibody 9E10.

JNK and MAPK/ERK assays. Transfections of Rat-1 cells were performed similarly to those for the transformation assays described above. Five micrograms of total DNA (1 μg of hemagglutinin-JNK1 [HA-JNK1], HA-ERK1, or HA-p38 and 2 μg of each test DNA; when single plasmids were tested, 2 μg of pUC19 plasmid) was mixed with 0.125 ml of 0.25 M $CaCl_2$ and 0.125 ml of 2× BES-buffered saline and incubated for 10 to 20 min at room temperature. The mixture was then added dropwise to a 50 to 60% confluent and freshly fed 35-mm-diameter dish of cells, mixed gently, and incubated for 18–24 h. The cells were then washed twice with growth medium, refed, and incubated for 24 to 48 h. Next, transfected Rat-1 cells were washed two times with cold PBS, lysed in lysis buffer, and centrifuged at 12,000 × *g* for 30 min at 4°C. Extracts were incubated with HA-antibody (12CA5) and protein A beads for 3 to 3.5 h at 4°C. Precipitates were washed three times with lysis buffer and two times with 2× phosphorylation buffer. The precipitates were then incubated with 5 μg of GST–c-Jun (for Jun kinase), myelin basic protein (for ERK), or GST-ATF2 (for p38 kinase), 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol), and 20 μM ATP (final concentration) at 30°C for 30 min. Mixtures were washed three times with lysis buffer and two times with 2× phosphorylation buffer. Reactions were stopped by adding 2× SDS sample buffer and heating to 95°C. All experiments were performed at least twice with similar results.

RESULTS

We utilized four Myc-tagged Pak mutants in the studies described here. The first was Pak1^{R299}, carrying a mutation which converts lysine 299 to arginine and renders the enzyme catalytically inactive (52, 57). This was confirmed by performing kinase assays on extracts from COS cells transfected with the expression vectors (Fig. 1A). While anti-Pak immunoprecipitates from Pak1-transfected cells efficiently phosphorylated myelin basic protein, no kinase activity was detected in cells transfected with Pak1^{R299}. Expression was confirmed by probing Western blots with antibody 9E10, which recognizes the Myc tag on the amino terminus (Fig. 1B and C). Another mutant tested was Pak1^{L83,L86}, which has leucines substituted for highly conserved histidines in the Cdc42 and Rac binding domain. This mutant failed to bind either Cdc42 or Rac. Binding assays were performed by precipitating with the appropriate Rac- or Cdc42-GST fusion protein and Western blotting the precipitates for Pak1 with antibody 9E10 (Fig. 1B and C). Pak1^{L83,L86} also failed to bind either Rac or Cdc42 in overlay assays and the yeast two-hybrid assay (data not shown). Other mutant proteins shown in Fig. 1 are discussed below.

