MOLECULAR AND CELLULAR BIOLOGY, Mar. 1992, p. 936–945
0270-7306/92/030936-10$02.00/0

Vol. 12, No. 3

Regulation of Tetradecanoyl Phorbol Acetate-Induced Responses in NIH 3T3 Cells by GAP, the GTPase-Activating Protein Associated with p21$^{c\text{-}ras}$

MUKUND NORI, GILLES L'ALLEMAIN,† AND MICHAEL J. WEBER*

Department of Microbiology, Box 441, University of Virginia School of Medicine, Charlottesville, Virginia 22908

Received 22 October 1991/Accepted 5 December 1991

Proteins of the *ras* family of oncogenes have been implicated in signal transduction pathways initiated by protein kinase C (PKC) and by tyrosine kinase oncogenes and receptors, but the role that *ras* plays in these diverse signalling systems is poorly defined. The activity of *ras* proteins has been shown to be controlled in part by a cellular protein, GAP (GTPase-activating protein), that negatively regulates p21$^{c\text{-}ras}$ by enhancing its intrinsic GTPase activity. Thus, overexpression of GAP provides a tool for determining the step(s) in signal transduction dependent on p21$^{c\text{-}ras}$ activity. In this paper, we report that overexpression of GAP blocks the phorbol ester (tetradecanoyl phorbol acetate [TPA])-induced activation of p42 mitogen-activated protein kinase (p42^{mapk}), c-*fos* expression, and DNA synthesis. GAP overexpression did not block responses to serum or fluoroaluminate. Moreover, not all biochemical events elicited by TPA were affected by GAP overexpression, as increased glucose uptake and phosphorylation of MARCKS, a major PKC substrate, occurred normally. Reduction of GAP expression to near normal levels restored the ability of the cells to activate p42^{mapk} in response to TPA. These findings suggest that *ras* and GAP together play a key role in a PKC-dependent signal transduction pathway which leads to p42^{mapk} activation and cell proliferation.

The c-*ras* proto-oncogene encodes a 21,000-Da guanine nucleotide-binding protein (5, 11, 43) which is thought to be involved in the regulation of cell growth (5, 11, 46), differentiation (4, 6, 11, 31, 54, 64), and carcinogenesis (13, 21, 49, 56, 68). When microinjected into quiescent fibroblasts, the p21^{ras} proteins have been shown to be potent mitogens (46), and when microinjected into pheochromocytoma PC12 cells, they have been shown to induce neurite outgrowth (31, 54). Activating point mutations in the p21^{ras} protein have been found in a large number of human tumors (1, 5, 19, 60), thus implicating this protein in the biochemical and cellular events involved in the pathogenesis of these malignancies. Despite these advances, the function of p21$^{c\text{-}ras}$ in the regulation of normal cell growth, mitogenic signal transduction, and cell differentiation is still unknown.

It has been well established that the p21$^{c\text{-}ras}$ protein undergoes a cyclic association with guanosine nucleotides (5, 11, 40, 43). The conversion from the active GTP-bound form to the inactive GDP-bound form is catalyzed by the intrinsic GTPase activity of the *ras* protein itself. Studies on the effect of activating point mutations of *ras* on *ras* function led to the discovery of a cytosolic protein that could enhance the GTPase activity of the p21$^{c\text{-}ras}$ protein (62). This GTPase-activating protein (GAP) had no GTPase activity of its own (42, 62), but it could stimulate the GTPase activity of the normal c-*ras* gene product by over 100-fold. Since GAP increased the rate at which p21$^{c\text{-}ras}$ was converted from the active GTP-bound state to the inactive GDP-bound state, it could be considered to be a negative regulator of c-*ras*. Studies that explore the function and activity of the *ras*/GAP complex should, therefore, lead to a better understanding of the role of *ras* in cell metabolism.

A characteristic response of quiescent mammalian cells triggered by a variety of mitogens to enter the cell cycle is phosphorylation of proteins on tyrosine (12, 23, 30, 63). One of these proteins is GAP (16, 28, 37, 44). Activation of tyrosine kinase receptors, such as the platelet-derived growth factor receptor or the epidermal growth factor receptor, with the appropriate ligands, or transformation of cells with the tyrosine kinase oncogene product pp60$^{v\text{-}src}$, resulted in the phosphorylation of GAP on tyrosine (16, 28, 37, 44), suggesting that the *ras*/GAP complex is a component of or is regulated by the tyrosine kinase signal transduction cascade. Anti-*ras* antibodies, when microinjected into the appropriate cells, could block v-*src*-induced transformation of fibroblasts (56) and v-*src*-induced differentiation of PC12 cells (31). We and others have previously shown that overexpression of GAP could also block transformation of fibroblasts by v-*src* (13, 49). Furthermore, the tyrosyl-phosphorylated GAP, unlike the unmodified protein, is found to preferentially associate with the cell membrane (28, 44), the normal cellular location for *ras*, *src*, and tyrosine kinase receptors. Taken together, this evidence strongly supports the concept that the *ras*/GAP complex plays an integral role in the tyrosine kinase signal transduction cascade.

Several laboratories have presented evidence showing a link between *ras*/GAP and protein kinase C (PKC)-mediated signalling pathways (14, 32, 45, 64). It was recently shown that treatment of T cells with tetradecanoyl phorbol acetate (TPA) increased the relative amount of active GTP-bound *ras* in the cell membranes (14). It was demonstrated that this increase was due to a PKC-mediated reduction in GAP activity. Expression of oncogenic *ras* has also been shown to block TPA-induced neuronal differentiation of PC12 cells (32), and expression of a dominant negative *ras* mutant was shown to block TPA-induced DNA synthesis in fibroblasts (10). There appears, therefore, to be a strong connection between the PKC and the *ras*/GAP signalling pathways.

One early signalling step common to both tyrosine kinases and PKC is the activation of p42/mitogen-activated protein

* Corresponding author.
† On leave from the Centre de Biochimie, Centre National de la Recherche Scientifique, Nice, France.

kinase (p42^{mapk}). p42^{mapk} is a serine/threonine protein kinase that becomes activated following treatment of quiescent cells with a variety of mitogens, including insulin, platelet-derived growth factor, epidermal growth factor, fibroblast growth factor, thrombin, and phorbol esters (22, 34, 35, 51, 52). The enzyme is also activated following treatment of nonmitogenic, differentiated cells with stimulatory agonists (17), suggesting that this enzyme plays a fundamental role in several signal transduction pathways (reviewed in references 11a and 59). p42^{mapk} becomes phosphorylated on both tyrosine and threonine during enzymatic activation, and both phosphorylations are essential for maximal activity (3). p42^{mapk} is a member of a family which have also been termed MAP kinases, ERKs, and MBP kinases (reviewed in reference 11a).

Since the activity of p42^{mapk} is regulated by activation of both tyrosine kinases and PKC, and since the *ras*/GAP complex has been shown to be involved in the transmission of signals generated upon activation of tyrosine kinases and PKC, we speculated that *ras*/GAP could be involved in regulation of p42^{mapk}. Furthermore, since agonists that activate p42^{mapk} and *ras*/GAP also induce cell proliferation, we thought that a block or inhibition of the proposed *ras*/GAP/p42^{mapk} pathway might result in a corresponding block of DNA synthesis in response to the specific agonist. To test this hypothesis, we used a cell line that overexpresses GAP, the negative regulator of p21$^{c\text{-}ras}$, and investigated the ability of various agonists to phosphorylate p42^{mapk} on tyrosine and to activate it.

In this paper, we report that overexpression of GAP inhibits the tyrosyl phosphorylation of p42^{mapk} and its activation in response to TPA. Furthermore, overexpression of GAP also blocks the mitogenic response of the cells to TPA but not to serum. Phosphorylation and activation of p42^{mapk} in response to TPA were restored when GAP levels were reduced to nearly that observed in control cells. However, the cells continued to be mitogenically refractory to TPA. These results strongly suggest that *ras*/GAP participates in the regulation of p42^{mapk}.

MATERIALS AND METHODS

Materials. Sources of commercial products were as follows: myelin basic protein (MBP), TPA, deferoxamine mesylate, aluminum chloride, and sodium fluoride, Sigma Chemical Co., St. Louis, Mo.; Tween 20, Bio-Rad, Richmond, Calif.; bovine serum albumin (BSA) fraction V, Boehringer Mannheim Biochemicals, Indianapolis, Ind.; fetal bovine serum, HyClone Laboratories, Inc., Logan, Utah; DE52 (DEAE cellulose), Whatman Biosystems Ltd., Maidstone, England; the phenyl-Superose column, Pharmacia-LKB, Piscataway, N.J.; ^{125}I-labeled protein A, Amersham, Arlington Heights, Ill.; and [^{32}P]H_3PO_4 (9,000 Ci/mmol), [γ-^{32}P]ATP (6,000 Ci/mmol), [α-^{32}P]dCTP (3,000 Ci/mmol), [^{3}H]2-deoxyglucose, and [^{3}H]thymidine, NEN-DuPont Research Products, Wilmington, Del. Specific antiphosphotyrosine antibodies were generated in this laboratory as described previously (26). Rabbit anti-GAP antiserum 637 (65), generated against a peptide spanning residues 139 through 152 of the GAP protein, was the kind gift of J. B. Gibbs (Merck Sharp & Dohme Research Laboratories, West Point, Pa.), and rabbit anti-MARCKS protein antiserum 94 was the kind gift of P. J. Blackshear (Duke University Medical Center, Durham, N.C.).

