Ras Isoprenylation Is Required for ras-induced But Not for NGF-induced Neuronal Differentiation of PC12 Cells

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Abstract. We have used compactin, an inhibitor of mevalonate biosynthesis, to block $p21^{ras}$ posttranslational modification and membrane association in PC12 cells. Previous studies have demonstrated a requirement for isoprenylation for mitogenic effects of activated $p21^{ras}$ in mammalian cells and for function of *RAS* gene products in yeast. Immunoprecipitation of [^{35}S]methionine-labeled $p21^{ras}$ from PC12 cell homogenates confirmed that the processed $p21^{ras}$ species is missing from compactin-treated PC12 cells. Immunoprecipitation from particulate and cytosolic fractions of PC12 cells confirmed that compactin blocks $p21^{ras}$ membrane association: $p21^{ras}$ is confined to the cytosol fraction. Induction of neuronal differentiation and ornithine decarboxylase (ODCase) transcription by oncogenic $p21^{N-ras}$ does not occur in compactin-treated cells indicating that activity of oncogenic $p21^{N-ras}$ expressed in PC12 cells is abolished by compactin treatment.

Thus, $p21^{ras}$ isoprenylation or association with the membrane appears to be required for early responses and neuronal differentiation attributable to $p21^{ras}$ activation. In contrast, blockade of $p21^{ras}$ isoprenylation and membrane association by compactin treatment did not significantly reduce PC12 cell responses to NGF. Responses examined included rapid phosphorylation of tyrosine hydroxylase, rapid induction of ODCase expression, survival in serum-free medium and neuronal differentiation. Compactin blocked growth factor-induced rapid changes in cell surface morphology but did so whether this response was induced by NGF or by EGF. These results indicate that functional $p21^{ras}$ is not necessary for responses to NGF which in turn implies that if a *ras*-dependent NGF signal transduction pathway exists, as has been previously suggested, at least one additional *ras*-independent pathway must also be present.

THE *ras* protooncogene proteins, $p21^{H-ras}$, $p21^{K-ras}$, and $p21^{N-ras}$, are members of a family of small membrane-associated GTP-binding proteins. Although the precise mechanism of action is unclear, $p21^{ras}$ proteins appear to be involved in signal transduction: coupling of intercellular signals to cellular response mechanisms (4, 6, 32, 52 for recent reviews). In particular, $p21^{ras}$ proteins appear to be involved in transduction of mitogenic signaling. More recent studies (5, 26, 49, 57, 58) have indicated that $p21^{ras}$ proteins may also play a role in the transduction of signals that induce cell differentiation, specifically, neuronal differentiation induced by nerve growth factor (NGF)[1] in PC12 cells.

$p21^{ras}$ proteins are associated with the cytoplasmic surface of the plasma membrane (63). This association is due to posttranslational modifications which involve covalent attachment of lipid to the protein. Palmitic acid is attached to

one or more cysteines near the COOH-terminus (9) of most $p21^{ras}$ proteins, although not $p21^{K-ras}$ transcribed with exon 4B (33). Since $p21^{K-ras(B)}$ proteins are not palmitoylated yet can associate with the membrane and transform cells (33), palmitoylation is apparently not necessary for *ras* function. However, all $p21^{ras}$ proteins are isoprenylated: isoprenoid, probably a farnesyl moiety (11, 56), is covalently attached to the COOH-terminal cysteine (33, 53). The signal for isoprenylation is the COOH-terminal 4-amino acid CAAX motif (C=cysteine, A=any aliphatic amino acid, X=any amino acid), which all *ras* proteins possess. Other proteins that possess this COOH-terminal sequence, such as nuclear lamin (64), G protein γ subunit (18, 19), or *Saccharomyces cerevisiae* mating hormone a-factor (2), are also isoprenylated. Recombinant heterologous proteins to which the CAAX COOH-terminal sequence has been added are also isoprenylated, indicating that this motif is sufficient, as well as necessary, for isoprenylation (33). The isoprenoid used for protein isoprenylation is derived from mevalonate as part of a metabolic pathway that also gives rise to sterols, ubiquinone and dolichol (7). Thus, drugs such as lovastatin (1, 17) or compactin (17) which inhibit HMG-CoA (hydroxymethylglutaryl coenzyme A) reductase and thus block mevalonate synthesis also inhibit protein isoprenylation (42, 51). Isoprenylation of

1. *Abbreviations used in this paper*: FGF, fibroblast growth factor; NGF, nerve growth factor; ODCase, ornithine decarboxylase.

p21^{ras}, in particular, is blocked by these drugs (33, 45, 53) and, in *Saccharomyces cerevisiae*, also by mutations which abolish mevalonate synthesis (53). Posttranslational modification of p21^{ras} also involves two additional steps (13, 29): (*a*) proteolytic removal of the COOH-terminal three amino acids, AAX; (*b*) carboxyl methylation, in addition to isoprenylation, of the cysteine now at the COOH-terminal. These steps too appear to require isoprenylation (33, 35, 45, 53).

Isoprenylation of p21^{ras} appears to be necessary for its localization to the membrane. Inhibition of mevalonate synthesis by mutation or drug treatment results in cytosolic localization of *ras* proteins in yeast and mammalian cells (33, 53) and mutations that eliminate the CAAX cysteine abolish membrane localization of mammalian p21^{ras} (33, 35). Inhibition of *ras* isoprenylation in *S. cerevisiae* results in loss of *ras* biological activity (53). Transforming activity of oncogenic mutant *ras* in mammalian cells and *Xenopus* oocytes, is abolished in the absence of isoprenylation (35, 53). In metazoan cells the functions of *ras* are not understood as well as they are in yeast and the requirement for isoprenylation for normal *ras* biological activity, as opposed to transforming activity of oncogenic p21^{ras} has hitherto not been investigated.