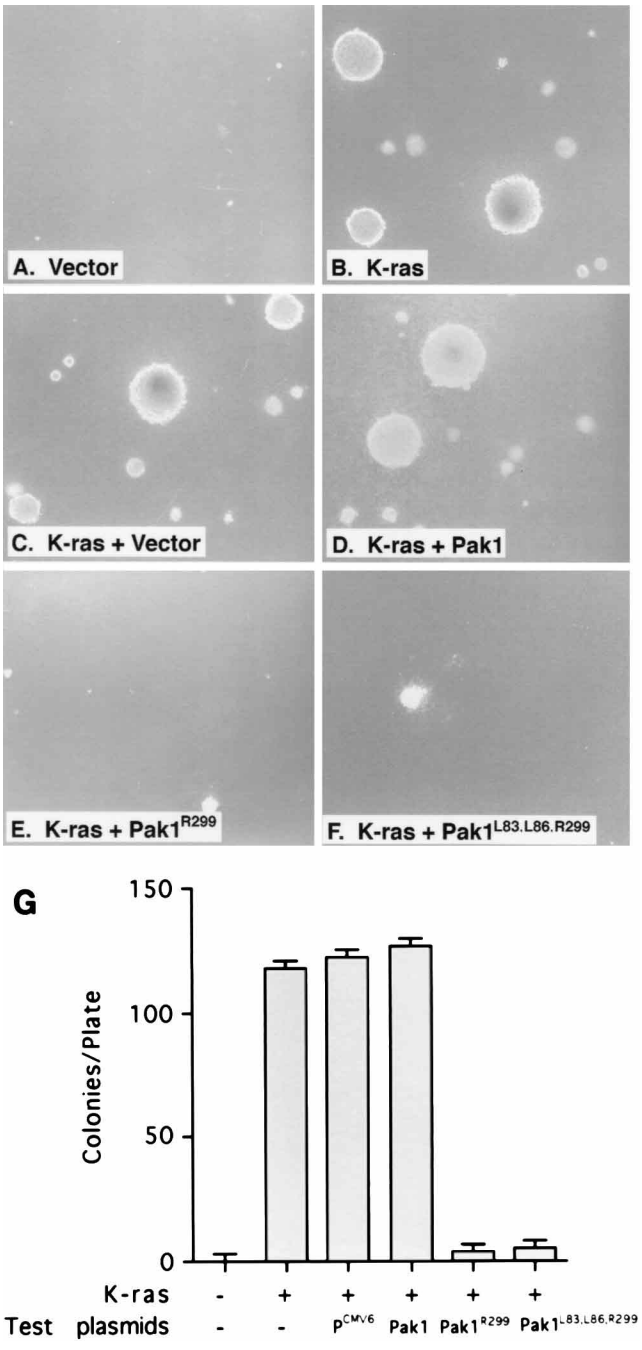

FIG. 3. Effect of Pak1 and Pak1^{R299} on K-*ras* transformation in soft-agar assays. Cells were transfected as described in the legend to Fig. 1 and then plated on soft agar. (A to F) Representative microscopic views of colonies. (G) Quantification of the soft-agar assays. Error bars indicate standard deviations. Similar results were seen in more than five independent experiments.

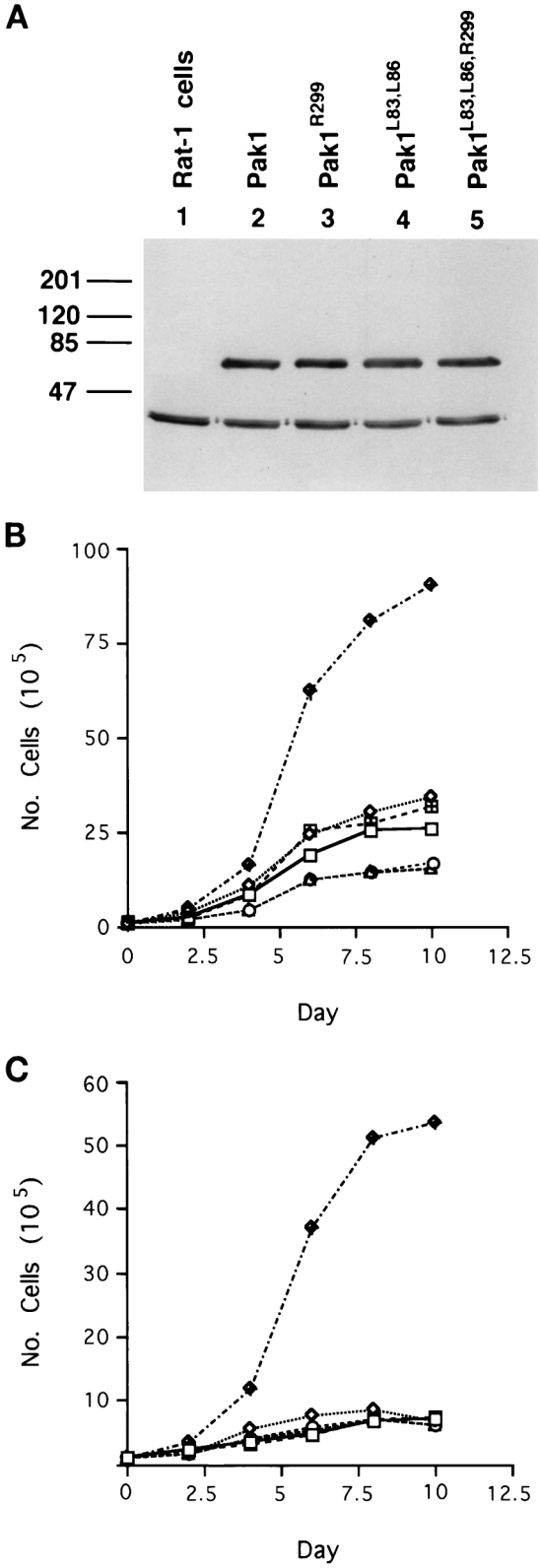

FIG. 4. Stable expression of Pak1 in Rat-1 cells. Rat-1 cells were cotransfected with pCDNA and the various Pak1 expression plasmids. G418-resistant colonies were isolated, expanded into cell lines, and tested as described below. (A) Western blot of 10 μl of extracts from Rat-1 cells probed with antibody 9E10, which recognizes the Myc tag on the Pak1 constructs. Pak1 is seen as an ~65-kDa band. Numbers on the left are molecular masses in kilodaltons. (B) Growth rates of stable cell lines and an H-*ras*-transformed cell line. (C) Growth of cells in 1% serum. Similar results were obtained in more than two independent experiments. Symbols: □, Rat-1-Pcmv; ◇, Rat-1-Pak1; ○, Rat-1-Pak1^{R299}; △, Rat-1-Pak1^{L83,L86,R299}; ⊞, Rat-1-Pzeo; ◆, Rat-1-H-*ras*.