Cell culture. 3T3GAP4 cells, which overexpressed bovine GAP by about 100-fold (20), and control 3T3V8 cells were obtained from J. B. Gibbs. These cells were grown in minimal essential medium alpha supplemented with 10% dialyzed fetal calf serum (FCS; HyClone) and 1 μM sodium methotrexate (Adria Laboratories). To downmodulate the expression of GAP, 3T3GAP4 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% defined/supplemented bovine calf serum (HyClone) for 6 to 8 weeks before testing. This time was previously determined as the period necessary for enough downmodulation of GAP to allow transformation by v-*src* (49). Cells were rendered quiescent by growing them to confluence.

Purification and analysis of p42^{mapk}. Prior to stimulation with agonists, cells were cultured in the appropriate serum-free medium for 1 h. The dishes were rinsed twice with prewarmed KRB-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (51) (5 ml per rinse) and incubated with 3 ml of KRB-HEPES containing 500 μM deferoxamine mesylate for 20 min at 37°C.

Agonists were added directly to the dishes, and the cells were incubated at 37°C for a further 10 min; FCS was used at a final concentration of 10%, TPA was used at 50 ng/ml, and a freshly prepared stock solution of aluminum fluoride (10 μl of 20 mM aluminum chloride plus 25.14 mg of sodium fluoride in a total of 1 ml of KRB-HEPES) was used at 5 μl/ml. Agonist stimulation was stopped by rapidly chilling the cells on ice and rinsing them twice with ice-cold phosphate-buffered saline.

The cells were lysed either by addition of hot 2× electrophoresis sample buffer for immediate electrophoresis or by the method described by Ray and Sturgill (51). Cell lysates obtained with the latter method were used to partially purify p42^{mapk} as described before (51, 52). The collected fractions, except for 20 μl saved for enzyme assays, were precipitated with deoxycholate-trichloroacetic acid (34, 35). The precipitates were dissolved in 1× hot (90°C) electrophoresis sample buffer, resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and Western immunoblotted with antiphosphotyrosine antibodies (26) as described before (35, 52).

Analysis of whole cell lysates. Lysates of cells treated in a manner identical to that used for purification of p42^{mapk} were made by using hot electrophoresis sample buffer. Lysates of sister plates were made in CIPA (49) for protein determination (41). Equal amounts of total cell protein (75 μg) from each lysate were resolved by SDS-PAGE and Western blotted onto nitrocellulose filters (7). The filters were then immunoblotted for detection of either GAP, using rabbit antiserum 637 (65), or of tyrosyl phosphorylated proteins, using affinity-purified rabbit antiphosphotyrosine antibodies (26), as described before (49).

Cytoplasmic RNA analysis. Cells from several confluent 100-mm^2 tissue culture dishes were harvested, and the cytoplasmic RNAs were extracted by the guanidinium isothiocyanate-cesium chloride method (39). Five micrograms of RNA was loaded into each well and separated by electrophoresis through an agarose-formaldehyde gel (39). The RNA was transferred to a Nytran (Schleicher & Schuell) nylon membrane, immobilized, and hybridized with a DNA probe in buffer containing 50% formamide. The filter was rinsed free of excess probe, dabbed dry, sealed in Saran Wrap, and visualized by autoradiography. The 28S and 18S rRNAs, clearly visible under shortwave UV light, were used as internal size markers. The probe for c-*fos* was the 2-kb *Nco*I-*Xho*I fragment of pc-fos (mouse)-3 from the American Type Culture Collection (catalog no. 41041), and that for chicken glyceraldehyde-3-phosphate dehydrogenase

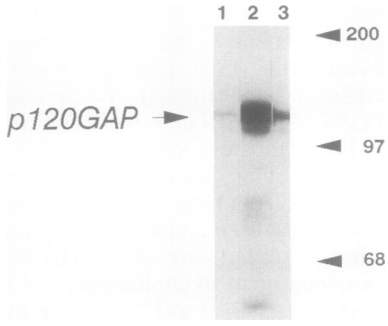

FIG. 1. GAP levels in control cells (3T3V8; lane 1), cells overexpressing GAP (3T3GAP4; lane 2), and cells that have downmodulated GAP expression (3T3GDM; lane 3). Whole cell extracts (75 μg) were subjected to SDS-PAGE, electroblotted onto nitrocellulose, and probed with rabbit anti-GAP antiserum 637 and [^{125}I]protein A. Sizes in kilodaltons are indicated on the right.

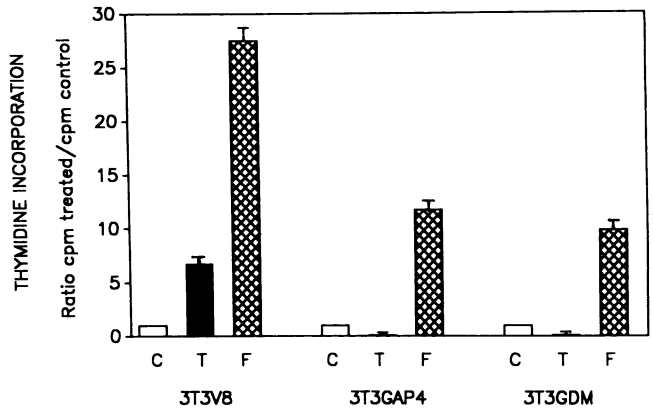

FIG. 2. Mitogenic responses of 3T3V8, 3T3GAP4, and 3T3GDM cells to no mitogen (C), 50 ng of TPA per ml (T), or 10% FCS (F) as measured by [^{3}H]thymidine incorporation. Each bar represents the mean of five experiments, each performed in triplicate.

(GAPDH) was the 1.1-kb *Pst*I fragment isolated from pGAD28 (15). Both probes were radioactively labeled with [α-^{32}P]dCTP by nick translation (39).

Analysis of PKC activity. The PKC activity in cells was determined by the level of phosphorylation of the MARCKS protein in response to various agonists. MARCKS protein phosphorylation was determined by a modification of the method described by Lobaugh and Blackshear (38). Prior to labeling, confluent dishes of the various cell lines were made quiescent by first rinsing them with warm (37°C) serum-free medium and then culturing them for 1 h in the appropriate serum-free medium supplemented with 1% BSA. The cells were then rinsed three times with warm (37°C) KRB-HEPES and then incubated with KRB-HEPES supplemented with 1% BSA and containing [^{32}P]H_3PO_4 (1.39 mCi/ml) for 1 h at 37°C. Agonists, at the concentrations described above for activation of p42^{mapk}, were added directly to the dishes, and the cells were incubated at 37°C for a further 10 min. Agonist stimulation was stopped by rapidly chilling the cells on ice and rinsing them three times with ice-cold KRB-HEPES. The cells were lysed with cold lysis buffer (38). The lysates were passed 10 times through a tuberculin syringe fitted with a 23-gauge needle to break up the membrane fragments and then cleared by centrifugation at 50,000 rpm (Beckman Ti 50 rotor) for 30 min at 4°C. An aliquot (3 μl) of each cell extract supernatant was precipitated with ice-cold trichloroacetic acid. Volumes corresponding to equal numbers of trichloroacetic acid-precipitable counts from each cell extract were used for immunoprecipitation of the MARCKS protein (58). The immunoprecipitates were resuspended in electrophoresis sample buffer (33) and analyzed by 10% SDS-PAGE. The gel was fixed, dried, and visualized by autoradiography.

Other methods. The mitogenic responses of various cell populations to different stimuli were analyzed in a [^{3}H]thymidine incorporation assay (8). The increase in 2-deoxyglucose uptake was measured as described before (66).

RESULTS

Inhibition of TPA-induced DNA synthesis. To establish that the 3T3GAP4 cells used here overexpressed GAP, equal amounts of protein from whole cell extracts were resolved by SDS-PAGE and subjected to Western immunoblotting (7), using rabbit anti-GAP antiserum 637 and [^{125}I]protein A to detect GAP (Fig. 1). The 3T3GAP4 cells exhibited about 100-fold more GAP (lane 2) than did the control 3T3V8 cells (lane 1), as described earlier (20, 49).