In PC12 cells, expression of oncogenic *ras* genes results, not in transformation or mitogenic responses, but in neuronal differentiation similar to that which occurs after treatment with NGF or fibroblast growth factor (FGF). Since inhibition of *ras* function can inhibit PC12 responses to NGF and FGF (30, 49, 57), it has been suggested that *ras* proteins may function in NGF signal transduction pathway. This system allows experimental study of the function of *ras* in the induction of differentiation, as opposed to induction of transformation by oncogenic *ras* mutants. In the present study we have used compactin treatment of PC12 cells to assess the role of PC12 cells to assess the role of p21^{ras} isoprenylation and membrane localization in its differentiation-inducing activity. We have also exploited this system to more closely examine the function of p21^{ras} in NGF signal transduction. Our results indicate that isoprenylation is required for neuronal differentiation induced by a transforming mutant p21^{N-ras} but not by NGF. This implies that the role of p21^{ras} in NGF signal transduction is not a necessary one, an idea consistent with previous suggestions that NGF signal transduction involves multiple parallel pathways.

Materials and Methods

Materials

Unless otherwise noted, reagents were obtained from VWR Scientific Corp. (Philadelphia, PA), Fisher Scientific (Fair Lawn, NJ) or Sigma Chemical Co. (St. Louis, MO). Compactin was provided by Dr. Akira Endo and lovastatin by Dr. Alfred Alberts. These HMG-CoA reductase inhibitors were dissolved in ethanol which was alkalinized with an equal volume of 0.1 M NaOH. The drugs were dissolved by heating to 50°C for 2 h. They were then neutralized with 0.1 M HCl and diluted to stock concentration (1,000× working concentration) for storage.

Cell Culture and Microscopy

PC12 cells and cells of the PC12-derived UR6IJ line (provided by David Burstein) were maintained, as described in detail in Greene et al. (24), on collagen-coated tissue culture dishes in 85% RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) + 5% FBS (Gibco Laboratories) + 10% heat-inactivated donor horse serum (Hazelton Biologics, Inc., Herndon,

VA). NGF was prepared as described in Mobley et al. (48) Y13-259 hybridoma cells (obtained from American Type Culture Collection, Rockville, MD) were maintained in DMEM plus 10% FBS. All culture media were supplemented with 100 U/ml penicillin plus 100 μg/ml streptomycin (Gibco Laboratories).

Cells were generally observed by phase-contrast microscopy. Phase contrast photomicrographs were taken of cells immediately after removal from the 37°C/6.5% CO_2 incubator. Growth factor–induced cell surface movements were observed using Hoffman optics and contrast-enhanced video (Hamamatsu Corp., Hamamatsu City, Japan). For these experiments, because the cells had to be maintained for several minutes outside of the incubator, the culture medium was replaced with iso-osmolar RPMI 1640 medium containing 25 mM Hepes, pH 7.4 and the cells were kept on a microscope stage heated to 37°C.

Metabolic Radiolabeling of Cells

Cellular proteins were labeled by adding 50–100 μCi/ml [^{35}S]Met/Cys (Tran^{35}S-label; ICN Radiochemicals, Irvine, CA) directly to the culture medium for 24 h (10^7 cells in a 60-mm culture dish). Phosphoproteins were labeled by incubating 2×10^6 cells in a 35-mm dish in 1 ml HBSG (HEPES-buffered saline plus glucose = 137 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mg/ml glucose, 25 mM HEPES, pH 7.4) plus 100 μCi [^{32}P]Na$_3$ (PO$_4$) (ICN Radiochemicals) for 2 h at 37°C. The incubation was terminated by removal of the culture medium and washing with PBS (137 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 9.6 mM sodium phosphate, pH 7.1). Cholesterol uptake was assessed by the addition of 5 μCi/ml [^{3}H]cholesterol to the culture medium, washing the cells with PBS, solubilizing the cells in PBS plus 1% Triton X-100 plus 0.1% SDS, and assaying cell-associated radioactivity in a scintillation counter.

Immunoprecipitation of p21^{ras} Proteins

Rabbit anti-rat conjugated protein A-Sepharose (RAR-PAS) was made by adding 10 mg of BSA in 1 ml PBS to 100 mg protein A-Sepharose beads (Pharmacia Fine Chemicals, Piscataway, NJ) and incubating overnight on a rotator at 4°C in a microfuge tube. The beads were recovered by a 3-min centrifugation at 13,000 g, washed four times with PBS and resuspended in 1 ml PBS. 150 λ of rabbit anti-rat IgG (Zymed Corp., San Francisco, CA) at 1 mg/ml was added and the beads were incubated overnight on a rotator at 4°C. The beads were recovered by a 3-min centrifugation at 13,000 g, washed five times with PBS and resuspended in 150 λ PBS plus 0.05% NaN$_3$.

Cells were solubilized in 400 λ PBS-TDS (150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 0.2% NaN$_3$, 10 mM sodium phosphate pH 7.3, 0.5 μg/ml leupeptin, 1 mM EDTA, 0.7 μg/ml pepstatin, 0.2 mM PMSF) and centrifuged at 13,000 g for 60 min at 4°C. RAR-PAS (20 λ) was added to the supernatant, the mixture was incubated overnight on a rotator at 4°C in a microfuge tube, and centrifuged at 13,000 g for 3 min. The supernatant was transferred to a fresh tube, 40 λ Y13-259 culture supernatant or control medium was added, and the mixture was incubated on ice for 60 min. RAR-PAS (20 λ) was added and the mixture was incubated overnight on a rotator at 4°C. The beads were recovered by centrifugation at 13,000 g for 3 min and washed twice with PBS-TDS, once with PBS-TDS plus 1 M NaCl, and three times with PBS-TDS. The washed beads were boiled for 5–10 min in SDS-PAGE SB (5% 2-mercaptoethanol, 2% SDS, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 62.5 mM Tris-Cl, pH 6.8), loaded onto 4-cm 17% polyacrylamide gels, and run according to Laemmli (36). Fixed, Coomassie blue–stained gels were soaked for in 1 M sodium salicylate/40% methanol, dried, and exposed to preflashed Kodak XAR-5 film (37). Autoradiograms were scanned on a Hoefer densitometer (Hoefer Scientific Instruments, San Francisco, CA) for quantitation of radioactivity in bands.

