Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 6924–6928, August 1992
Biochemistry

Evidence for a Ras-dependent extracellular signal-regulated protein kinase (ERK) cascade

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Communicated by Edwin G. Krebs, May 4, 1992 (received for review March 10, 1992)

ABSTRACT **The small GTP-binding protein Ras appears to be required for transformation and differentiation induced by tyrosine kinases. The Ras requirement may be limited to a few tyrosine kinase-regulated signaling pathways or may be universal for all tyrosine kinase actions. Because both Ras and the microtubule-associated protein 2 kinases ERK1 and ERK2 have been implicated in events that lead to neurite outgrowth, we explored the possibility that Ras and ERKs may lie on the same signaling pathway. Utilizing PC-12 rat adrenal pheochromocytoma cell lines that contain a dominant inhibitory Ras mutant (S17N-Ras^H), we found that Ras was required for stimulation of the ERK cascade by nerve growth factor but apparently not by the heterotrimeric G protein activator AlF_4^-. Within this cascade, Ras appears to be upstream of an ERK activator, raising the intriguing possibility that Ras may directly regulate a serine/threonine protein kinase.**

Nerve growth factor (NGF), like many other factors that influence growth and differentiation, activates a tyrosine kinase (1) that mediates its physiological actions. The sequence of signaling events downstream from this or any other tyrosine kinase remains uncertain, but the small GTP-binding protein Ras is thought to be involved in the actions of tyrosine kinases. Ras homologs were originally identified as the transforming components of certain retroviruses and have received even greater attention since the discovery that activated Ras proteins are products of oncogenes associated with human tumors (2). Ras proteins interact with GTPase-activating proteins (GAPs), which enhance their GTP hydrolytic activity, and guanine-nucleotide exchange factors, which allow them to exchange GDP for GTP, to regulate the extent to which they are in the active GTP-liganded states (3). Although neither the mechanisms by which the active states of Ras are modulated by receptors nor the downstream targets of Ras are understood, Ras has been implicated in changes in the cellular program such as those that occur during transformation or differentiation elicited by tyrosine kinases. For example, microinjection of antibodies that inhibited Ras function blocked transformation by the tyrosine kinase-encoding oncogenes *src*, *fms*, and *fes* but did not inhibit transformation by the serine/threonine kinase-encoding oncogenes *mos* and *raf* (4). In *Drosophila* a Ras-like protein and a GTP/GDP exchange protein are necessary for the function of the sevenless (*sev*)-encoded tyrosine kinase and the faint little ball (*flb*) product, an epidermal growth factor receptor-like molecule (5). Finally, microinjection of an activated Ras induced neurite outgrowth in PC-12 rat adrenal pheochromocytoma cells independent of NGF (6), and expression of a dominant inhibitory Ras mutant blocked neuronal differentiation of PC-12 cells promoted by NGF (7).

Three sequential components in an NGF-stimulated protein kinase cascade are believed to lead to the phosphorylation of ribosomal protein S6, a widely used marker for tyrosine kinase-regulated phosphorylation pathways (8): (*i*) one or more S6 protein kinases (9); (*ii*) the S6 protein kinase kinases (9, 10), extracellular signal-regulated protein kinases (ERKs) 1 and 2 (11, 12); and (*iii*) one or more activators of these latter kinases [ERK/microtubule-associated protein 2 (MAP2) kinase activators (13–15)]. Volonte *et al.* (16) have suggested that inhibition of this cascade, like inhibition of Ras, blocks neurite outgrowth. We therefore reasoned that Ras and the three-component protein kinase cascade may lie on the same signaling pathway. Thus, we examined which, if any, steps of this kinase cascade were affected by a dominant interfering mutant of Ras (S17N-Ras^H) in PC-12 cells. This inhibitory form of Ras is constitutively inactive (17, 19) and is thought to block the activation of endogenous Ras by competing for guanine-nucleotide exchange factor (19). In PC-12 cells inhibition of Ras function blocks the function of endogenous Ras, without interfering with cell division (7).