Both cell lines were also tested for the ability to synthesize DNA in response to various mitogens as previously described (8). The results (Fig. 2) showed that the control 3T3V8 cells exhibited a 7-fold increase in [^{3}H]thymidine incorporation in response to TPA, whereas the cells overexpressing GAP, 3T3GAP4, consistently exhibited a 7- to 10-fold inhibition of [^{3}H]thymidine incorporation compared with unstimulated controls. When FCS was used as the agonist, the 3T3GAP4 cells showed almost a 12-fold increase in [^{3}H]thymidine incorporation (Fig. 2), indicating that they were not completely defective in the ability to respond to mitogens. Under the same conditions, the control 3T3V8 cells exhibited a 25- to 30-fold increase in [^{3}H]thymidine incorporation. The possible reasons for this difference in the responses of the two cell lines to FCS will be discussed below.

Phosphorylation and activation of p42^{mapk}. Acute (10-min) treatment with TPA at 50 ng/ml induced tyrosyl phosphorylation of a 42-kDa band in the control 3T3V8 cells (Fig. 3, lane 2) but not in cells overexpressing GAP, 3T3GAP4 (lane 5), as detected by using antiphosphotyrosine antibodies to immunoblot whole cell lysates separated by SDS-PAGE. The untreated control 3T3V8 and 3T3GAP4 cell lysates are shown in lanes 1 and 4, respectively. This 42-kDa band has been previously identified by us as p42^{mapk} (52).

The lack of rapid tyrosyl phosphorylation of pp42 in TPA-treated 3T3GAP4 cells is specific to this agonist, since treatment of both cell lines with FCS induced tyrosyl phosphorylation of p42^{mapk} (Fig. 3, lanes 3 and 6). However, the magnitude of the response of 3T3GAP4 cells (lane 6) was less than that of 3T3V8 cells (lane 3). This decreased phosphorylation of pp42 in 3T3GAP4 cells treated with FCS compared with similarly treated 3T3V8 cells probably reflects the fact that phosphorylation of pp42 is at least partially dependent on PKC, even when the stimulating agent is FCS (35). These results indicate that pp42 is present in 3T3GAP4 cells but that the portion of its tyrosyl phosphorylation that is dependent on PKC-mediated signalling is defective.

The p42^{mapk} enzyme can be partially purified from agonist-stimulated cells by sequential anion-exchange and hydrophobic-interaction chromatography (52). The partially purified preparation has been shown to phosphorylate MBP (18)

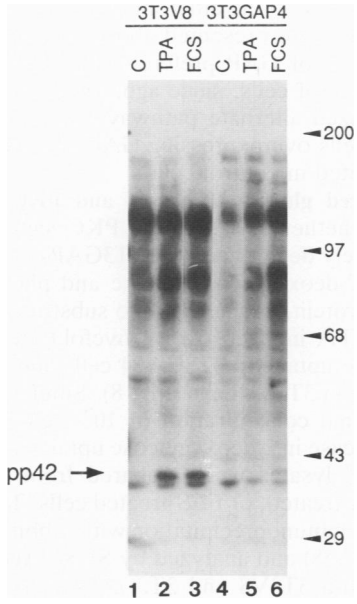

FIG. 3. Phosphorylation of a 42-kDa protein in control cells (3T3V8) and cells overexpressing GAP (3T3GAP4). Whole cell extracts (75 μg) prepared from untreated (C), TPA-treated (T), or FCS-treated (F) cells were subjected to SDS-PAGE, electroblotted onto nitrocellulose, and probed with affinity-purified rabbit antiphosphotyrosine antibodies and [^{125}I]protein A. Sizes in kilodaltons are indicated on the right.

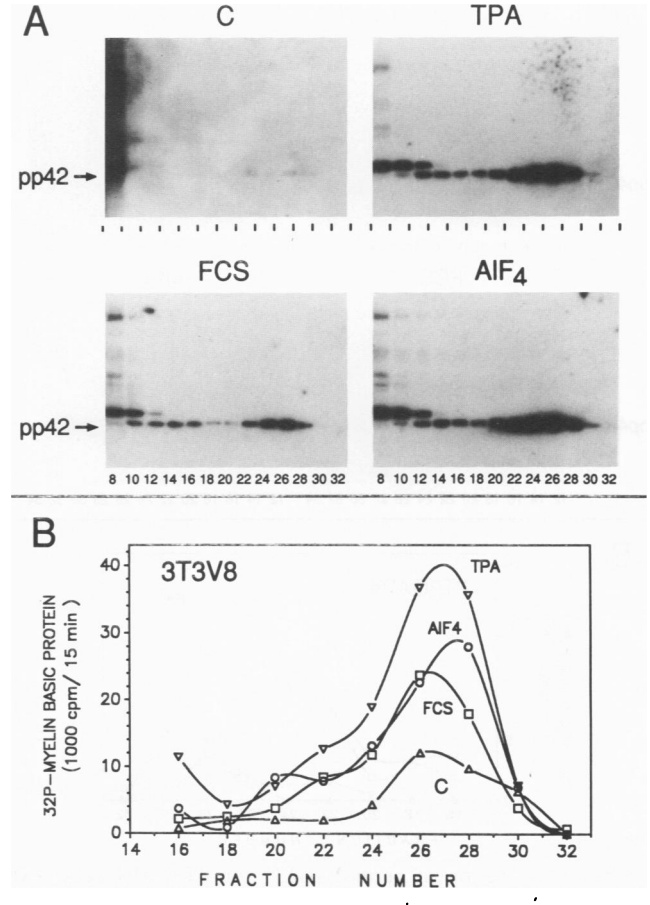

FIG. 4. Activation of purified p42^{mapk} in 3T3V8 cells. (A) Antiphosphotyrosine Western immunoblots of phenyl-Superose-eluted fractions of extracts prepared from untreated (C), TPA-treated (TPA), FCS-treated (FCS), and AlF_4-treated (AlF_4) 3T3V8 cells. (B) p42^{mapk} activity as measured by ^{32}P incorporation, using MBP as an exogenous substrate.

and to migrate as a 42-kDa tyrosyl-phosphorylated protein in one-dimensional SDS-PAGE (52). Under the conditions used, p42^{mapk} is the only kinase and the only phosphoprotein in the peak fractions (50). p42^{mapk} was thus purified from untreated and TPA-, FCS-, or AlF_4-treated 3T3V8 (Fig. 4) and 3T3GAP4 (Fig. 5) cells. AlF_4 has been shown to be capable of activating heterotrimeric G proteins in a ligand-independent (57, 61) manner, and it does not activate small GTP-binding proteins, e.g., p21^{ras} (24). Column fractions were separated by SDS-PAGE and immunoblotted with antiphosphotyrosine antibodies (Fig. 4A and 5A) and were also assayed for MBP kinase activity (Fig. 4B and 5B). Activated p42^{mapk} elutes from phenyl-Superose between fractions 24 and 30 under our conditions. Treatment of cells with TPA, FCS, or AlF_4 stimulated phosphorylation and enzymatic activation of p42^{mapk} as well as an earlier-eluting protein of 44 kDa, which may be the related p44^{mapk}/ERK1 (53).

In the cells overexpressing GAP, unstimulated cultures displayed an early-eluting tyrosyl-phosphorylated 42-kDa protein that displayed little or no MBP kinase activity (Fig. 5A). Stimulation of these cells with FCS or AlF_4 increased the tyrosyl phosphorylation and MBP kinase activity of the p42^{mapk} fractions, but treatment with TPA had no effect (Fig. 5). These results demonstrated that in cells that overexpress GAP, p42^{mapk} was present and activatable by both growth factor-dependent and -independent pathways. Furthermore, they also suggest that the TPA-induced phosphorylation and activation of p42^{mapk} was specifically blocked in these cells.

To facilitate direct comparison of the responses of the different cell lines to the various agonists, the results obtained in the MBP kinase assays were replotted as a histogram (Fig. 6), using the aggregate of kinase activities from fractions 22 to 30. The values were normalized for each cell line, with the response to AlF_4, an agonist that does not activate p21$^{c\text{-}ras}$, given the value of 100%. The results show that the relative MBP kinase activity induced by FCS in 3T3GAP4 cells was similar to that observed in 3T3V8 cells to the same agonist. On the other hand, the relative basal MBP kinase activity in 3T3GAP4 cells was severely reduced compared with that of 3T3V8 cells. Furthermore, in cells overexpressing GAP, there was no increase over relative basal activity in response to treatment with TPA. By contrast, in 3T3V8 cells, TPA induced an increase in relative MBP kinase activity better than did even AlF_4.