Preparation of Particulate and Cytosolic Fractions

All steps were performed on ice with ice-cold solutions unless otherwise noted. Cells were washed twice with H_2O, scraped up into 400 λ lysis buffer (5 mM $MgCl_2$, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 0.2 mM PMSF, 10 mM Hepes, pH 7.3) with a rubber policeman and transferred to a prechilled Dounce homogenizer. After swelling for 15 min the cells were disrupted with 15 strokes of the B pestle. Nuclei and unbroken cells were removed by centrifugation in a microcentrifuge at 3,000 rpm for 2 min. The supernatant was transferred to a Beckman airfuge and centrifuged at 100,000 g for 20 min. For immunoprecipitation, an equal volume of 2× PBS-TDS was added to the cytosolic fraction (supernatant) and the particu-
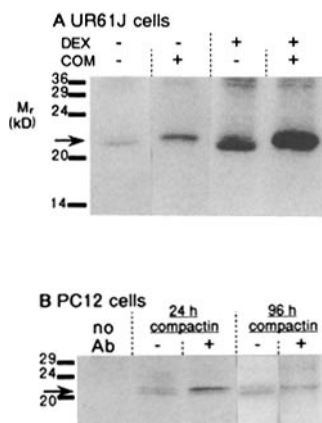
A UR61J cells

B PC12 cells

Figure 1. Compactin inhibits posttranslational modification of p21^{ras}. PC12 or UR61J cells were subjected to the indicated experimental or control treatments. During the final 24 h of the incubation [^{35}S]Met was added, the cells were solubilized, and p21^{ras} immunoprecipitated, as described in Materials and Methods. Cell homogenates contained approximately equal amounts of TCA-precipitable radioactivity. In this and other figures, autoradiograms were exposed for equal times to show the relative amount of p21^{ras} protein in the cells under all of the conditions used. Positions of molecular weight standards are indicated adjacent to each autoradiogram. An arrow indicates the position of p21^{ras}. (*A*) UR61J cells were labeled with [^{35}S]Met for 24 h in the presence (+) or absence (−) of 5 μM dexamethasone (*DEX*) to show, respectively, endogenous p21^{ras} or induced oncogenic p21^{N-ras}. In either case, the cells were incubated additionally in the presence or absence of 3 μg/ml compactin, as indicated, to show effects of compactin on endogenous p21^{ras} and on induced oncogenic p21^{N-ras}. Compactin, if present, was added to cell cultures 6 h before initiation of [^{35}S]Met labeling to deplete cellular mevalonate pools. DEX, if present, was added at the same time as [^{35}S]Met. In all cases stock solutions of compactin and DEX were added at a dilution of at least 1,000×. (*B*) PC12 cells were cultured for 24 or 96 h in the presence (+) or absence (−) of 3 μg/ml compactin (*COM*) as indicated. The lane labeled "no Ab" is a control immunoprecipitation performed in the same way as the other immunoprecipitations but with no Y13-259 antibody, demonstrating that no proteins are nonspecifically immunoprecipitated with our protocol.

late fraction (pellet), which includes the membranes, was solubilized in 300 λ PBS-TDS.

RNA Preparation and Northern Blotting

Total RNA was prepared by the method of Chomczynski and Sacchi (12) and fractionated on formaldehyde-agarose gels (10 μg RNA/slot) essentially as described by Ausubel et al. (3) but with 0.5 μg/ml ethidium bromide added to the RNA to allow subsequent visualization of the RNA and verification of equal loading among samples. The RNA was transferred to ZetaProbe (Bio-Rad Laboratories, Richmond, CA) in 10× SSC (150 mM NaCl, 1.5 mM sodium citrate, pH 7.0), UV-cross-linked, and prehybridized in 5× SSC, 4× Denhardt's, 50% formamide, 10% dextran sulfate, 1% SDS, 12.5 μg/ml sonicated calf thymus DNA. cDNA probes were radiolabeled using random primers (Bethesda Research Laboratories, Gaithersburg, MD) according to the manufacturer's protocol and added to the prehybridization buffer for an overnight incubation at 42°C. Filters were briefly rinsed in 2× SSC/0.2% SDS and then washed three times for 30 min each at 42°C in 0.1× SSC/0.1% SDS, exposed to preflashed XAR-5 film using an intensifying screen (Dupont Corp., Wilmington, DE). Autoradiograms were scanned on a Hoefer densitometer for quantitation of radioactivity in bands.

Analysis of Phosphoproteins

Growth factors, if present, were added to the culture dishes 1 h before solubilizing the cells. ^{32}P-labeled cells were solubilized in 200 λ SDS-PAGE buffer added directly to the 35 mm dish and scraped up with a rubber policeman. Samples containing equal amounts of TCA-precipitable radioactivity were loaded on 20-cm 7.5–15% polyacrylamide gradient gels. Gels were Coomassie blue–stained, fixed and dried, and exposed to preflashed XAR-5 film using an intensifying screen. Autoradiograms were scanned on a Hoefer densitometer for quantitation of radioactivity in bands. Quantita-

tion of optical density was corrected for differences among lanes in loading or exposure by scanning two regions of the autoradiogram unaffected by experimental treatment and normalizing density of the bands of interest to this background.

Results

Compactin Blocks p21^{ras} Posttranslational Modification in UR61J Cells and PC12 Cells

The UR61J cell line, described in detail in Guerrero et al. (27), is derived from the PC12 subline U7. U7 cells exhibit some responses to NGF, e.g., enhanced proliferation (8) and c-*fos* induction (58), but, unlike PC12 cells, undergo NGF-induced neuronal differentiation and neurite outgrowth only under growth-arresting conditions (8). UR61J cells share these properties (27). An oncogenic N-*ras* gene with a MMTV (murine mammary tumor virus) promoter was stably introduced into U7 cells to generate the UR61J cell line (27). The MMTV promoter is activated by glucocorticoids; thus expression of oncogenic N-*ras* is induced in UR61J cells by addition of dexamethasone (DEX) to the culture medium, resulting in neuronal differentiation. DEX does not induce neurite outgrowth or c-*fos* transcription in U7 cells nor does it inhibit these responses if induced by NGF (27, 58). Fig. 1 *A* shows p21^{ras} immunoprecipitated from UR61J cells. Comparison of protein immunoprecipitated from DEX-treated cells with that from DEX-untreated cells shows a large increase due to the addition of DEX-induced p21^{N-ras} to the endogenous pool of p21^{ras}. Data from Northern blot analysis (27) confirm that the DEX-induced protein is p21^{N-ras}.