Kinase-deficient Pak inhibits Ras transformation. To test the role of Pak in Ras transformation, we performed cotransfection experiments with human K-*ras* and Rat-1 fibroblasts. As expected, when we fixed the cells 14 to 18 days later and stained them with crystal violet, we observed more than 100 foci in the plates transfected with K-*ras* alone. However, when we included the Pak1^{R299} expression plasmid in the transfections, we observed about 90% fewer foci in the plates (Fig. 2A). Wild-type Pak1 or the vector plasmid did not inhibit K-*ras* transformation. To test whether Pak1^{R299} was a nonspecific inhibitor of transformation of Rat-1 cells, we performed cotransfection experiments with v-Raf (Fig. 2B). We found that Pak1^{R299} did not inhibit Raf transformation. Interestingly, the inhibition was specific for Rat-1 cells, as no inhibition was observed when NIH 3T3 cells were substituted for Rat-1 cells (Fig. 2C). To test whether Pak altered levels of Ras or Raf expression, we prepared extracts from transfected cells and performed Western blotting for Ras, Raf, and Pak. None of the mutant Paks affected expression of either Ras or Raf (Fig. 2D). Therefore, Pak1^{R299} specifically inhibits Ras transformation of Rat-1 cells without affecting transformation by Raf or affecting Ras expression.

We also measured transformation by assessing growth on soft agar after cotransfection with K-*ras* and Pak1 plasmids. We observed numerous colonies on soft agar plates with K-*ras* transfections, and, as seen in the focus assays, we observed very few colonies when cells were cotransfected with Pak1^{R299} (Fig. 3). Furthermore, most of the rare colonies seen in the presence of Pak1^{R299} were substantially smaller than those seen with K-*ras* alone. As with the focus assays, no inhibition was observed when wild-type Pak1 was substituted for the mutant Pak1^{R299} or when NIH 3T3 cells were tested instead of Rat-1 cells (data not shown for NIH 3T3 cells). Typically, when equal concentrations of K-*ras* and Pak1^{R299} DNA were transfected into cells, transformation was inhibited by about 90% in focus assays and by about 95% in soft-agar assays (Fig. 3G). We found that Pak1^{R299} inhibited transformation by both K-*ras* and H-*ras* but not by Raf (data not shown for H-*ras*).

We further addressed the interaction between Ras, Raf, and Pak by using stable cell lines that expressed Pak1 and Pak1^{R299}. We cotransfected Rat-1 cells with the plasmid pCDNA3 and selected Geneticin (G418)-resistant cell lines. We tested expression of the wild-type and mutant Pak proteins by probing a Western blot for a Myc tag fused to the N terminus of Pak (Fig. 4A) and found that the stable cell lines each expressed a novel 65-kDa protein at comparable levels. Pak1 expression caused a small stimulation of the growth rate, while Pak1^{R299} expression caused a small inhibition of the growth rate, but these differences were within the margin of error of the experiment (Fig. 4B). None of the cell lines expressing the Pak1 mutants proliferated in 1% serum or on soft agar (Fig. 4C) (data not shown for soft agar). Stable expression of Pak1 and Pak1^{R299} affected the morphology of cells. Pak1-expressing cells were elongated, while Pak1^{R299}-expressing cells lost the spindle shape characteristic of fibroblasts (Fig. 5A to C). Pak1^{L83,L86} and Pak1^{L83,L86,R299} also caused changes in cellular morphology, but to a smaller extent (Fig. 5D and E).

Upon establishing the cell lines for stable expression, we tested them in Ras transformation assays by using both focus assays and soft-agar colony assays. As predicted from the co-

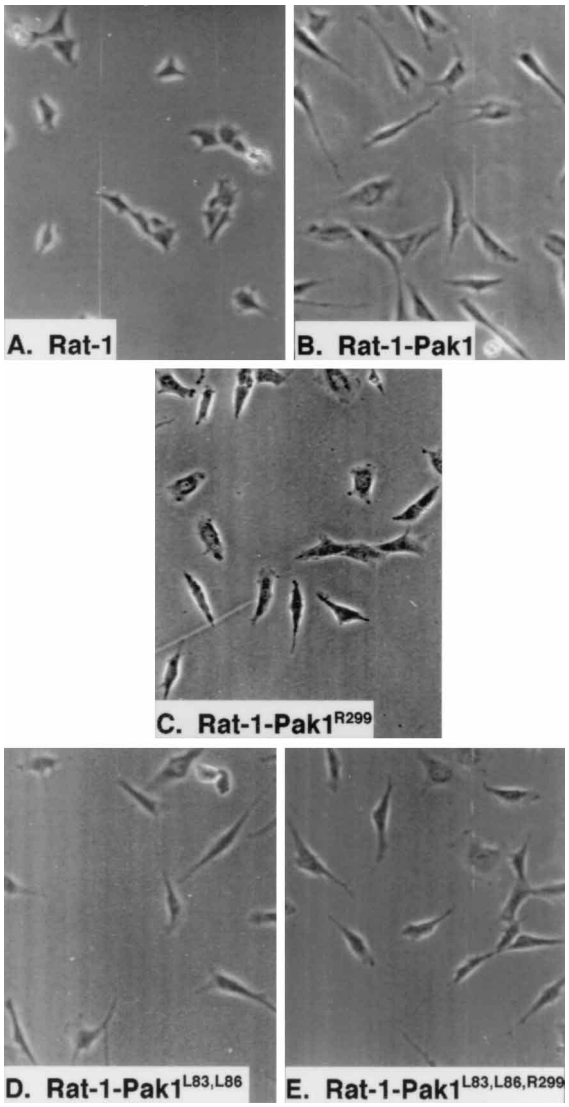

FIG. 5. Micrographs of Rat-1 cells expressing Pak1 and Pak1 mutants.