Inhibition of c-*fos* gene expression. Since overexpression of GAP blocked DNA synthesis in response to TPA, the 3T3GAP4 cells were tested for their ability to exhibit induction of c-*fos*, as a representative immediate-early gene, in response to various mitogens. Whole cell RNA was isolated from untreated cells and cells treated with TPA or FCS for 30 min and subjected to Northern (RNA) blot analysis (39) using either a c-*fos* probe or a chicken GAPDH probe as a control for the amount of RNA loaded. In both 3T3V8 and 3T3GAP4 cells, c-*fos* was induced within 30 min of treatment with either TPA or FCS (Fig. 7, upper panels). However, the level of c-*fos* induction in response to TPA was much lower in 3T3GAP4 cells than in 3T3V8 cells. In the GAP overex-

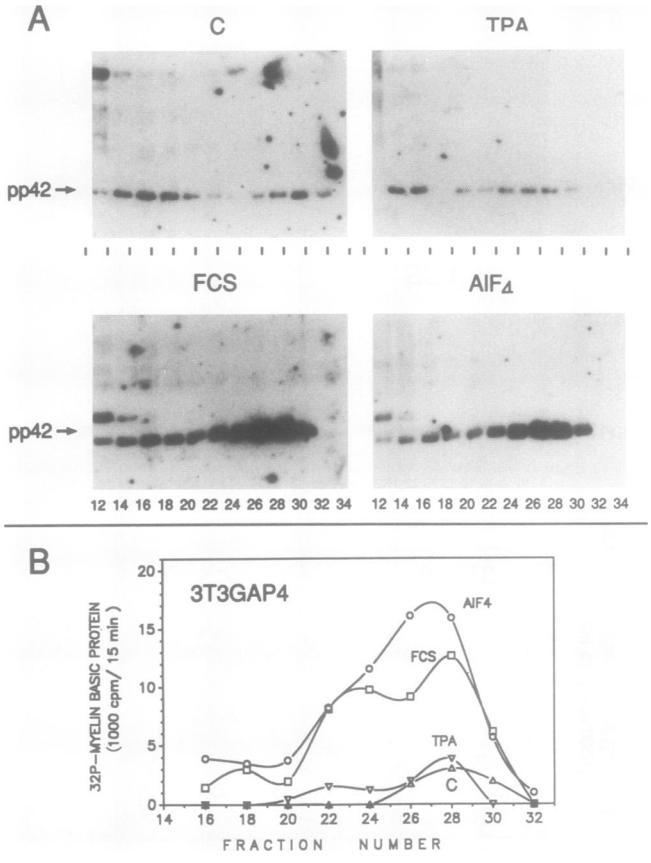

FIG. 5. Activation of purified p42^{mapk} in 3T3GAP4 cells. (A) Antiphosphotyrosine Western immunoblots of phenyl-Superose-eluted fractions of extracts prepared from untreated (C), TPA-treated (TPA), FCS-treated (FCS), and AlF_4-treated (AlF_4) 3T3GAP4 cells. (B) p42^{mapk} activity as measured by ^{32}P incorporation, using MBP as an exogenous substrate.

pressers, the ability of FCS to induce c-*fos* expression was partially blunted, but the induction was readily apparent. The lower panels of Fig. 7, showing the GAPDH mRNA levels, indicate that for a given cell line, similar amounts of RNA were loaded in the different lanes. These results, taken together with those presented above, strongly indicate that overexpression of GAP preferentially blocked PKC-mediated responses of cells, since agonists that either bypassed PKC or utilized alternate pathways continued to elicit responses in cells overexpressing GAP that were comparable to those elicited in control cells.

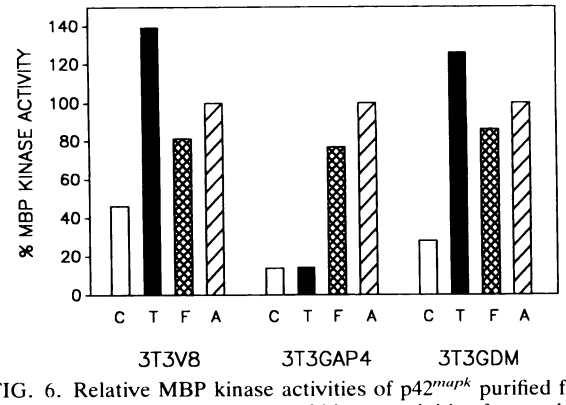

FIG. 6. Relative MBP kinase activities of p42^{mapk} purified from different cell lines. The aggregate of kinase activities from column fractions 22 to 30 was determined for untreated (C), TPA-treated (T), FCS-treated (F), and AlF_4-treated cells. The results for each cell line were normalized to the response to AlF_4 as 100%.

TPA-induced glucose transport and PKC activation. To determine whether PKC, or the PKC signalling pathway, was completely defective in the 3T3GAP4 cells, TPA induction of [^{3}H]2-deoxyglucose uptake and phosphorylation of MARCKS protein, a major in vivo substrate for PKC, were measured. TPA induced over a fivefold increase in [^{3}H]2-deoxyglucose uptake in 3T3GAP4 cells and almost a threefold increase in 3T3V8 cells (Fig. 8). Similar treatment with FCS, at a final concentration of 10%, elicited a three- to fourfold increase in 2-deoxyglucose uptake in both cell lines.

Whole cell lysates were prepared from ^{32}P-labeled untreated, TPA-treated, or FCS-treated cells. The lysates were subjected to immunoprecipitation with rabbit anti-MARCKS antiserum 94 (38) and analyzed by SDS-PAGE as described above. In both 3T3V8 and 3T3GAP4 cells, there was increased MARCKS protein phosphorylation in response to either TPA or FCS (Fig. 9). There was no difference in the responses of either cell line to either agonist. These results, along with the results obtained for [^{3}H]2-deoxyglucose uptake (Fig. 8), indicate that PKC activity and at least some early PKC-mediated responses are intact in the 3T3GAP4 cells and that the blockage in tyrosyl phosphorylation of pp42 and mitogenesis does not reflect a generalized paralysis of PKC-mediated signalling in these cells.

Partial restoration of TPA-induced responses by downmodulation of GAP. The 3T3GAP4 cells were derived from NIH 3T3 cells by transfecting a plasmid carrying both the bovine GAP gene and a mutant dihydrofolate reductase gene on the same piece of DNA and selecting with methotrexate (20). By removing the selective pressure, it should be possible to deamplify the plasmid, resulting in reduced GAP expression. The success of this method of downmodulation of GAP gene expression was determined by analyzing whole cell extracts in an SDS-PAGE/immunoblot assay using rabbit anti-GAP antiserum 637 (65) and [^{125}I]protein A (Fig. 1). GAP protein expression was reduced considerably from that observed in 3T3GAP4 cells (lane 2; from over 100-fold compared with control 3T3V8 cells [lane 1]) to approximately 7-fold in the downmodulated cells, termed 3T3GDM (lane 3). Expression of the GAP protein did not decrease any further despite prolonged culture in the absence of methotrexate (data not shown).

The 3T3GDM cells were tested for their ability to respond to TPA with respect to p42^{mapk} activation. Like the 3T3GAP4 cells (Fig. 5A) from which they were derived, the 3T3GDM cells exhibited a low basal level of pp42 phosphorylation (Fig. 10A) and a basal level of p42^{mapk} activity (Fig. 10B). However, unlike the 3T3GAP4 cells, the 3T3GDM cells showed a marked increase in both pp42 phosphorylation (Fig. 10A) and p42^{mapk} activity (Fig. 10B) in response to acute treatment with TPA for 10 min. The restoration of the TPA-induced p42^{mapk} (MBP kinase) activity in 3T3GDM cells is easily seen in the direct comparison among the three cell lines in Fig. 6. By comparison, the relative responses to FCS are similar in all three cell lines. Since the only apparent difference between the 3T3GAP4 cells and the 3T3GDM cells was in the expression of the GAP protein, these results strongly suggested that overexpression of GAP was respon-

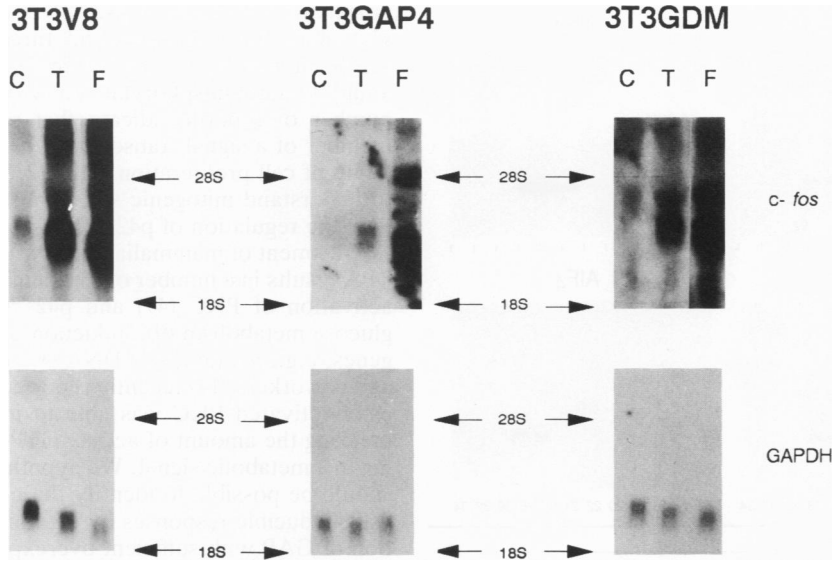

FIG. 7. Northern blot analysis of 5 μg of whole cell RNA isolated from 3T3V8, 3T3GAP4, and 3T3GDM cells. RNA was isolated from either untreated cells (C) or cells treated with 50 ng of TPA per ml (T) or 10% FCS (F) for 30 min. The filters were hybridized to a ^{32}P-labeled c-*fos* probe. Excess label was washed off, and the hybridized RNA was visualized by autoradiography as described previously (39). Filters were stripped of bound label by boiling for 10 min in 0.1× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS and rehybridized with ^{32}P-labeled chicken GAPDH to control for the amount of RNA loaded. The 28S and 18S rRNAs were used as internal size markers.

sible for the inability of the parental 3T3GAP4 cells to phosphorylate and activate p42^{mapk}.