Expression of mutant oncogenic p21^{ras} proteins in PC12 cells results in cessation of cell proliferation and neuronal differentiation including neurite outgrowth (5, 26, 49). Thus, induction of oncogenic p21^{N-ras} expression by DEX in UR61J cells results in neuronal differentiation of this PC12-derived line (27). Among the rapid responses to oncogenic p21^{N-ras} in UR61J cells is induction of ornithine decarboxylase (ODCase) gene expression and enzymatic activity (27, 58). However, rapid increase in c-*fos* expression, a typical cellular response to transforming and other signals, does not occur after expression of oncogenic p21^{N-ras} in UR61J cells (58; Qiu, M.-S., A. F. Pitts, and S. H. Green, unpublished observations). Thus, UR61J cells express a specific form of p21^{ras} that can be induced by DEX and that causes distinctive effects on the cells, effects that are rapidly and easily assessed.

p21^{ras} appears as a double band on polyacrylamide gels as a result of posttranslational processing; the lower, faster-running species is the processed form (33, 35, 53). As can be seen in Fig. 1 *A*, this lower band represents the predominant cellular form of p21^{ras} in control, compactin-untreated cells. Addition of 3 μg/ml compactin to the culture prevents posttranslational modification of all newly synthesized p21^{ras}, including the induced oncogenic p21^{N-ras}. This can be seen in Fig. 1 *A* as a shift of radiolabeled material from the lower to upper band of the doublet. In cells treated with ≥3 μg/ml compactin the lower band is no longer prominent and all, or nearly all, of the newly synthesized p21^{ras} is in the upper band. The lanes from cells untreated with DEX show endogenous p21^{ras} and the lanes from DEX-treated cells show primarily induced p21^{N-ras}. Both forms of p21^{ras} are similarly affected by compactin treatment.
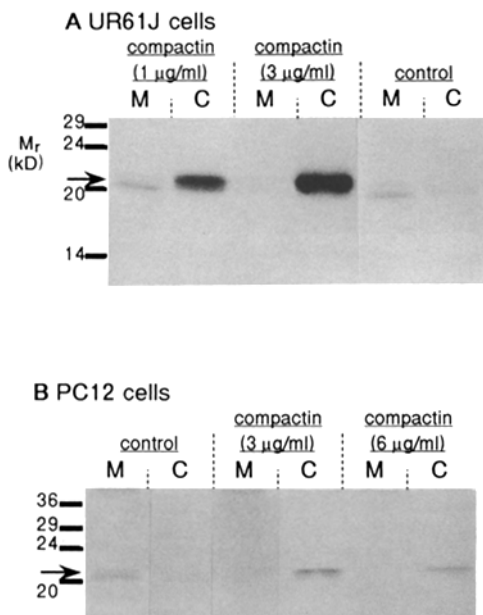
A UR61J cells

B PC12 cells

Figure 2. Compactin inhibits association of p21^{ras} with the membrane. PC12 or UR61J cell proteins were ^{35}S-labeled for 24 h as in Fig. 1, fractionated into nuclear, cytosolic, and particulate fractions and p21^{ras} was immunoprecipitated from particulate and cytosolic fractions as described under Materials and Methods. The figure shows p21^{ras} in the particulate fraction (*M*), which includes the membranes, and cytosolic fractions (*C*) of compactin-treated and untreated (*control*) cells: (*A*) UR61J cells treated for 30 h with compactin at 1 or 3 μg/ml, as indicated, or control compactin-untreated cells. [^{35}S]Met and 5 μg/ml dexamethasone were present during the final 24 h of incubation so the immunoprecipitated protein is primarily oncogenic p21^{N-ras}. (*B*) PC12 cells treated for 120 h with compactin at 3 or 6 μg/ml, as indicated, or control untreated cells.

Posttranslational processing of the endogenous p21^{ras} protein of PC12 cells is similarly inhibited by compactin (Fig. 1 *B*). Control, compactin-untreated PC12 cells exhibit both fast- and slow-migrating forms of p21^{ras} that give rise to a double band on PAGE of immunoprecipitates. Compactin treatment during ^{35}S-labeling causes a shift of radiolabeled p21^{ras} to the upper band or unprocessed form.

Compactin Transiently Increases Levels of p21^{ras} Protein Synthesis in UR61J and PC12 Cells

Fig. 1 shows p21^{ras} immunoprecipitated from cellular homogenates containing approximately equal amounts of ^{35}S-labeled protein. In addition to showing a DEX-induced increase in p21^{ras} in UR61J cells and a shift of p21^{ras} to the upper species, Fig. 1 *A* shows an increase in p21^{ras} in compactin-treated UR61J cells. This is true for both the endogenous p21^{ras} protein in DEX-untreated cells as well as for the DEX-induced oncogenic p21^{N-ras}. Densitometric scans of autoradiograms from five separate experiments indicate that induction of oncogenic p21^{N-ras} by DEX causes a 4.5 ± 1.1-fold increase in ^{35}S-labeled p21^{ras} protein. Compactin induces a 2.4 ± 0.3-fold ($n = 3$) further increase in ^{35}S-labeled p21^{ras} protein over the DEX-induced increase. In UR61J cells not treated with DEX, compactin induces a 3.7 ± 1.2-fold ($n = 4$) increase in ^{35}S-labeled p21^{ras} protein. Since p21^{ras} has a 24-h half-life in PC12 cells (Qiu, M.-S, and S. H.

Green, manuscript in preparation), the increase in p21^{ras} protein over the 24-h labeling period must be due mainly to an increase in p21^{ras} synthesis and not to inhibition of p21^{ras} degradation. Even if degradation of p21^{ras} were completely blocked by the addition of compactin, p21^{ras} would exhibit only a 2-fold increase in compactin-treated cells relative to control cells, not the 3.7-fold increase that we observe. However, the possibility does remain that a decrease in p21^{ras} degradation may make a contribution to the protein increase.

Fig. 1 *B* shows PC12 cells incubated for 24 or 96 h in the presence or absence of 3 μg/ml compactin. After a 24-h incubation, the compactin treated cells have an increased level of p21^{ras} protein synthesis relative to controls. The extent of this increase is similar to that in non–DEX-treated UR61J cells. However, this increase in p21^{ras} protein synthesis is transient. After a 96-h incubation, the level of p21^{ras} protein synthesis is approximately equal in compactin-treated and untreated PC12 cells.