MATERIALS AND METHODS

PC-12 Cell Lines. The PC-12 cells used for this study were isolated after infection of PC-12 cells with replication-defective retroviruses carrying either 17N-*ras*^{H} or no *ras* gene and selection in G418. The virus stocks were prepared by transfecting the packaging cell line ψ-2 (20) with *ras* genes subcloned in the retrovirus vector pZipneoSV(X) (21). Since expression of the 17N-*ras*^{H} product, p21, inhibits fibroblast proliferation, ψ-2 cells were transformed by transfection with v-*raf* DNA (22) prior to transfection with pZipneoSV(X)17N-*ras*^{H} to make them resistant to the growth-inhibitory effects of mutant Ras (17). Two PC-12 isolates that expressed S17N-Ras^H (17N-1 and 17N-3) and one that did not (Zip) were selected for further study. The three types of PC-12 cells were maintained, treated with NGF (75 ng/ml) or AlF_4^- (30 mM NaF plus 10 μM $AlCl_3$), and harvested for activity measurements as in ref. 23. Cells were labeled with ^{32}P, and phosphorylated amino acids recovered from immunoprecipitated ERK1 were analyzed as in ref. 24.

Measurement of ERK Activity. Assays of MAP2 and myelin basic protein (MBP) kinase activity were performed as described (12). Immune complex kinase assays were performed with the ERK1-selective antiserum 837 (12) in the presence of 0.1 μM staurosporine, 10 mM $MnCl_2$, 10 μM [γ-^{32}P]ATP (4060 cpm/pmol), 1 mM benzamidine, 1 mM dithiothreitol, MBP at 0.3 mg/ml, and 10 mM Hepes, pH 8, for 30 min at 30°C.

Preparation of ERK/MAP2 Kinase Activator Fractions. Cell extracts were passed through 0.5-ml columns of DEAE-

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Abbreviations: NGF, nerve growth factor; GAP, GTPase-activating protein; ERK, extracellular signal-regulated protein kinase; MAP2, microtubule-associated protein 2; MBP, myelin basic protein.

cellulose (Whatman DE-52) equilibrated in 50 mM β-glycerol phosphate/10 mM Hepes, pH 8/70 mM NaCl to resolve ERK activator activity from ERKs, which are retained on DEAE-cellulose. The activator fractions were collected by washing the columns with 1 vol of the column buffer.

RESULTS

We first examined the amount of the Ras mutant expressed in the three cell lines by immunoprecipitation of the protein from ^{35}S-labeled cells. The largest amount of the protein was present in 17N-1 cells (Fig. 1*A*), a smaller amount was present in 17N-3 cells, and none was detected in the control isolate Zip. Thus 17N-1 and 17N-3 cells overexpress the mutant protein relative to normal Ras^H. Next we assessed the ability of the three PC-12 lines, the control isolate (Zip) that had functional Ras proteins and the two clonal isolates (17N-1 and 17N-3) whose endogenous Ras proteins were inhibited by the expression of the dominant interfering Ras mutant S17N-Ras^H (17), to extend neurite-like processes in response to NGF. While Zip cells formed processes like other PC-12 lines, 17N-1 cells were unable to extend neurites in the presence of NGF (not shown), in agreement with results of others (7). The response of 17N-3 cells was intermediate, neither like wild-type nor like 17N-1. Thus, the Ras mutant impaired NGF-induced neurite outgrowth in proportion to its amount in the PC-12 isolates.