The recovery of TPA-induced p42^{mapk} phosphorylation and activation in 3T3GDM cells raised the possibility that these cells had also recovered their TPA-induced mitogenic response. To test this possibility, the 3T3GDM cells, with the 3T3V8 cells as controls, were assayed for [^{3}H]thymidine incorporation in response to TPA or FCS (Fig. 2). The control 3T3V8 cells exhibited a TPA-induced sevenfold increase of thymidine incorporation. However, the 3T3GDM cells, like the parental 3T3GAP4 cells, were still not stimulated into DNA replication by a similar treatment and exhibited a TPA-induced sevenfold inhibition of thymidine incorporation. The response to FCS was also similar to that of the 3T3GAP4 cells and lower than that observed for the 3T3V8 cells. These results suggest that despite approximately a 10-fold reduction in GAP expression compared with 3T3GAP4 cells, the higher basal level of GAP expression in 3T3GDM cells than in control 3T3V8 cells was sufficient to block TPA-induced mitogenic responses. The lower response to FCS further supported the idea that the PKC-dependent mitogenic pathway was either blocked or defective even in the GAP-downmodulated cells. These results will be treated in greater detail in Discussion.

As before, to ensure that the defect or block was not in all TPA-induced, PKC-dependent responses, the 3T3GDM cells were tested for the ability to exhibit increased c-*fos* gene expression, 2-deoxyglucose uptake, and MARCKS protein phosphorylation upon TPA treatment. There was little or no difference between 3T3GDM cells and 3T3V8

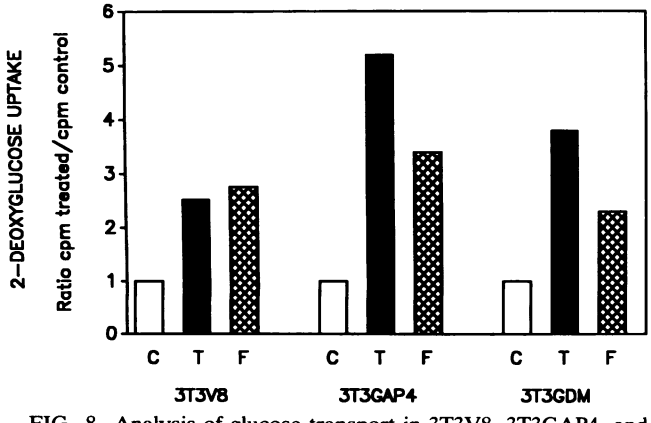

FIG. 8. Analysis of glucose transport in 3T3V8, 3T3GAP4, and 3T3GDM cells. Glucose transport was measured by [^{3}H]2-deoxyglucose uptake (counts per minute per microgram of protein) in response to either no agonist (C), 100 ng of TPA per ml (T), or 10% FCS (F) for 6 h. Each bar represents the mean of two experiments, each performed in triplicate.

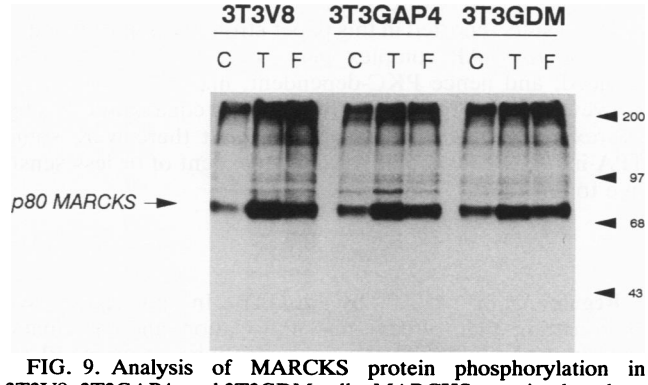

FIG. 9. Analysis of MARCKS protein phosphorylation in 3T3V8, 3T3GAP4, and 3T3GDM cells. MARCKS protein phosphorylation was determined in response to either no agonist (C) or treatment of the cells with 50 ng of TPA per ml (T) or 10% FCS (F) for 10 min. Sizes in kilodaltons are indicated on the right.

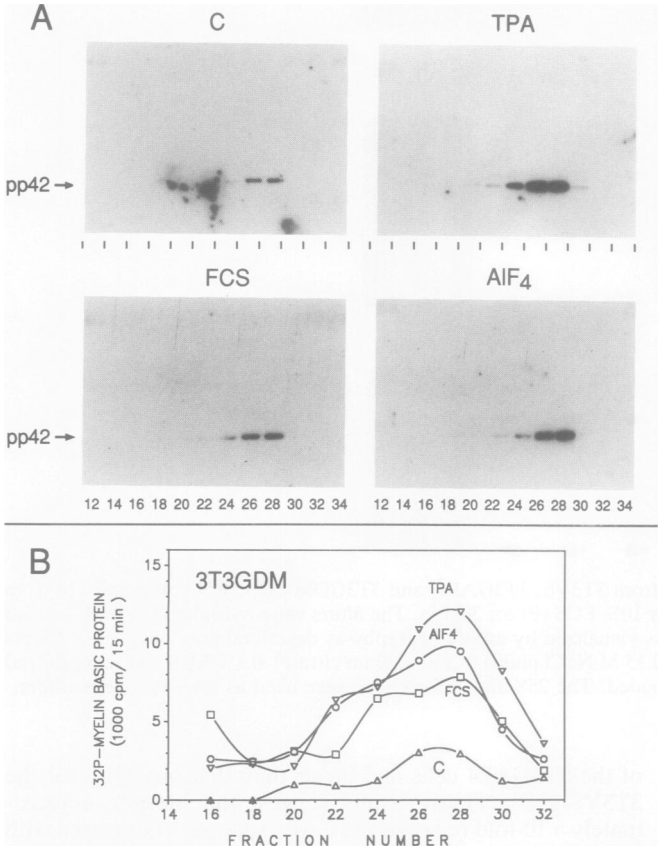

FIG. 10. Activation of purified p42^{mapk} in 3T3GDM cells. (A) Antiphosphotyrosine Western immunoblots of phenyl-Superose-eluted fractions of extracts prepared from untreated (C), TPA-treated (TPA), FCS-treated (FCS), and AlF_4-treated (AlF_4) 3T3GDM cells. (B) p42^{mapk} activity as measured by ^{32}P incorporation, using MBP as an exogenous substrate.

cells with respect to TPA or FCS induction of [^{3}H]2-deoxyglucose uptake (Fig. 8) or MARCKS protein phosphorylation (Fig. 9), indicating that PKC activity and at least part of its signalling pathway were unaffected by manipulations of GAP expression. Furthermore, TPA induction of the c-*fos* gene expression was largely restored in the 3T3GDM cells (Fig. 7), suggesting that part of the blockage of the PKC-dependent signalling mechanism was relieved by the reduction in GAP expression.

The results reported in this paper strongly support the idea that the *ras*/GAP complex plays a central role in TPA-induced, and hence PKC-dependent, mitogenic responses. However, not all responses to TPA were equally affected by overexpression of GAP, suggesting that there were some TPA-induced effects that were independent of or less sensitive to *ras*/GAP.

DISCUSSION

Regulation of p42^{mapk} by *ras*/GAP. In this report, we demonstrate that tyrosyl phosphorylation and enzymatic activation of p42^{mapk} by phorbol esters, which activate PKC, are regulated through p21$^{c\text{-}ras}$. p42^{mapk} is a 42-kDa serine/threonine protein kinase that becomes phosphorylated on tyrosine and activated in cells treated with a wide variety of mitogens (22, 34, 35, 51, 52). It has been shown that enzymatic activation of p42^{mapk} is dependent on phosphorylation on both tyrosine and threonine residues (3). Recently, it has been reported that p42^{mapk} also exhibits an ability to autophosphorylate on tyrosine residues (55, 67). A number of reports indicate that p42^{mapk} is an important member of a signal transduction pathway involved in regulation of cell proliferation (11a, 22, 52, 59). Hence, in order to understand mitogenic signalling, it is important to elucidate the regulation of p42^{mapk}.