Compactin Blocks p21^{ras} Membrane Localization in UR61J and PC12 Cells

In control, compactin-untreated UR61J cells, ≈80% of the oncogenic p21^{N-ras} protein synthesized over a 24-h labeling period was associated with the cell membrane (Fig. 2 *A*). Oncogenic p21^{N-ras} synthesized in the presence of compactin was not membrane associated (Fig. 2 *A*): ≈85% of oncogenic p21^{N-ras} synthesized in 1 μg/ml compactin was associated with the cytosolic fraction and >95% of oncogenic p21^{N-ras} synthesized in 3 μg/ml compactin was associated with the particulate fraction.

Similarly, in control, compactin-untreated PC12 cells, ≈70% of the p21^{ras} protein synthesized over a 24-h labeling period was associated with the cell membrane (Fig. 2 *B*). In contrast, in compactin-treated cells, >95% of p21^{ras} synthesized in 3 μg/ml compactin was associated with the cytosolic fraction and essentially all of the p21^{ras} synthesized in 6 μg/ml compactin was associated with the cytosolic fraction (Fig. 2 *B*). In the cells used for Fig. 2 *B* compactin treatment included a 96-h pretreatment before the 24-h [^{35}S]methionine labeling in the presence of compactin.

Effects of compactin on p21^{ras} synthesis, post-translational processing and membrane localization are entirely reversed by the addition of 100 μM mevalonate during compactin treatment (not shown). Thus, these effects are due to inhibition of mevalonate biosynthesis by compactin and not to direct effects of compactin on protein isoprenylation.

Compactin Blocks ras-induced Neuronal Differentiation in UR61J Cells

Since compactin treatment blocks both p21^{ras} posttranslational modification and association with the membrane, it was expected that such treatment would inhibit neuronal differentiation induced by oncogenic p21^{ras} in PC12 cells just as such treatment inhibits the activity of transforming *ras* oncoproteins in other systems. Fig. 3 shows the effects of compactin treatment on *ras*-induced neuronal differentiation and UR61J cell morphology. Compactin treatment results in cell rounding and the formation of small processes or "spikes." Both of these morphological changes become prominent within 24 h of compactin treatment (Fig. 3 *B*). However, the "spikes" are transient and disappear within 48 h of
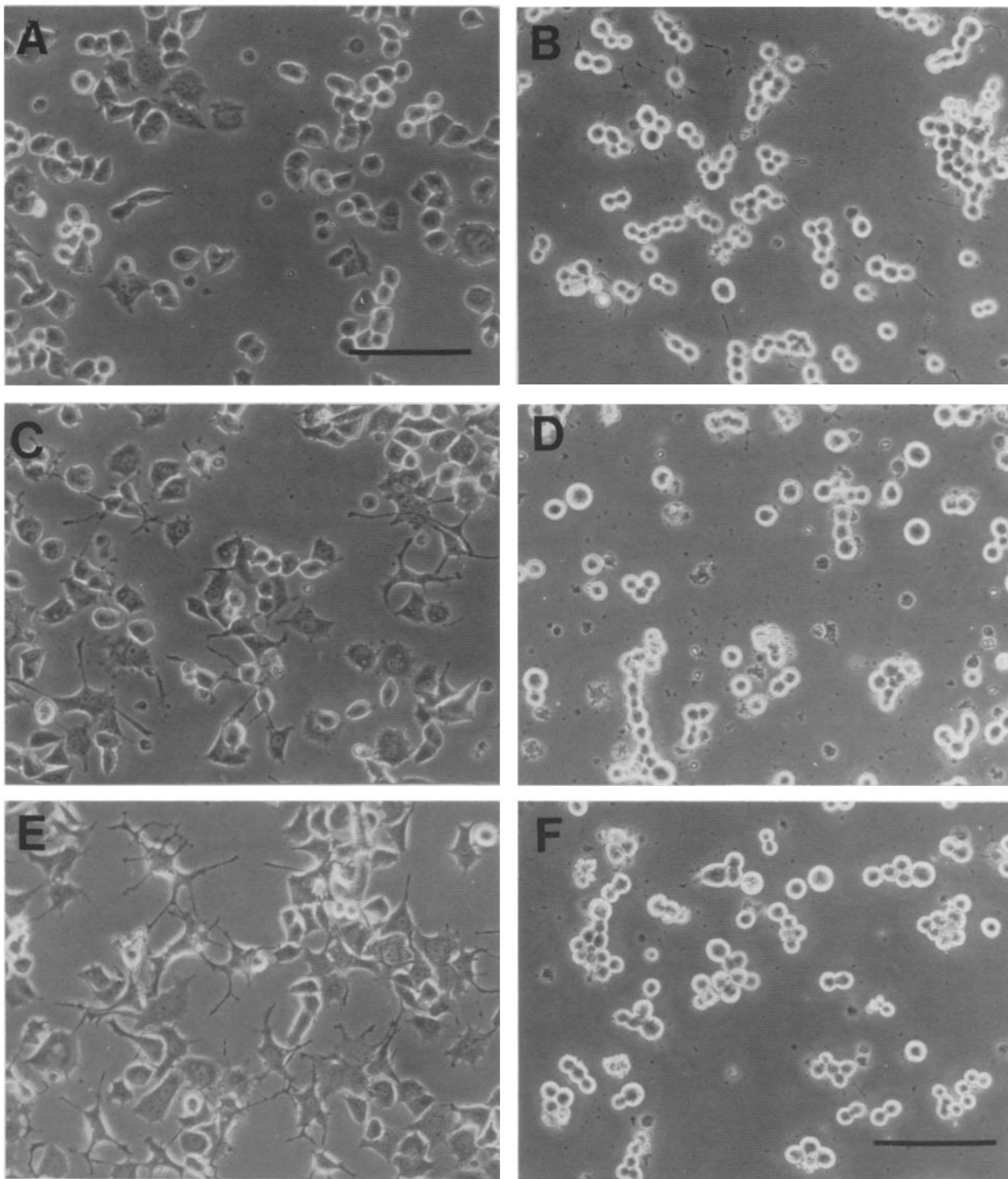
Figure 3. Effects of compactin on UR61J cell morphology. Cells were photographed using phase-contrast microscopy. (*A*) UR61J cells in control conditions. (*B*) Cells treated with 3 μg/ml compactin for 24 h showing compactin-induced rounding and "spikes." (*C*) Cells treated with 5 μM dexamethasone for 24 h showing initiation of *ras*-induced neurite outgrowth. (*D*) Cells treated with 3 μg/ml compactin for 48 h showing loss of compactin-induced "spikes" apparent at 24 h of compactin treatment. (*E*) Cells treated with 5 μM dexamethasone for 48 h showing *ras*-induced neurite outgrowth. (*F*) Cells treated with 5 μM dexamethasone + 3 μg/ml compactin for 48 h showing lack of *ras*-induced neurite outgrowth. Bar, 100 μm.