transfection experiments, we found that cells expressing Pak1 were transformed as efficiently as control cells, while cells expressing Pak1^{R299} were highly resistant to K-*ras* transformation (Fig. 6). In a dose-response experiment, we determined that about 10 to 100 times as much K-*ras* was required to transform the Pak1^{R299}-expressing cells as was required to transform Rat-1 cells or Rat-1 cells expressing wild-type Pak1. Similar dose-response curves were obtained in both focus formation (Fig. 6A) and soft-agar (Fig. 6B) assays. As expected from the focus assays described above, all cell lines were efficiently transformed by Raf (6C). Expression of wild-type and mutant Pak1 proteins did not affect transfection efficiencies (data not shown).

A functional Cdc42 and Rac binding domain is not required for Ras inhibition. Since Rac is essential for Ras transformation, it was possible that the dominant-negative Pak1 mutant inhibited Ras transformation by sequestering Rac or Cdc42 into an inactive complex. To address this mechanism, we tested a Pak1 mutant that fails to bind either Cdc42 or Rac and is also defective in kinase activity. The mutant, Pak1^{L83,L86,R299}, has substitutions of leucines for conserved histidines at positions 83 and 86, along with the original R299 in the kinase domain (Fig. 1). The new mutations lie within the PBD, a region which is necessary and sufficient for both Rac and Cdc42 binding. Western blots of extracts from transfected COS cells confirmed expression at levels comparable to those for other mutants, and no Rac or Cdc42 binding was detected when measured directly (Fig. 1C). We found that the Pak1^{L83,L86,R299} mutant was as potent in Ras inhibition as the original Pak^{R299} (Fig. 3). In dose-response experiments, cells expressing Pak1^{L83,L86,R299} were as resistant to K-*ras* transformation as cells expressing Pak1^{R299} (Fig. 6). We also tested Pak1^{L83,L86}, which expressed a hyperactive kinase that was not further stimulated by Rac or Cdc42 (Fig. 1A). Mutations within other conserved residues in the PBD of Pak3 also cause hyperactivity (2). No effect of Pak1^{L83,L86} on K-*ras* or Raf transformation was observed, suggesting that, despite its essential role in Ras transformation, constitutively active Pak1 does not appear to be an oncogene product, nor does it cooperate with Ras or Raf in transforming cells (data not shown).

Ras inhibition is uncoupled from JNK but not MAPK signaling. In order to address whether the signaling pathways of Rac and Ras were affected by Pak, we measured the effect of Pak on JNK and MAPK activation in Rat-1 cells. We cotransfected an HA-tagged JNK with Rac and the various Pak constructs described above, immunoprecipitated JNK with the HA antibody, and measured phosphorylation of a GST-Jun fusion protein (Fig. 7A). The activated Rac^{L61} stimulated JNK activity almost 40-fold, as did the activated Pak1^{L83,L86}, relative to results with the vector control (Fig. 7A, lanes 3, 7, and 8). No stimulation was observed with Pak1, Pak1^{R299}, or Pak1^{L83,L86,R299} (Fig. 7A, lanes 1, 2, and 9). To test whether the mutant Paks inhibited activation by Rac, we cotransfected them with Rac^{L61}. We observed no changes when the active Pak1 constructs (Pak1 and Pak1^{L83,L86}) were cotransfected with Rac^{L61}. We found that the Pak1^{R299} construct inhibited JNK activation by ~75%, while no inhibition was observed with the Pak1^{L83,L86,R299} construct (Fig. 7A, lanes 5 and 6). Similar levels of activation were also observed when p38 kinase was tested in place of JNK (Fig. 7D) and when COS-7 cells were used in place of Rat-1 cells (data not shown). Similarly, we found that Pak1^{R299}, but not Pak1^{L83,L86,R299}, inhibited Ras activation of JNK (Fig. 7B). No stimulation of JNK by Raf was observed (Fig. 7C). These observations support a Ras-to-Rac-to-Pak/p38 activation model and suggest that JNK inhibition is not obligatory for Pak mutants to inhibit Ras transformation.

To measure if Pak1 interacted with the MAPK/ERK pathway, we cotransfected Rat-1 cells with HA-tagged ERK1, K-*ras*, and the various Pak constructs described above, immunoprecipitated ERK1 with the HA antibody, and measured phosphorylation of myelin basic protein (Fig. 8). K-*ras* stimulated ERK1 activity about 35-fold relative to that with the vector control (Fig. 8A, lanes 8 and 9). No stimulation was observed with Pak1, Pak1^{R299} Pak1^{L83,L86}, or Pak1^{L83,L86,R299}, either alone or in the presence of Rac^{L61} (Fig. 8A, lanes 1 to 7, and B, lanes 1 and 2). To test whether the mutant Paks inhibited activation by Ras, we cotransfected them with K-*ras*. We observed no changes when the active Pak1 constructs (Pak1 and Pak1^{L83,L86}) were cotransfected with K-*ras* (Fig. 8B, lanes 3 and 5). We found that the Pak1^{R299} construct inhibited ERK activation by about 50% and that Pak1^{L83,L86,R299} also inhibited ERK activation to a similar extent (Fig. 8B, lanes 4 and 6). We also observed similar levels of activity when experiments were performed with COS-7 cells (data not shown). To test whether these mutants also inhibited Raf activation, we determined their effects on Raf activation of ERK. None of the Pak constructs inhibited Raf activation of ERK (Fig. 8C).