Treatment of mammalian cells with phorbol esters such as TPA results in a number of biochemical responses, including activation of PKC (47) and p42^{mapk} (34, 35), increase in glucose metabolism (9), induction of various early-response genes, e.g., *fos* (36), and DNA synthesis (8, 47). Downward and coworkers (14) recently reported that in T cells, phorbol ester-activated PKC was able to inactivate GAP, thus increasing the amount of active, GTP-bound c-*ras* and resulting in a metabolic signal. We hypothesized, therefore, that it should be possible to identify the c-*ras*-dependent, phorbol ester-inducible responses by titrating out the PKC inactivation of GAP with sufficient overexpression of GAP.

To test this hypothesis, we used a cell line, 3T3GAP4, which expresses GAP at 100-fold-greater levels than does the control cell line, 3T3V8. Both the basal and platelet-derived growth factor-induced levels of active GTP-bound c-*ras* are threefold lower in 3T3GAP4 cells than in similarly treated control 3T3V8 cells (20). Hence, c-*ras* is predominantly in the inactive GDP-bound state in cells overexpressing GAP.

As expected, overexpression of GAP inhibited the phosphorylation and activation of p42^{mapk} in response to TPA. When agonists that could activate the PKC-independent signalling pathways, such as FCS, were used, phosphorylation and activation of p42^{mapk} were comparable to those observed in the control 3T3V8 cells (Fig. 6). Moreover, the inhibitory effects of GAP on p42^{mapk} activation could be overcome by prolonged (approximately 1 h) treatment with high concentrations (>1 μg/ml) of TPA, consistent with the idea that the high level of GAP in 3T3GAP4 cells was responsible for the block in TPA responsiveness (data not shown). These results supported our hypothesis and identified tyrosyl phosphorylation and enzymatic activation of p42^{mapk} as components of a PKC-mediated, c-*ras*-dependent signal transduction pathway.

A prediction of this hypothesis is that decreasing the expression of GAP should restore the PKC-mediated response. We have previously shown that the level of GAP expression could be reduced to near that seen in the control cells by culturing the 3T3GAP4 cells in the absence of methotrexate (49). The reduction of GAP expression also restored the susceptibility of these cells to transformation by v-*src* (49). By culturing the 3T3GAP4 cells in the absence of methotrexate, we obtained a cell line, 3T3GDM, that expressed considerably lower levels of GAP. Although the level of GAP expression was over 10-fold lower than that in the parental 3T3GAP4 cells, we were unable, even after prolonged culture, to lower the GAP levels to those of the control 3T3V8 cells (data not shown). The 3T3GDM cells used in these experiments retained GAP levels five- to sevenfold higher than those in the 3T3V8 cells. The significance of the higher basal levels of GAP expression in the 3T3GDM cells will be discussed later.

As predicted, the 3T3GDM cells exhibited TPA-induced phosphorylation and activation of p42^{mapk} similar to that seen with the control 3T3V8 cells. Since the only apparent difference between the 3T3GDM cells and the 3T3GAP4

cells from which they were derived was in the level of GAP expression, we inferred that overexpression of GAP was responsible for blocking the phosphorylation and activation of $p42^{mapk}$, thereby supporting our hypothesis. Hence, in normal cells, TPA activates $p42^{mapk}$ in a PKC- and $p21^{c\text{-}ras}$-dependent manner with GAP as a negative regulator of the system. Whether PKC directly activates $p21^{c\text{-}ras}$ (14) or whether *ras* is required in some other way for aspects of PKC-mediated signalling remains to be determined.

Although our hypothesis is that overexpression of GAP caused an arrest of signals derived from activated PKC, the observations presented above could also result from a defect in TPA-induced activation of PKC or a general paralysis of PKC-mediated signalling. To test this possibility, we examined other cellular responses to TPA in the GAP overexpressers. The 3T3GAP4 cells showed TPA-induced glucose transport and MARCKS protein phosphorylation at levels that were comparable to those observed in control cells. Similar results were also obtained from cells in which GAP expression levels were reduced, 3T3GDM, indicating that these signals were mediated in a *ras*/GAP-independent manner. These results strongly suggested that there was no significant difference between the control cells and those overexpressing GAP with respect to expression of PKC and its activation by TPA.

We have reported parallel results in studies using a fibroblast cell line that was isolated for its inability to respond mitogenically to TPA (8, 48). These cells, called 3T3-TNR9, were also defective in their ability to phosphorylate and activate $p42^{mapk}$ in response to TPA (35). Furthermore, like the 3T3GAP4 cells (49), the 3T3-TNR9 cells were refractory to transformation by v-*src* but not v-*ras* (48). The results reported in this paper, along with our previous results with 3T3-TNR9 cells (35, 48) and those reported by other groups (10, 14, 25, 32), strongly support the model that there is a subset of PKC-initiated signals, one of which is tyrosyl phosphorylation and enzymatic activation of $p42^{mapk}$, that are dependent on c-*ras* and which can be blocked by overexpression of GAP.

Regulation of phorbol ester-induced DNA synthesis. There are several reports showing tyrosyl phosphorylation and activation of $p42^{mapk}$ in response to a variety of mitogens (22, 35, 51, 52). These studies suggested that $p42^{mapk}$ may play an important role in regulation of cell proliferation. Since PKC-mediated activation of $p42^{mapk}$ could be regulated by GAP, we speculated that PKC-dependent mitogenesis and immediate-early gene expression may be similarly regulated. On the basis of the model suggested above, we predicted that TPA-induced DNA synthesis and c-*fos* gene expression would be blocked in cells overexpressing GAP.

We found that overexpression of GAP inhibited both c-*fos* gene expression and TPA-induced DNA synthesis in these cells. Both responses were induced by FCS, indicating that the cells were defective only in a PKC-dependent pathway for these responses. The mitogenic response of 3T3GAP4 cells to FCS was also lower than that seen in the control 3T3V8 cells, suggesting that the mitogenic response of 3T3GAP4 cells to FCS was due solely to PKC-independent mechanisms and that the PKC-dependent pathways were blocked.

According to the model, DNA synthesis in response to TPA treatment should be blocked by overexpressing GAP; i.e., there should be no difference in [^{3}H]thymidine incorporation in 3T3GAP4 cells treated with TPA compared with untreated cells. Our results show that there was not only a prevention of DNA synthesis but an inhibition of mitogenic response to TPA, indicated by a decrease in [^{3}H]thymidine incorporation in the 3T3GAP4 cells treated with TPA compared with untreated cells. Furthermore, there was no restoration of TPA-induced mitogenic response when GAP levels were reduced. TPA-induced DNA synthesis in 3T3GDM cells was still impaired and comparable to that observed in the parental 3T3GAP4 cells. These results suggested two possibilities: (i) that the level of GAP expression was still high enough to inhibit the mitogenic response of 3T3GDM cells to TPA (although low enough for activation of $p42^{mapk}$ and induction of c-*fos* mRNA), or (ii) that there was another mutation in the 3T3GAP4 cells that was responsible for the inhibition of mitogenic response to TPA. In either case, there is an implied existence of a TPA/PKC-specific mitogenic signal that is separate from induction of glucose transport, MARCKS protein phosphorylation, immediate-early gene expression, and activation of $p42^{mapk}$. The nature of such a mitogenic signal is completely unknown. The first possibility suggests that GAP can interact with such a signal and behave not just as a negative regulator but as an inhibitor of the signal; the second possibility suggests that the mutation was independent of GAP and was a dominant inhibitor specific to the PKC-mediated mitogenic signal. Neither possibility can be ruled out at present. Furthermore, the observations reported in this paper strongly suggest that activation of $p42^{mapk}$ was not sufficient for induction of mitogenesis since in 3T3GDM cells, $p42^{mapk}$ could be activated by TPA in the absence of DNA synthesis. So far, there have been no observations reported of induction of DNA synthesis in the absence of $p42^{mapk}$ activation. Hence, our results are consistent with the possibility that activation of $p42^{mapk}$ is a necessary intermediate for mitogenic responses.