compactin treatment (Fig. 3 *D*), although the cells remain rounded. DEX treatment induces neurite outgrowth in UR61J cells which is visible by 24 h (Fig. 3 *C*) and prominent by 48 h of treatment (Fig. 3 *E*) as has been previously reported

(38). This *ras* response is entirely abolished by the addition of 3 μg/ml compactin to the culture medium during the DEX treatment (Fig. 3 *F*). Extended pretreatments with compactin up to 4 d before addition of DEX similarly inhibit subsequent

induction of neurite outgrowth by p21$^{N\text{-}ras}$ (data not shown).

One alternative interpretation is that the compactin treatment does not block ras-induced differentiation but rather blocks DEX induction of ras transcription from the MMTV promoter construct. This is unlikely because UR61J cells treated with compactin and then with DEX do exhibit increased p21^{ras} synthesis relative to cells treated with compactin alone (Figs. 1 A and 2 A). A second alternative interpretation of this experiment is that compactin affects UR61J cells more strongly than it affects PC12 cells because of some as-yet-unknown metabolic difference between these clones. To test these two alternatives we transiently expressed oncogenic p21$^{N\text{-}ras}$ in PC12 cells using a constitutive promoter. The construct tl1-N-ras(T) (26) contains a Lys^{61} mutant transforming ras gene, which is expressed constitutively by an LTR promoter in PC12 cells. When transfected into PC12 cells this induces neuronal differentiation at low frequency (26). We typically see a neuronal appearance like that in Fig. 3 E in 1-5% of cells transfected with tl1-N-ras(T) within 48 h after transfection. Under control conditions we observed between 10^6 and 5 × 10^6 neuronal cells among 10^8 cells transfected with tl1-N-ras(T); no neuronal cells were observed among 10^8 similarly transfected PC12 cells shifted to medium containing 3 μg/ml compactin. This indicates that compactin directly inhibits the function of transforming ras genes in PC12 cells.

Compactin Blocks ras-induced Early Gene Expression in UR61J Cells

A possible explanation for the results described above is that compactin specifically blocks neurite outgrowth but does not actually block ras activity. To test this possibility, we chose to assay a rapid response to oncogenic ras expression in DEX-treated UR61J cells. Oncogenic ras does not induce c-fos in UR61J cells but does induce a large increase in ODCase expression (58). Also, DEX treatment has only a minimal inhibitory effect on neurite outgrowth or ODCase expression in PC12 cells (59). We treated UR61J cells with DEX for 18 h in the presence and absence of 3 μg/ml compactin and assayed ODCase expression by Northern blot (Fig. 4). DEX treatment in the absence of compactin caused a large increase in ODCase expression. Compactin treatment alone caused a small but significant increase in ODCase expression. However, compactin treatment completely abolished the large DEX-induced increase in ODCase expression. Thus compactin apparently blocks even rapid responses to oncogenic p21$^{N\text{-}ras}$.

Compactin Does Not Block NGF-induced Neuronal Differentiation in PC12 Cells

PC12 cell p21^{ras} protein has a half-life of ≈24 h (Qiu, M.-S., and S. H. Green, manuscript in preparation). The isoprenyl moiety on the COOH-terminal cysteine appears to be stable (33; Qiu, M. S., and S. H. Green, manuscript in preparation). Since compactin only blocks isoprenylation of newly synthesized p21^{ras} and can have no effect on p21^{ras} present before addition of compactin, PC12 cells were treated for 120 h and 3 μg/ml compactin before assessing their responses to NGF. With this treatment protocol, >98% of the p21^{ras} present in the cells at the time of NGF addition was synthesized in the presence of 3 μg/ml compactin and was thus nei-

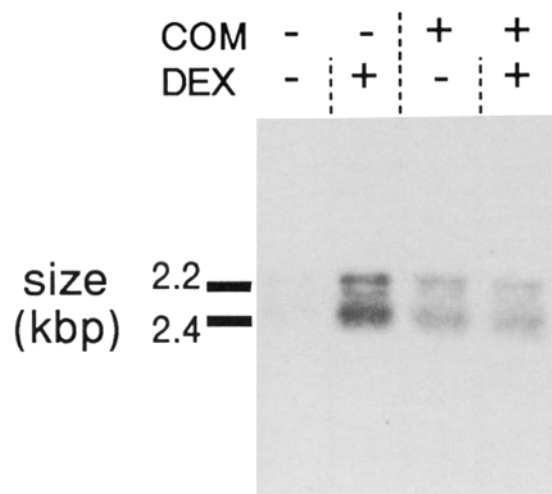
COM −   − + +
DEX −   + − +

size   2.2
(kbp) 2.4

Figure 4. Effects of compactin on ODCase mRNA expression in UR61J cells. Total RNA from UR61J cells was probed for ODCase mRNA as described in Materials and Methods. DEX treatment was with 5 μM dexamethasone for 18 h. COM treatment was with 6 μg/ml compactin for 24 h (cells treated with dexamethasone therefore received a 6-h pretreatment with compactin). Averaged densitometer scans of three autoradiograms indicates that DEX treatment alone caused a sevenfold increase in ODCase expression; COM, a threefold increase; DEX + COM, a threefold increase.

ther isoprenylated nor membrane-associated. An extended pretreatment was not necessary in experiments performed with UR61J cells because synthesis of oncogenic p21^{ras} was not initiated until after compactin had been added.