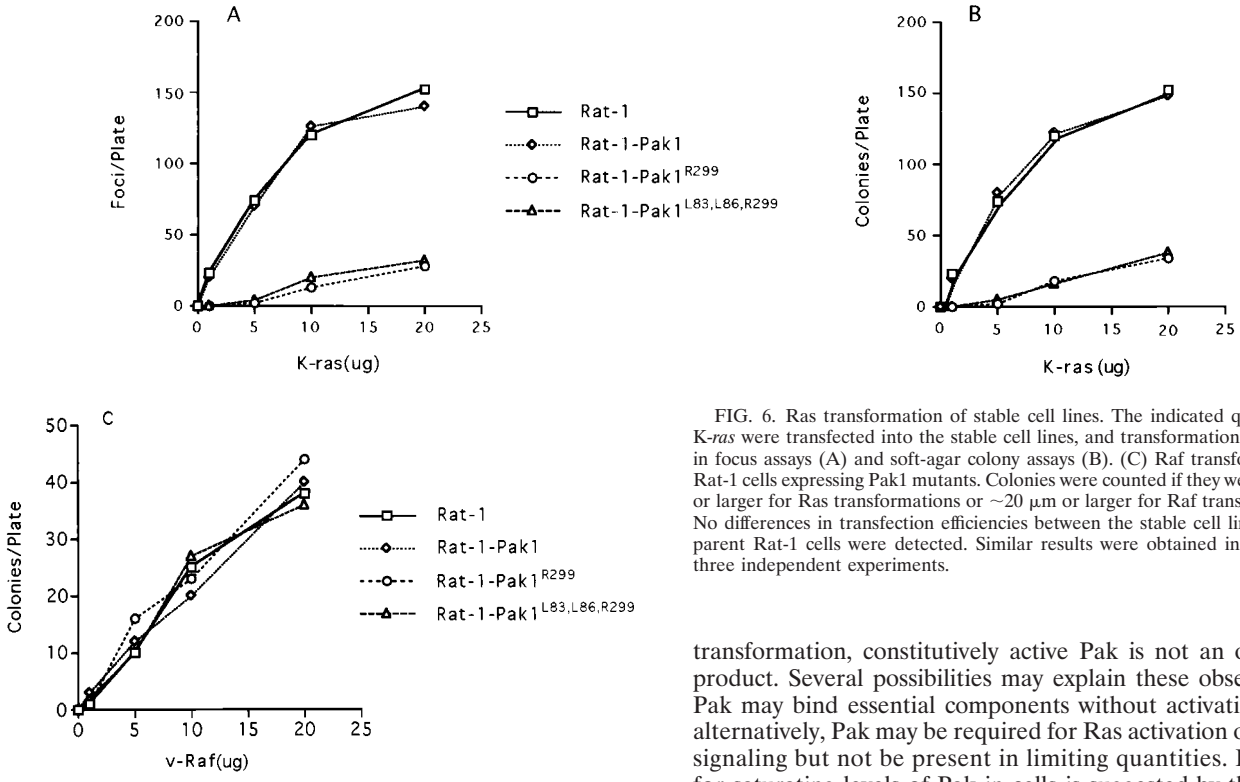

FIG. 6. Ras transformation of stable cell lines. The indicated quantities of K-*ras* were transfected into the stable cell lines, and transformation was scored in focus assays (A) and soft-agar colony assays (B). (C) Raf transformation of Rat-1 cells expressing Pak1 mutants. Colonies were counted if they were ~50 μm or larger for Ras transformations or ~20 μm or larger for Raf transformations. No differences in transfection efficiencies between the stable cell lines and the parent Rat-1 cells were detected. Similar results were obtained in more than three independent experiments.

These observations suggest that the dominant-negative Pak1 mutants may inhibit Ras transformation by interfering with the MAPK/ERK cascade.

DISCUSSION

We have demonstrated that Pak1 interacts with an essential component of the Ras signaling pathway in addition to Rac and Cdc42. A catalytically inactive Pak1 mutant inhibited Ras transformation of Rat-1 fibroblasts in both focus assays and soft-agar assays, two well-established assays for Ras transformation. Neither wild-type Pak1 nor a hyperactive mutant transformed cells, nor did either significantly affect the transformation frequencies of Ras or Raf. To extend these studies, we have begun surveying cell lines to determine which ones are sensitive to Pak^{R299} inhibition. We found that Pak^{R299} had no effect on Ras transformation of NIH 3T3 cells but did inhibit Ras transformation of a rat Schwann cell line (reference 44 and unpublished observations). Thus, dominant-negative Pak1 does not inhibit Ras in all cells, but nevertheless, our observations here are not unique to Rat-1 cells.