To examine the protein kinase cascade involving ERKs, we assessed kinase activity due to ERK1 and ERK2 in PC-12 cells with and without NGF treatment (Fig. 2). NGF stimulated ERK activity, as detected with the substrates MAP2 or MBP, 4- to 5-fold in Zip cells. On the other hand, in the two clonal lines expressing the S17N-Ras^H mutant, NGF had a reduced effect on ERK activity. The suppression of the NGF effect was less pronounced in 17N-3 cells (Fig. 2*A Left*), which express less of the Ras mutant (Fig. 1*A*), but the effect of NGF was consistently blocked in 17N-1 cells (Fig. 2*A Right*). The lack of effect of NGF on ERK activity was not due to a loss of ERK1 or ERK2 from the lines harboring the

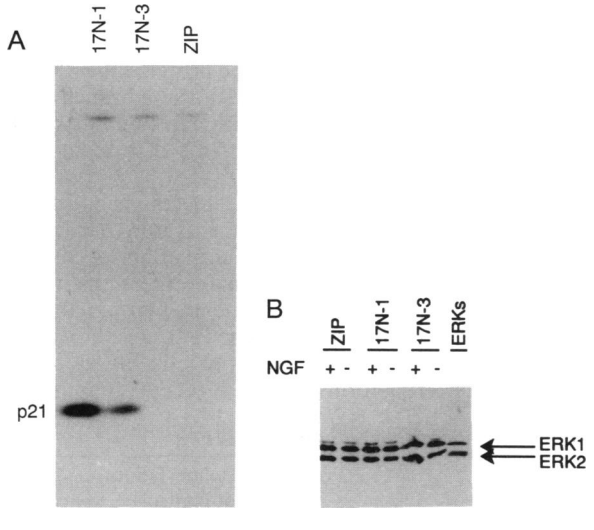

FIG. 1. S17N-Ras^H p21 and ERKs 1 and 2 in PC-12 cell lines. (*A*) PC-12 clones expressing either S17N-Ras^H (17N-1 and 17N-3) or no exogenous Ras gene (Zip) were labeled with [^{35}S]methionine for 18 hr. Cell extracts were immunoprecipitated with the Ras^H-specific antibody YA6-172 as described (19) and the proteins were separated by SDS/PAGE and autoradiographed. (*B*) Amounts of ERK1 and ERK2 in 20 μg of soluble protein from Zip, 17N-1, and 17N-3 lines before and after NGF treatment were compared by Western blotting with antiserum 691 (12) and chemiluminescent detection (Amersham ECL system). A sample of partially purified ERK1 and ERK2 (last lane) was included as standard.

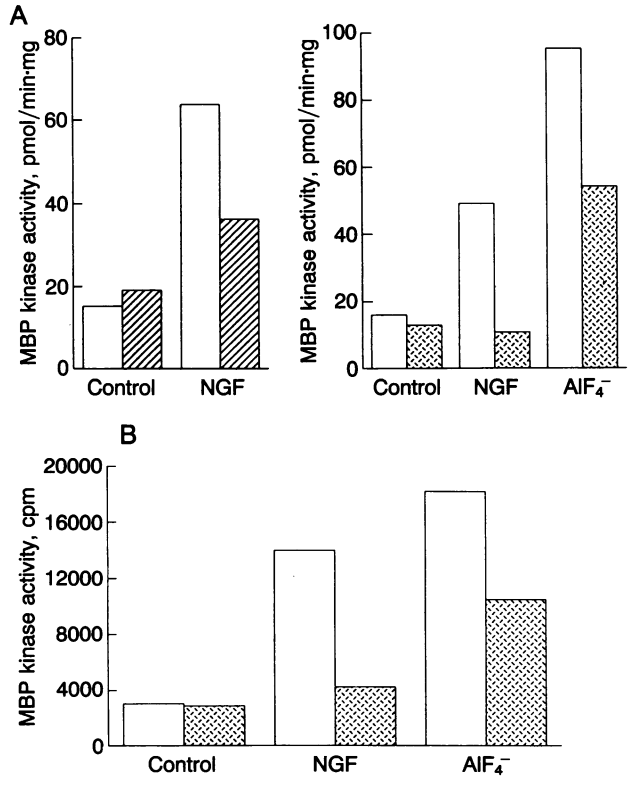

FIG. 2. ERK activity in PC-12 cell lines. (*A*) ERK activity was assayed with MBP as substrate (11) in extracts of Zip and 17N-3 cells either untreated or treated with NGF (75 ng/ml, 5 min) (*Left*) and in Zip and 17N-1 cells untreated or treated with NGF (75 ng/ml, 5 min) or AlF_4^- (30 mM NaF plus 10 μM $AlCl_3$ for 15 min) (*Right*). One of four similar experiments is shown. Open bars, Zip; hatched bars, 17N-3; stippled bars, 17N-1. (*B*) Immune complex kinase assays of these extracts with the ERK1-selective antiserum 837.