Treatment of PC12 cells with NGF leads, within 24 h, to flattening and extension of short processes (28). Extension of neurites is apparent within 48 h and prominent by 72 h of NGF treatment (Fig. 5 B). Within 24 h, 3 μg/ml compactin induced cell rounding and transient "spikes" in PC12 cells similar to those induced in UR61J cells (Fig. 5 C). Possibly associated with the morphological change was a reduction in adhesion of PC12 cells to the substrate observed as an increase in the number of cells in suspension in the culture. These contrast with the well-described flattening, substrate adhesion, and long, permanent processes induced by NGF. As with UR61J cells, the spikes disappear after 24-48 h of treatment. After 120 h of compactin treatment, the spikes are gone and the toxicity of compactin becomes evident (Fig. 5 D): 21% of the original population of PC12 cells is dead. Treatment with compactin for an additional 72 h results in death of >95% of the original population (Fig. 5 E). In contrast, if NGF is added with compactin for this 72-h period then continued cell death is greatly reduced (Fig. 5 F). Fig. 5 F also demonstrates that NGF promotion of cell survival was not compromised by compactin. In the absence of serum, NGF is sufficient to maintain PC12 cell survival (23). PC12 cells that carry mutations which eliminate their ability to respond to NGF do not survive in NGF-supplemented serum-free medium (21). However, compactin-pretreated PC12 cells maintained for 72 h in NGF-supplemented serum-free medium survive, thus demonstrating that the neurotrophic effect of NGF is not blocked by compactin. These NGF-treated PC12 cells, which represent 79% of the original population, were pretreated with 3 μg/ml compactin for
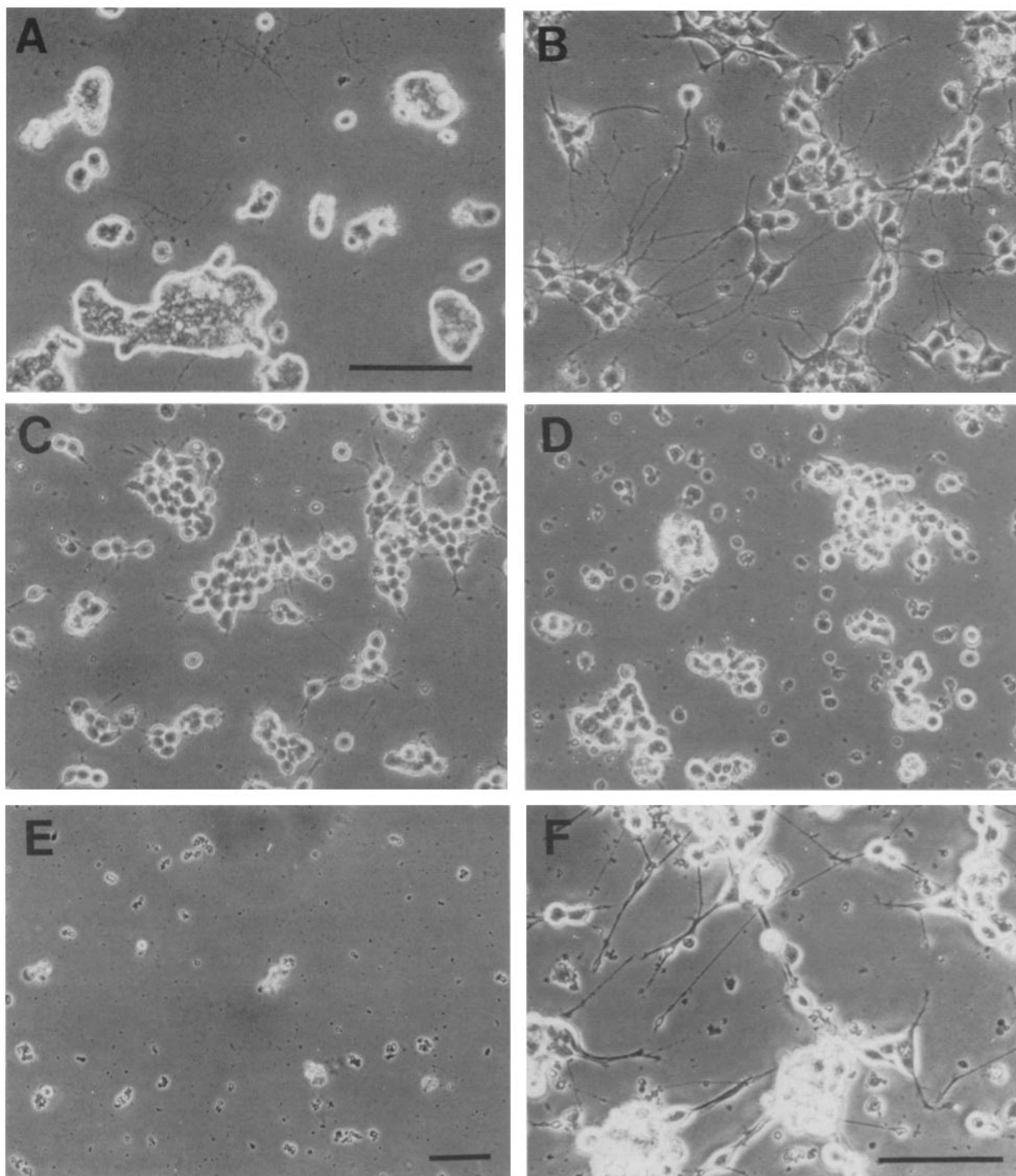
Figure 5. Effects of compactin on morphology of NGF-treated and -untreated PC12 cells. Cells were photographed using phase-contrast microscopy. (*A*) PC12 cells in control conditions: no NGF, no compactin. (*B*) Cells treated with 50 ng/ml NGF for 72 h showing initiation of NGF-induced neurite outgrowth. (*C*) Cells treated with 3 μg/ml compactin for 24 h showing compactin-induced rounding and "spikes." (*D*) Cells treated with 3 μg/ml compactin for 120 h showing progress of compactin-induced cell death. (*E*) Cells treated with 3 μg/ml compactin for 192 h showing compactin-induced cell death. (*F*) Cells treated with 3 μg/ml compactin for 120 h, then 3 μg/ml compactin plus 50 ng/ml NGF for 72 h in serum-free medium showing (*1*) NGF-induced neurite outgrowth typical of PC12 cells treated with NGF for 72 h, and (*2*) prevention of the compactin-induced cell death evident after 192 h of compactin treatment in the absence of NGF. All pictures except *D* are at the same magnification; bar, 100 μm.
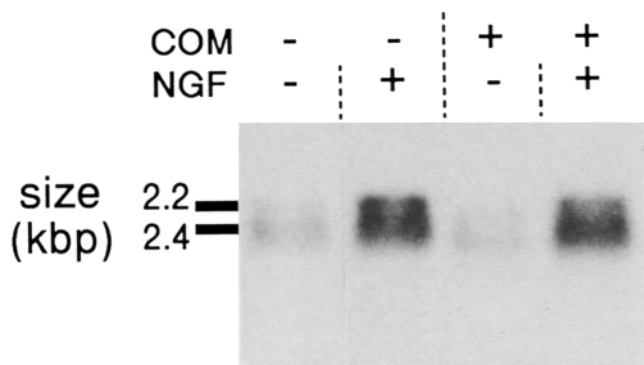
COM   −   −   +   +
NGF   −   +   −   +