The major Ras signaling pathway in most organisms is the MAPK cascade (37). Since only Pak1 mutants that inhibit Ras activation of MAPK inhibit transformation, our studies support a role for MAPK inhibition as relevant for Pak1 inhibition. $Pak1^{R299}$ inhibits both JNK activation and MAPK activation, but since $Pak1^{L83,L86,R299}$ inhibits transformation without inhibiting JNK or p38 activation, our studies suggest that JNK inhibition is not necessary for Ras inhibition. Interestingly, we have not been able to measure activation of MAPK by $Pak1^{L83,L86}$, which activates JNK to about the same extent as Rac^{L61}. We also have not detected any evidence of cell transformation by $Pak1^{L83,L86}$, either by itself or in cooperation with Ras, Rac, or Raf. Thus, despite very strong inhibition of Ras transformation, constitutively active Pak is not an oncogene product. Several possibilities may explain these observations. Pak may bind essential components without activating them; alternatively, Pak may be required for Ras activation of MAPK signaling but not be present in limiting quantities. Evidence for saturating levels of Pak in cells is suggested by the observation that maximum levels of JNK and p38 activation are obtained with Rac^{L61} alone; cotransfection of Rac^{L61} with Pak1 or $Pak1^{L83,L86}$ does not further stimulate JNK or p38 (Fig. 7). Additionally, activation of MAPK via Pak may require translocation as well as enzymatic activation; recently, activation of the platelet-derived growth factor receptor was shown to recruit the adapter protein Nck to the membrane through an SH2 domain binding site. Nck, in turn bound and activated Pak through one of its SH3 domains translocating the complex to the membrane. Interestingly, both JNK/p38 and MAPK were activated by Pak translocation (17, 34). Thus, translocation of Pak may be important for activation of MAPK, which could explain why the hyperactive kinase $Pak1^{L83,L86}$ does not activate MAPK. Roles for Rho, Rac, and Cdc42 have also been suggested in MAPK signaling because, although none will activate MAPK alone, all will synergize with an activated Raf to activate MAPK (16). Our data is consistent with this receptor-Nck-Rac-Pak-MAPK pathway playing an essential role in Ras transformation.

Expression of $Pak1^{R299}$ or the N-terminal half of Pak inhibits Rac and Cdc42 activation of JNK in cotransfection experiments (39, 57) (Fig. 7). Additionally, the N-terminal half of Pak inhibits ERK activation (16). The mechanism of inhibition was proposed to be through sequestering of Rac and Cdc42 via the PBD. These same mutants also inhibit JNK activation, which prevented determination of whether JNK signaling was required. Our studies confirm that mutant Paks inhibit both ERK and JNK. Furthermore, they suggest that Rac/Cdc42 sequestering is only required for JNK inhibition; neither Rac/Cdc42 sequestering nor JNK inhibition is required for ERK inhibition.

The Ras-Raf-MEK-ERK signaling pathway has been established in many cell types, but other routes to transformation occur in most cells, and in some cells the alternate pathways may predominate over the Ras-Raf pathway. Cells in which Ras signaling is largely Raf independent include a certain line