Ras mutant, as Western blotting revealed comparable amounts of the kinases in Zip, 17N-1, and 17N-3 lines (Fig. 1*B*). The increased activity was attributable to ERKs rather than protein kinases A or C, because 0.1 μM staurosporine, a potent inhibitor of protein kinases A and C, did not block the changes in activity in supernatants from cells stimulated by either NGF or AlF_4^- (not shown). Further, activity of ERK1 (12) in immune complex kinase assays (Fig. 2*B*) reflected activity measured in extracts.

To ascertain if ERKs could be activated by any mechanism in 17N-1 cells, we tested the ability of the heterotrimeric G protein activator AlF_4^- to activate ERKs. AlF_4^- has been shown to stimulate MAP2/MBP kinase activity in fibroblasts (25) and did so in Zip cells (Fig. 2 *A Right* and *B*). While NGF had little effect on activity in 17N-1 cells, AlF_4^- stimulated kinase activity well in both Zip and 17N-1 cells, although its effect was modestly reduced in 17N-1 cells. The finding with AlF_4^- is in support of the idea that certain heterotrimeric G proteins do not require Ras to stimulate ERKs (26). We conclude that the ERKs in these cells are normal because they are present in normal amounts and because they can be stimulated by a pathway apparently independent of Ras.

ERKs 1 and 2 must be phosphorylated on both tyrosine and threonine residues to be fully active (12, 13, 27). One possible explanation for the loss of stimulation of the ERKs in 17N-1 and -3 cells would be that phosphorylation on tyrosine residues occurs normally, while phosphorylation on threonine residues does not occur. To determine the effect of the Ras mutant on the phosphorylation of the ERKs, we immunoprecipitated the kinases from untreated and NGF-treated PC-12 Zip and 17N-1 lines labeled with ^{32}P. A 5-min exposure

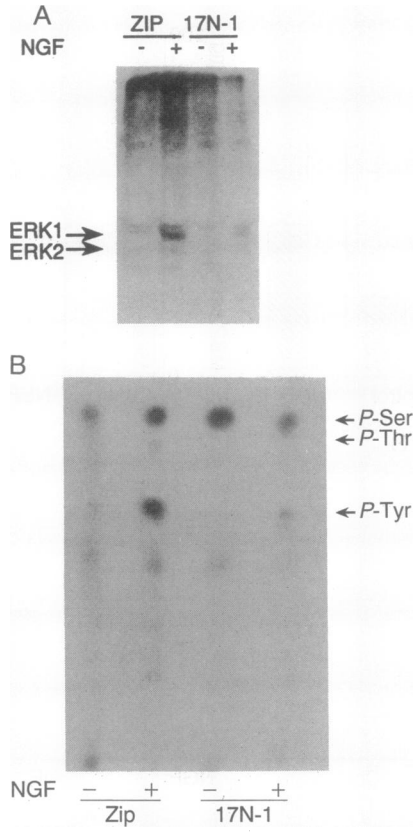

FIG. 3. Immunoprecipitation of ^{32}P-labeled ERKs from PC-12 cells treated with NGF. (*A*) ERKs were immunoprecipitated with antiserum 837 from ^{32}P-labeled Zip and 17N-1 cells with or without treatment for 5 min with NGF at 75 ng/ml as described (24) and analyzed by SDS/PAGE and autoradiography. The positions of ERK1 and ERK2 are indicated with arrows. One of four experiments is shown. (*B*) Phosphoamino acid analysis of immunoprecipitated ERK1. ERK1 was excised from the gel shown in *A* and subjected to phosphoamino acid analysis. As noted previously (24), phosphoserine was detected in ERK1 from NGF-treated 17N-1 and Zip cells and untreated 17N-1 cells, although its relationship to enzyme activity is unclear. Phosphotyrosine was consistently recovered in excess of phosphothreonine in these cells; however, the reason for the large relative recovery of phosphotyrosine is unknown.