There is at least one additional possible explanation for the observations reported in this paper. There have been several reports implicating the *src*-homology 2 (SH2) regions of proteins in intermolecular interactions (2, 27, 29). The SH2 peptide fragments have been shown to mimic the protein-protein interactions exhibited by the intact protein (2, 29), and mutations in the SH2 regions have been shown to block such interactions (27). The amino-terminal half of GAP has two such SH2 regions, while the GTPase-activating property resides in the carboxy-terminal half of the protein. Therefore, a formal possibility could be that when GAP is highly overexpressed, as in 3T3GAP4 cells, a portion of the overexpressed GAP acts as a negative regulator of c-*ras* and the rest acts as an SH2 peptide serving to interfere in and inhibit SH2-dependent protein-protein interactions. However, our own results (unpublished data) and those reported by DeClue et al. (13) suggest otherwise. Overexpression of the amino-terminal half of GAP, which contains the SH2 domains, did not block transformation by v-*src*, whereas overexpression of the carboxy-terminal half of GAP, which contains the GTPase-activating activity, did (13). These results strongly support our belief that the overexpressed GAP is acting primarily as a negative regulator of c-*ras* and not as an SH2 sink. Hence, we conclude that GAP, by blocking c-*ras* activity, is also blocking the arms of the tyrosine kinase and PKC signal transduction pathways that are dependent on c-*ras*.

In this paper, we have proposed a model for a PKC-derived signal transduction pathway that is dependent on active $p21^{c\text{-}ras}$ for its transmission. We have also established that a member of this pathway is $p42^{mapk}$, a molecule implicated in playing a central role in cell proliferation and other cell functions. As studies on the regulation of $p42^{mapk}$

proceed, opportunities should arise to investigate biochemically the roles of c-*ras* and GAP in this process.

ACKNOWLEDGMENTS

This work was supported by grants CA 39076, CA 40042, and CA 47815 from the National Institutes of Health. G.L. was a Fogarty postdoctoral fellow (grant TWO 4196) supported by INSERM.

We thank Jay Gibbs for the generous gifts of anti-GAP antisera and cells overexpressing GAP, and we thank Perry Blackshear for kindly providing the anti-MARCKS antiserum.

REFERENCES

1. **Almoguera, C., D. Shibata, K. Forrester, J. Martin, N. Arnheim, and M. Perucho.** 1988. Most human carcinomas of the exocrine pancreas contain mutant c-K-*ras* genes. Cell **53:**549–554.
2. **Anderson, D., C. A. Koch, L. Grey, C. Ellis, M. F. Moran, and T. Pawson.** 1990. Binding of SH2 domains of phospholipase $C_\gamma 1$, GAP, and src to activated growth factor receptors. Science **250:**979–982.
3. **Anderson, N. G., J. L. Maller, N. K. Tonks, and T. W. Sturgill.** 1990. Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. Nature (London) **343:**651–653.
4. **Balch, W. E.** 1990. Small GTP-binding proteins in vesicular transport. Trends Biochem. Sci. **15:**473–477.
5. **Barbacid, M.** 1987. *ras* genes. Annu. Rev. Biochem. **56:**779–827.
6. **Benito, M., A. Porras, A. R. Nebreda, and E. Santos.** 1991. Differentiation of 3T3-L1 fibroblasts to adipocytes induced by transfection of *ras* oncogenes. Science **253:**565–568.
7. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112:**195–203.
8. **Butler-Gralla, E., and H. R. Herschman.** 1981. Variants of 3T3 cells lacking mitogenic response to the tumor promoter tetradecanoyl-phorbol-acetate. J. Cell. Physiol. **107:**59–67.
9. **Butler-Gralla, E., and H. R. Herschman.** 1983. Glucose uptake and ornithine decarboxylase activity in tetradecanoyl phorbol acetate non-proliferative variants. J. Cell. Physiol. **114:**317–320.
10. **Cai, H., J. Szeberényi, and G. M. Cooper.** 1990. Effect of a dominant inhibitory Ha-*ras* mutation on mitogenic signal transduction in NIH 3T3 cells. Mol. Cell. Biol. **10:**5314–5323.
11. **Chardin, P.** 1991. Small GTP-binding proteins of the ras family: a conserved functional mechanism? Cancer Cells **3:**117–126.

11a. **Cobb, M. H., T. G. Boulton, and D. J. Robins.** 1991. Extracellular signal-regulated kinases: ERKs in progress. Cell Reg. **2:**965–978.

12. **Cooper, J. A., B. M. Sefton, and T. Hunter.** 1984. Diverse mitogenic agents induce the phosphorylation of two related 42,000-dalton proteins on tyrosine in quiescent chick cells. Mol. Cell. Biol. **4:**30–37.
13. **DeClue, J. E., K. Zhang, P. Redford, W. C. Vass, and D. R. Lowy.** 1991. Suppression of *src* transformation by overexpression of full-length GTPase-activating protein (GAP) or of the GAP C terminus. Mol. Cell. Biol. **11:**2819–2825.
14. **Downward, J., J. D. Graves, P. H. Warne, S. Rayter, and D. A. Cantrell.** 1990. Stimulation of $p21^{ras}$ upon T-cell activation. Nature (London) **346:**719–723.
15. **Dugaiczyk, A., J. A. Haron, E. M. Stone, O. E. Dennison, K. N. Rothblum, and R. J. Schwartz.** 1983. Cloning and sequencing of a deoxyribonucleic acid copy of glyceraldehyde-3-phosphate dehydrogenase messenger ribonucleic acid isolated from chicken muscle. Biochemistry **22:**1605–1613.
16. **Ellis, C., M. Moran, F. McCormick, and T. Pawson.** 1990. Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases. Nature (London) **343:**377–381.
17. **Ely, C. M., K. M. Oddie, J. S. Litz, A. J. Rossomando, S. B. Kanner, T. W. Sturgill, and S. J. Parsons.** 1990. A 42-kD tyrosine kinase substrate linked to chromaffin cell secretion exhibits an associated MAP kinase activity and is highly related to a 42-kD mitogen-stimulated protein in fibroblasts. J. Cell Biol. **110:**731–742.
18. **Erickson, A. K., D. M. Payne, P. Martino, A. J. Rossomando, J. Shabanowitz, M. J. Weber, D. F. Hunt, and T. W. Sturgill.** 1990. Identification by mass spectrometry of Thr-97 in bovine myelin basic protein as a specific phosphorylation site for MAP (mitogen-activated protein) kinase. J. Biol. Chem. **265:**19728–19735.
19. **Forrester, K., C. Almoguera, K. Han, W. E. Grizzle, and M. Perucho.** 1987. Detection of high incidence of K-*ras* oncogenes during human colon tumorigenesis. Nature (London) **327:**298–303.
20. **Gibbs, J. B., M. S. Marshall, E. M. Scolnick, R. A. F. Dixon, and U. S. Vogel.** 1990. Modulation of guanine nucleotides bound to ras in NIH3T3 cells by oncogenes, growth factors, and the GTPase activating protein (GAP). J. Biol. Chem. **265:**20437–20442.
21. **Gilbert, P. X., and H. Harris.** 1988. The role of *ras* oncogene in the formation of tumours. J. Cell Sci. **90:**433–446.
22. **Hoshi, M., E. Nishida, and H. Sakai.** 1988. Activation of a Ca^{2+}-inhibitable protein kinase that phosphorylates microtubule-associated protein 2 *in vitro* by growth factors, phorbol esters, and serum in quiescent cultured human fibroblasts. J. Biol. Chem. **263:**5396–5401.
23. **Hunter, T., and J. A. Cooper.** 1984. Rapid tyrosine phosphorylation of a 42,000 dalton protein is a common response to many mitogens. Cancer Cells **2:**61–68.
24. **Kahn, R. A.** 1991. Fluoride is not an activator of the smaller (20–25 kDa) GTP-binding proteins. J. Biol. Chem. **266:**15595–15597.
25. **Kamata, T., N. F. Sullivan, and M. W. Wooten.** 1987. Reduced protein kinase C activity in a *ras*-resistant cell line derived from Ki-MSV transformed cells. Oncogene **1:**37–46.
26. **Kamps, M. P., and B. M. Sefton.** 1988. Identification of multiple novel polypeptide substrates of the v-*src*, v-*yes*, v-*fps*, v-*ros*, and v-*erb*-B oncogenic tyrosine protein kinases utilizing antisera against phosphotyrosine. Oncogene **2:**305–315.
27. **Kanner, S. B., A. B. Reynolds, H.-C. R. Wang, R. R. Vines, and J. T. Parsons.** 1991. The SH2 and SH3 domains of $pp60^{src}$ direct stable association with tyrosine phosphorylated proteins p130 and p110. EMBO J. **10:**1689–1698.
28. **Kaplan, D. R., D. K. Morrison, G. Wong, F. McCormick, and L. T. Williams.** PDGF β-receptor stimulates tyrosine phosphorylation of GAP and association of GAP with a signaling complex. Cell **61:**125–133.
29. **Koch, C. A., D. Anderson, M. F. Moran, C. Ellis, and T. Pawson.** 1991. SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. Science **252:**668–674.
30. **Kohno, M.** 1985. Diverse mitogenic agents induce rapid phosphorylation of a common set of cellular proteins at tyrosine in quiescent mammalian cells. J. Biol. Chem. **260:**1771–1779.
31. **Kremer, N. E., G. D'Arcangelo, S. M. Thomas, M. DeMarco, J. S. Brugge, and S. Halegoua.** 1991. Signal transduction by nerve growth factor and fibroblast growth factor in PC12 cells requires a sequence of src and ras actions. J. Cell Biol. **115:**809–819.
32. **Lacal, J. C., A. Cuadrado, J. E. Jones, R. Trotta, D. E. Burstein, T. Thomson, and A. Pellicer.** 1990. Regulation of protein kinase C activity in neuronal differentiation induced by the N-*ras* oncogene in PC-12 cells. Mol. Cell. Biol. **10:**2983–2990.
33. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
34. **L'Allemain, G., J. Pouyssegur, and M. J. Weber.** 1991. p42/mitogen-activated protein kinase as a converging target for different growth factor signaling pathways: use of pertussis toxin as a discrimination factor. Cell Regul. **2:**675–684.
35. **L'Allemain, G., T. W. Sturgill, and M. J. Weber.** 1991. Defective regulation of mitogen-activated protein kinase activity in a 3T3 cell variant mitogenically nonresponsive to tetradecanoyl phorbol acetate. Mol. Cell. Biol. **11:**1002–1008.
36. **Lim, R. W., B. C. Varnum, and H. R. Herschman.** 1987. Cloning