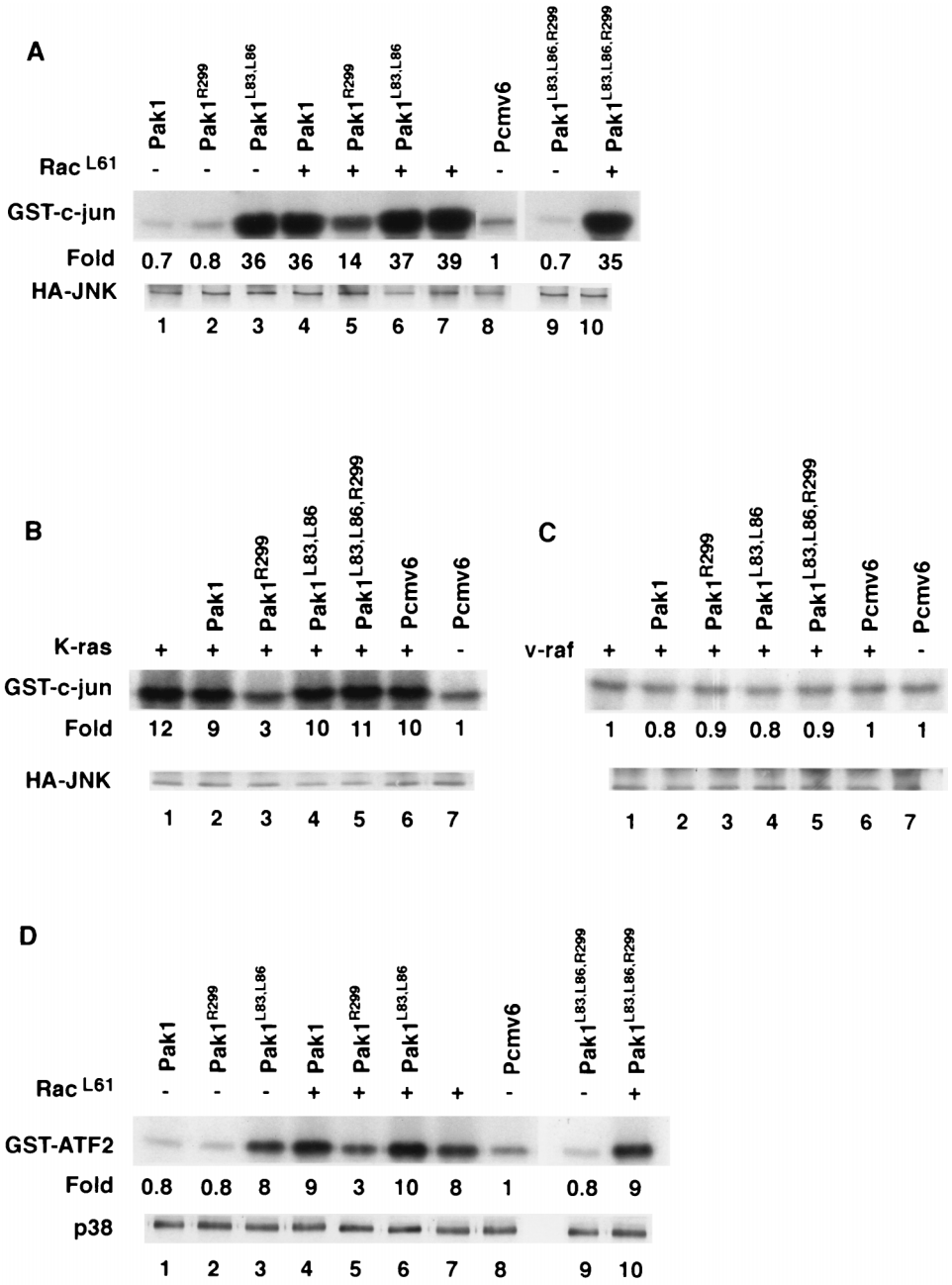

FIG. 7. Effect of Pak mutants on JNK1 and p38 activation. Rat-1 cells were cotransfected with either HA-JNK (A, B, and C) or HA-p38 (D) and the plasmids encoding the proteins shown. Fold, fold increase in substrate phosphorylation over that occurring in the Pcmv6 lanes, as determined through phosphorimager analysis. At the bottom of each panel is a Western blot showing expression of HA-JNK or HA-p38, as indicated.

of NIH 3T3 fibroblasts, rat intestinal epithelial cells, and Wistar rat thyroid cells (1, 27, 42). In each of these cell types Ras transduces mitogenic signals independent of Raf activation. Since our Rat-1 cells can be transformed by both Ras and Raf (Fig. 2 and 6), they are not Raf independent. Yet since they are sensitive to Pak inhibition, there may be a major role for Raf-independent transformation. The growing number of experimental systems in which Ras transformation is uncoupled from Raf activation suggests that the components of Ras alternate pathways are possible targets for novel antineoplastic drugs.

The possibility that JNK is involved in mediating an alternate Ras transformation signal is supported by the observations that Rac stimulates JNK and that dominant-negative Rac mutants inhibit Ras transformation. However, several groups recently constructed Rac mutants that failed to interact with PAK and subsequently failed to activate JNK (25, 29, 55). Both groups found that their mutant Rac proteins still transformed cells and caused membrane ruffling. Thus, Pak and JNK activation are not required for Rac transformation. To address the role of JNK in Ras transformation, Clark et al. tested a dominant-negative mutant of SEK and found that it inhibited JNK activation and Ras transformation but not Ras activation of MAPK, suggesting a critical role for JNK in Ras transforma-