to NGF resulted in a dramatic increase in ^{32}P incorporation into ERK1 in Zip cells (Fig. 3*A*). In contrast, there was a less pronounced increase in ^{32}P incorporation into ERK1 elicited by NGF in 17N-1 cells. Phosphorylation of ERK2 was also decreased, although the ERK2 band is very light in the autoradiogram shown. Phosphoamino acid analysis of ERK1 (Fig. 3*B*) revealed phosphotyrosine and phosphothreonine in ERK1 from NGF-stimulated Zip cells but much less NGF-stimulated accumulation of phosphotyrosine and phosphothreonine in ERK1 from 17N-1 cells. These results demonstrate that the Ras mutant interferes with the NGF-dependent phosphorylation of ERKs on both kinds of residues.

Because NGF-stimulated ERK activity and phosphorylation were depressed in cells expressing the Ras mutant, we evaluated the capacity of fractionated cell extracts to bring about the phosphorylation of exogenous ERK2, as an assay of ERK/MAP2 kinase activator (13–15). Supernatants were fractionated by anion-exchange chromatography on DEAE-cellulose, and their ERK-phosphorylating activities were measured with purified recombinant ERK2 as substrate (Fig. 4*A*). Fractions from Zip cells treated with NGF and AlF_4^- displayed an enhanced ability, compared with fractions from untreated cells, to promote the phosphorylation of ERK2. ERK2 alone was not phosphorylated under these conditions (not shown), and heat-inactivated DE-52 fractions no longer caused ERK2 phosphorylation (Fig. 4*B*). Fractions from 17N-1 cells treated with NGF increased the phosphorylation of ERK2 very little relative to the comparable fraction from untreated cells. Thus, the dominant negative Ras mutant prevented stimulation of the ERK/MAP2 kinase activator by NGF. The activator fraction from 17N-1 cells treated with AlF_4^- increased ERK2 phosphorylation dramatically, indicating that this step in the cascade could be activated in a manner apparently independent of Ras. To document further the inhibitory effect of the interfering Ras mutant on ERK activator, the activator-stimulated incorporation of phosphate into ERK2 was measured as a function of ERK2 concentration (Fig. 4*C*) and time of incubation (not shown). ERK2 phosphorylation was significantly greater under all assay conditions when material from NGF-stimulated Zip cells was used compared with material from NGF-stimulated 17N-1 cells.

Finally, we measured the last of the three steps in the cascade, S6 protein kinase activity. As shown in Table 1, a 5-min exposure to NGF stimulated S6 kinase activity measured in supernatants from Zip cells 2-fold, while 30 min of NGF treatment resulted in a 5-fold increase in S6 kinase activity. On the other hand, in 17N-1 cell supernatant there was no detectable effect of NGF on S6 kinase activity at 5 min and only a 1.4-fold effect at 30 min, further demonstrating that impaired Ras function interfered with this cascade.

DISCUSSION

These studies indicate that Ras is required for the control of this ERK cascade by NGF. Inhibition of all three steps by the interfering Ras mutant suggests that Ras must participate downstream from the NGF receptor and upstream from the first of the three measured steps, ERK activator. It has been suggested that GAP may provide the communication between tyrosine kinases and Ras, because GAP is a tyrosine kinase substrate (3). However, without considering how Ras is linked to receptors, we can now propose the following order of steps in this pathway: NGF receptor → ? → Ras → ? → ERK activator → ERK → S6 kinase. The MAP2 kinases ERK1 and ERK2 not only stimulate S6 kinase but also may control Raf, another serine/threonine protein kinase (30, 31). Thus, Raf may be downstream of ERKs at a branch point in this cascade. This is consistent with the finding that Ras-neutralizing antibodies block the effects of tyrosine kinase oncogenes but not the effects of *raf* (4).