of tetradecanoyl phorbol ester-induced 'primary response' sequences and their expression in density-arrested Swiss 3T3 cells and a TPA non-proliferative variant. Oncogene **1:**263–270.

37. **Liu, X., and T. Pawson.** 1991. The epidermal growth factor receptor phosphorylates GTPase-activating protein (GAP) at Tyr-460, adjacent to the GAP SH2 domains. Mol. Cell. Biol. **11:**2511–2516.
38. **Lobaugh, L. A., and P. J. Blackshear.** 1990. Neuropeptide Y stimulation of myosin light chain phosphorylation in cultured aortic smooth muscle cells. J. Biol. Chem. **265:**18393–18399.
39. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. **Manne, V., S. Yamazaki, and H.-F. Kung.** 1984. Guanosine nucleotide binding by highly purified Ha-*ras*-encoded p21 protein produced in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **81:**6953–6957.
41. **Markwell, M. A. K., S. M. Haas, L. L. Bieber, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87:**206–210.
42. **McCormick, F.** 1990. The world according to GAP. Oncogene **5:**1281–1283.
43. **McGrath, J. P., D. J. Capon, D. V. Goeddel, and A. D. Levinson.** 1984. Comparative biochemical properties of normal and activated human *ras* p21 protein. Nature (London) **310:**644–649.
44. **Molloy, C. J., D. P. Bottaro, T. P. Fleming, M. S. Marshall, and J. B. Gibbs.** 1990. PDGF induction of tyrosine phosphorylation of GTPase activating protein. Nature (London) **342:**711–714.
45. **Morris, J. D. H., B. Price, A. C. Lloyd, A. J. Self, C. J. Marshall, and A. Hall.** 1988. Scrape-loading of Swiss 3T3 cells with ras protein rapidly activates protein kinase C in the absence of phosphoinositide hydrolysis. Oncogene **4:**27–31.
46. **Mulcahy, L. S., M. R. Smith, and D. W. Stacey.** 1985. Requirement for *ras* proto-oncogene function during serum-stimulated growth of NIH 3T3 cells. Nature (London) **313:**241–243.
47. **Nishizuka, Y.** 1984. The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature (London) **308:**693–698.
48. **Nori, M., L. K. Shawver, and M. J. Weber.** 1990. A Swiss 3T3 variant cell line resistant to the effects of tumor promoters cannot be transformed by *src*. Mol. Cell. Biol. **10:**4155–4162.
49. **Nori, M., U. S. Vogel, J. B. Gibbs, and M. J. Weber.** 1991. Inhibition of v-*src*-induced transformation by a GTPase-activating protein. Mol. Cell. Biol. **11:**2812–2818.
50. **Payne, D. M., A. J. Rossomando, P. Martino, A. K. Erickson, J.-H. Her, J. Shabanowitz, D. F. Hunt, M. J. Weber, and T. W. Sturgill.** 1991. Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). EMBO J. **10:**885–892.
51. **Ray, L. B., and T. W. Sturgill.** 1987. Rapid stimulation by insulin of a serine/threonine kinase in 3T3-L1 adipocytes that phosphorylates microtubule-associated protein 2 *in vitro*. Proc. Natl. Acad. Sci. USA **84:**1502–1506.
52. **Rossomando, A. J., D. M. Payne, M. J. Weber, and T. W. Sturgill.** 1989. Evidence that pp42, a major tyrosine kinase target protein, is a mitogen-activated serine/threonine protein kinase. Proc. Natl. Acad. Sci. USA **86:**6940–6943.
53. **Rossomando, A. J., J. S. Sanghera, L. A. Marsden, M. J. Weber, S. L. Pelech, and T. W. Sturgill.** 1991. Biochemical characterization of a family of serine/threonine protein kinases regulated by tyrosine and serine/threonine phosphorylations. J. Biol. Chem. **266:**20270–20275.
54. **Satoh, T., S. Nakamura, and Y. Kaziro.** 1987. Induction of neurite formation in PC12 cells by microinjection of proto-oncogenic Ha-*ras* protein preincubated with guanosine-5′-*O*-(3-thiotriphosphate). Mol. Cell. Biol. **7:**4553–4556.
55. **Seger, R., N. G. Ahn, T. G. Boulton, G. D. Yancopoulos, N. Panayotatos, E. Radziejewska, L. Ericsson, R. L. Bratlien, M. H. Cobb, and E. G. Krebs.** 1991. Microtubule-associated protein 2 kinases, ERK1 and ERK2, undergo autophosphorylation on both tyrosine and threonine residues: implications for their mechanism and activation. Proc. Natl. Acad. Sci. USA **88:**6142–6146.
56. **Smith, M. R., S. J. DeGudicibus, and D. W. Stacey.** 1986. Requirement for c-*ras* proteins during viral oncogene transformation. Nature (London) **320:**540–543.
57. **Smrcka, A. V., J. R. Hepler, K. O. Brown, and P. C. Sternweis.** 1991. Regulation of polyphosphoinositide-specific phospholipase C activity by purified G_q. Science **251:**804–807.
58. **Stumpo, D. J., J. M. Graff, K. A. Albert, P. Greengard, and P. J. Blackshear.** 1989. Molecular cloning, characterization, and expression of a cDNA encoding the "80- to 87-kDa" myristoylated alanine-rich C kinase substrate: a major cellular substrate for protein kinase C. Proc. Natl. Acad. Sci. USA **86:**4012–4016.
59. **Sturgill, T. W., and J. Wu.** 1991. Recent progress in characterization of protein kinase cascades for phosphorylation of ribosomal protein S6. Biochim. Biophys. Acta **1092:**350–357.
60. **Sukumar, S.** 1989. *ras* oncogenes in chemical carcinogenesis. Curr. Top. Microbiol. Immunol. **148:**93–114.
61. **Taylor, S. J., H. Z. Chae, S. G. Rhee, and J. H. Exton.** 1991. Activation of the β1 isozyme of phospholipase C by α subunits of the G_q class of G proteins. Nature (London) **350:**516–518.
62. **Trahey, M., and F. McCormick.** 1987. A cytoplasmic protein stimulates normal N-*ras* p21 GTPase, but does not affect oncogenic mutants. Science **238:**542–545.
63. **Ullrich, A., and J. Schlessinger.** 1990. Signal transduction by receptors with tyrosine kinase activity. Cell **61:**203–212.
64. **Vaidya, T. B., C. M. Weyman, D. Teegarden, C. L. Ashendel, and E. J. Taparowsky.** 1991. Inhibition of myogenesis by the H-*ras* oncogene: implication of a role for protein kinase C. J. Cell Biol. **114:**809–820.
65. **Vogel, U. S., R. A. F. Dixon, M. D. Schaber, R. E. Diehl, M. S. Marshall, E. M. Scolnick, I. S. Sigal, and J. B. Gibbs.** 1988. Cloning of bovine GAP and its interaction with oncogenic *ras* p21. Nature (London) **335:**90–93.
66. **Weber, M. J.** 1973. Hexose transport in normal and Rous sarcoma virus-transformed cells. J. Biol. Chem. **248:**2978–2983.
67. **Wu, J., A. J. Rossomando, J.-H. Her, R. Del Vecchio, M. J. Weber, and T. W. Sturgill.** 1991. Autophosphorylation *in vitro* of recombinant 42 kDa mitogen-activated protein kinase on tyrosine. Proc. Natl. Acad. Sci. USA **88:**9508–9512.
68. **Zhang, K., J. E. deClue, W. C. Vass, A. G. Papageorge, F. McCormick, and D. R. Lowy.** 1990. Suppression of c-*ras* transformation by GTPase-activating protein. Nature (London) **346:**754–756.