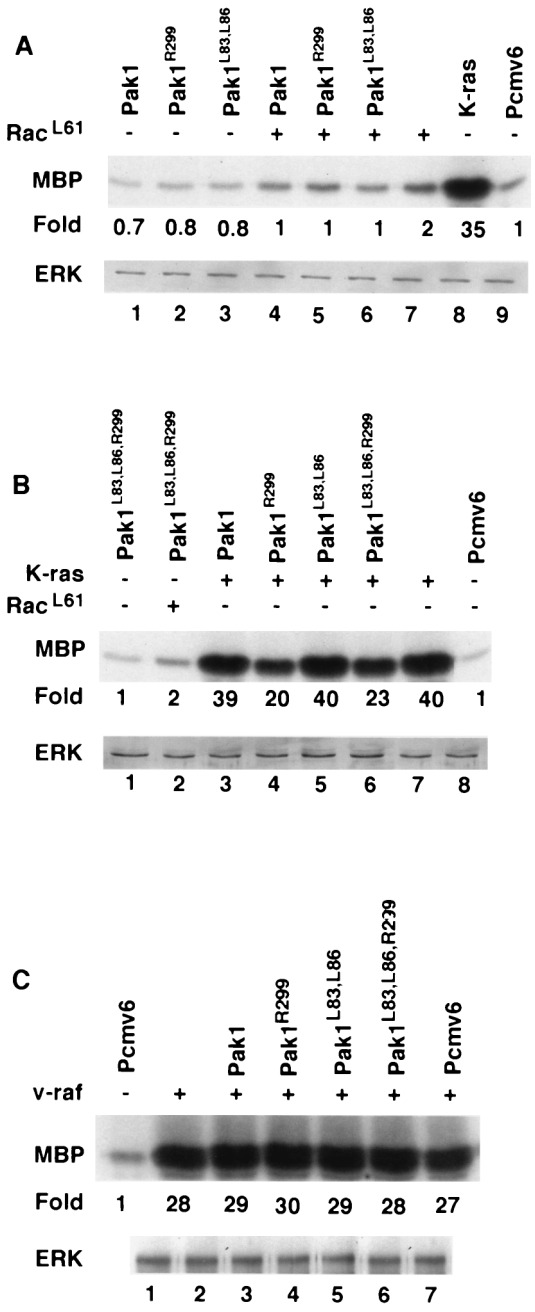

FIG. 8. Effect of Pak mutants on ERK1 activation. Rat-1 cells were cotransfected with HA-ERK and the test DNA. Fold, fold increase in substrate phosphorylation over that occurring in the Pcmv6 lanes, as determined through phosphorimager analysis. At the bottom of each panel is a Western blot showing expression of HA-ERK. MBP, myelin basic protein.

tion (9). Thus, while JNK activation may not be essential for Rac transformation, it appears to be essential for Ras transformation. Although we correlate MAPK and not JNK inhibition with the dominant-negative Pak mutants, our work does not necessarily exclude JNK from an essential role in Ras transformation, since $Pak1^{R299}$, the only mutant that inhibited JNK activation, also inhibited ERK activation. This prevented us from using Pak mutants to address the role of JNK exclusive of MAPK.

The mechanism by which Ras communicates to Pak presumably utilizes an effector that is activated by GTP-bound Ras, but the relevant protein has yet to be identified. Raf is a strong candidate for the relevant effector, since all mutants that inhibited Ras transformation also inhibited MAPK activation. If Raf is indeed the site of inhibition, the inhibition is bypassed by the activating mutant, v-Raf. Other potential Ras effectors that Pak dominant-negative mutants might interfere with include Ral GDS, Rin1, and phosphatidylinositol-3-OH kinase, all of which bind Ras-GTP (20, 23, 38, 50). Another potential target is p190 Rho GAP, because it associates with Ras GAP, an association proposed to mediate Ras activation of JNK (9, 53). The effects that the dominant-negative Pak mutant have on cell shape (Fig. 5) suggest that the actin cytoskeleton may also be involved in Pak's inhibition of Ras transformation (52).

Our observation that a kinase-deficient PBD mutant Pak still inhibits Ras transformation suggests that Pak interacts with the Ras signaling pathway independent of Cdc42 and Rac sequestering via the PBD. Two mechanisms may account for these observations: (i) multiple binding sites for Rac and Cdc42 on Pak1 and (ii) novel interactions between Pak and other proteins required for Ras signaling.

Raf is an example of a small G-protein effector with multiple binding sites. Two sites on Raf bind Ras, the first is apparently the primary binding site, while the second is a cryptic site that is unmasked only after Ras binds the first site (7, 14, 24, 58). Although we cannot detect Rac or Cdc42 binding to $Pak1^{L83,L86}$ and $Pak1^{L83,L86,R299}$, there is still a possibility that multiple binding sites for Rac and Cdc42 exist on Pak1. A recent study using chimeras between Rac and Rho found that two sites on Rac were required for Pak binding and membrane ruffling. The first site (amino acids 30 to 40) is equivalent to the major effector region on Ras, while the second region (amino acids 143 to 175) does not correspond to a known effector region of Ras (13). Since there are two effector regions for Pak on Rac, there may well be multiple binding sites on Pak for Rac and Cdc42. However, since we did not detect any Rac or Cdc42 binding by the L83 L86 mutant, it is likely that the primary Rac binding site on Pak is the PBD. If a second Rac binding site exists, it does not support significant binding by itself.

Another mechanism by which Pak may inhibit Ras is by sequestering other proteins, distinct from Rac and Cdc42, that are essential for Ras transformation. Such factors may interact with the kinase domain in the C terminus, causing, in the case of $Pak1^{L83,\ L86,\ R299}$, a nonproductive interaction. A dominant-negative Raf mutant that fails to bind Ras, which probably acts by sequestering the downstream MEK kinase, has also been constructed (7, 54). Similarly, $Pak1^{L83,\ L86,\ R299}$ may sequester downstream kinases such as MEK kinase or SEK. Other potential sites for protein-protein interactions are found in the N terminus of Pak1; these include several proline-rich regions that bind to SH3 domains and an acidic region (17, 51).

The identification of physiological targets for Pak1 may elucidate the mechanism of Ras inhibition. Although the protein-protein interactions of mammalian Pak kinases are not well understood, the yeast Pak homolog, Ste20p, interacts with several other components of the mating signaling complex in addition to Cdc42, including Ste5p, and Bem1p (31). Homologs of Ste5p and Bem1p have yet to be identified in mammals. Work in progress is aimed at determining the cellular target responsible for Pak inhibition of Ras signaling.

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