Formally, there are at least three mechanisms by which Ras may control this cascade. First, Ras may participate in this pathway by influencing the activation of the NGF receptor itself; second, Ras may enable the signaling intermediates to interact in the appropriate manner; or, third, Ras may act directly in this cascade. The first possibility can be tested by a careful analysis of NGF receptor function in cells expressing interfering forms of Ras. A recent study by Wood

Table 1. S6 kinase activity in PC-12 cells

Treatment	S6 phosphorylation, % of control	
	Zip	17N-1
No NGF	100	100
NGF 5 min	184	100
NGF 30 min	566	141

S6 protein kinase activity was assayed with 40S ribosomal subunits (9) with 10 μg of soluble protein from untreated cells or cells treated with NGF for 5 or 30 min. There are at least two types of S6 kinase, rsk and "70 K" enzymes (28). Because of recent evidence that ERKs may control both types (29), no effort to distinguish between them was made. One of three similar experiments is shown.

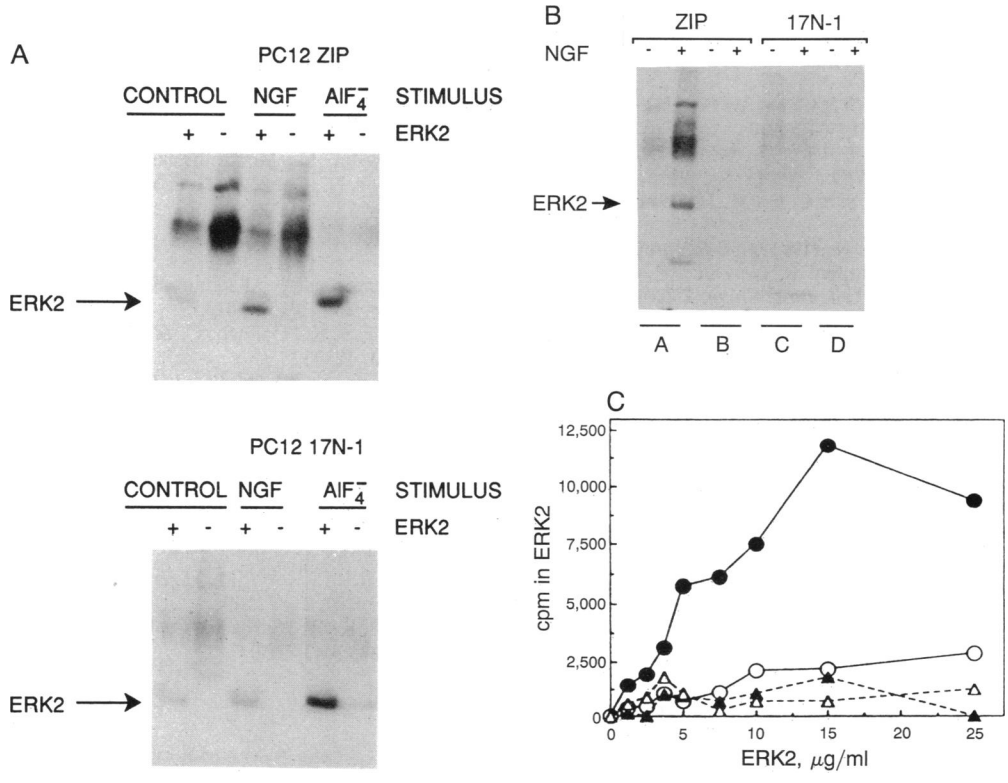

FIG. 4. ERK activator in PC-12 cells. Protein (12 μg) not bound to the DEAE-cellulose columns was assayed for the ability to promote phosphorylation of purified recombinant ERK2 in a mixture containing 10 μM [γ-^{32}P]ATP (4660 cpm/pmol), 10 mM $MgCl_2$, and 0.5 mM EGTA. (*A*) Assays with DEAE-cellulose fractions from the indicated cells included no exogenous ERK2 in lanes designated − and ERK2 at 15 μg/ml in lanes designated +. (*B*) Activator fractions were boiled for 5 min (lane pairs B and D) and mixed with ERK2 from both Zip (lane pairs A and B) and 17N-1 (lane pairs C and D) cells untreated (−) or treated with NGF at 75 ng/ml (+). Assays with DEAE-cellulose fractions that were not heated (lane pairs A and C) are also shown. (*C*) Assays of ERK2 phosphorylation by DEAE-cellulose fractions with increasing concentrations of ERK2. ○, Unstimulated Zip cells; ●, NGF-treated Zip cells; △, unstimulated 17N-1 cells; ▲, NGF-treated 17N-1 cells.

et al. (32) suggests that NGF receptors function normally in cells expressing this form of Ras. Testing the second possibility will await reconstitution of cascade components, as they become available *in vitro* in the absence of Ras. If the final possibility, that Ras participates directly in this cascade, is correct, there appears to be at least one intermediate in this NGF-dependent protein kinase cascade, Ras, that is not itself a protein kinase. Two reports (18, 32) that appeared after submission of this manuscript for review arrived at similar conclusions regarding the role of Ras in this cascade.

This cascade can also be activated in a manner apparently independent of Ras. Okadaic acid caused a significant activation of ERKs in cells expressing the Ras mutant (M.C., E.Z., and M.H.C., unpublished findings). The heterotrimeric G protein activator AlF_4^-, which has not been shown to activate Ras (T. Higashijima, personal communication), also stimulated the cascade in the absence of documentable Ras function, implying that certain G proteins might control the pathway in a Ras-independent manner. In cells expressing the Ras mutant, AlF_4^- elicited a significant but smaller effect than in cells without this mutant. AlF_4^- will activate many heterotrimeric G proteins, including those able to stimulate phospholipases and thereby the production of inositol phosphates and diacylglycerol. Thus, the inhibition noted may be due to a protein kinase C-dependent pathway. In support of this possibility, we find that the ability of phorbol esters to stimulate ERKs is also blocked in cells expressing the dominant-interfering Ras mutant.

The extracellular signals that regulate this three-component cascade most likely converge at or before the ERK activator. Because Ras may immediately precede the ERK activator in the cascade examined here, this latter protein may be one of the elusive Ras targets. Limited information regarding the activator (13–15) indicates that it is regulated by serine/threonine phosphorylation; either it phosphorylates itself or it is phosphorylated by a kinase upstream of it in the cascade. The possible juxtaposition of Ras and a kinase in this cascade raises the interesting question of whether protein kinases are targets controlled directly by Ras.

We thank Nikos Panayotatos (Regeneron Pharmaceuticals) for *Escherichia coli* containing recombinant ERK2, Rony Seger and Natalie Ahn (University of Washington) for valuable advice about the fractionation and assay of ERK activator, Doug Ebert for purification of recombinant ERK2, Paul Sternweis and Elliott Ross for helpful discussions and critical evaluation of the manuscript, and Jo Hicks for preparation of the manuscript. This work was supported by a grant from the Texas Advanced Research Program, by Research Grant DK34128 and Research Career Development Award DK01918 (to M.H.C.), Training Grant GM07062 (to D.J.R.), and Research Grant CA47391 from the National Institutes of Health, and by Grant JFRA-222 (to L.A.F.) from the American Cancer Society. This work was completed in partial fulfillment of the requirements for the Ph.D. degrees of D.J.R. and M.C. at the Southwestern Graduate School of Biomedical Sciences.

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