size   2.2
(kbp)   2.4

Figure 6. ODCase mRNA expression in compactin- and NGF-treated PC12 cells. Total RNA from PC1 cells was probed for ODCase mRNA as described in Materials and Methods. NGF treatment was 50 ng/ml NGF for 3 h. COM treatment was 3 μg/ml compactin for 120 h.

5 d, a condition in which ≤2% of the cellular p21^{ras} is iso-prenylated and membrane-associated. Thus p21^{ras} isoprenylation and membrane-association is not required for NGF maintenance of cell survival.

p21^{ras} isoprenylation and membrane-association is also not required for NGF induction of neurite outgrowth: PC12 cells treated with NGF plus 3 μg/ml compactin for 72 h, after pretreatment with 3 μg/ml compactin for 120 h (Fig. 5 *F*),

extend neurites similar in length and quality to those extended in 72 h the absence of compactin (Fig. 5 *B*). Under control conditions, NGF-induced neurites extend 45 ± 24 μm (*n* = 60) in 72 h; after compactin treatments that eliminate >98% of membrane-associated p21^{ras}, NGF-induced neurites extend 47 ± 26 μm (*n* = 70) in 72 h. The number of cells bearing neurites of length ≥2 cell diameters at 72 h is reduced from 69% in control conditions to 46% in compactin, possibly due to loss of substrate adhesion.

Compactin Does Not Block NGF-induced Early Gene Expression in PC12 Cells

NGF, like oncogenic p21^{ras}, induces ODCase expression in PC12 cells. However, induction by NGF, unlike that by oncogenic p21^{ras}, is not inhibited by compactin treatment. PC12 cells exposed to 3 μg/ml compactin for 120 h to eliminate membrane-associated p21^{ras} were treated with NGF for 3 h and total cellular RNA was probed for ODCase on a Northern blot. Expression of ODCase mRNA was equal in control and compactin-treated cells (Fig. 6). Fig. 6 also shows that the induction of ODCase by compactin, apparent after a 24-h treatment with the drug (Fig. 4), is no longer apparent after a 120-h treatment with compactin.

Compactin Does Not Block NGF- or EGF-stimulated Phosphorylation of Tyrosine Hydroxylase

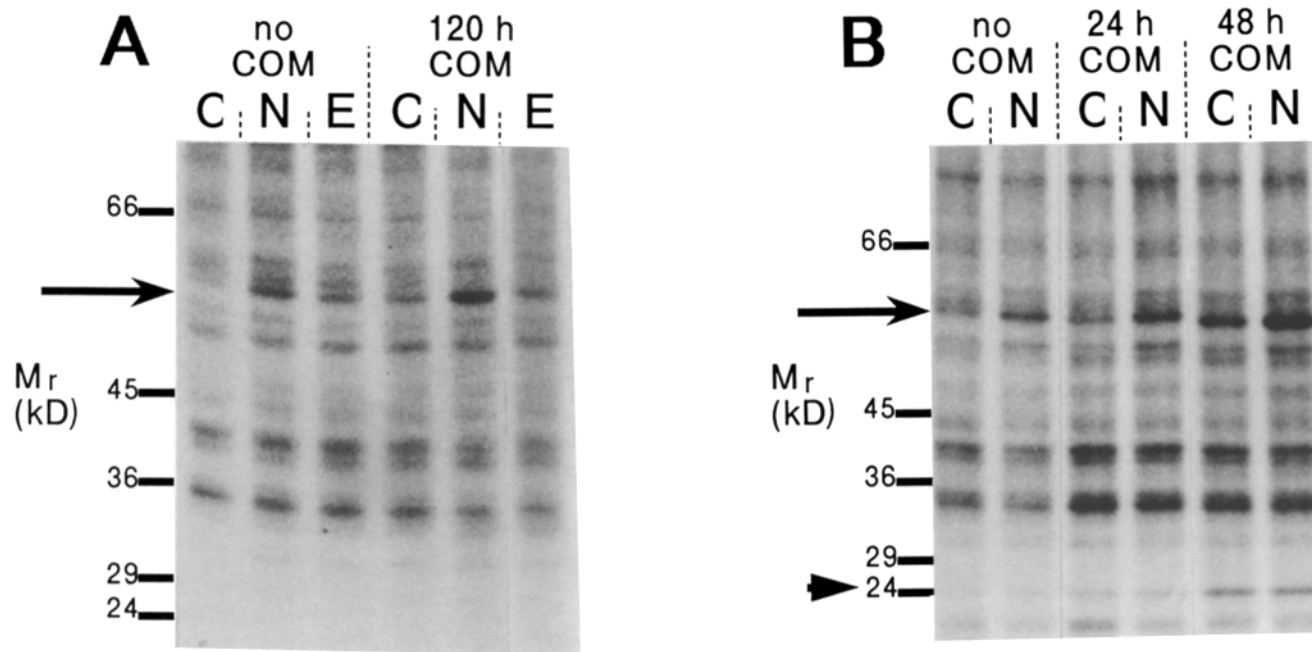NGF and EGF (epidermal growth factor) stimulate rapid

Figure 7. Phosphoproteins in PC12 cells treated with NGF, EGF, or compactin. Phosphoproteins were labeled with [^{32}P]orthophosphate under various conditions as described in Materials and Methods. Positions of molecular weight standards are indicated adjacent to each autoradiogram. (*A*) Compactin-treated cells were so treated for 120 h with 6 μg/ml compactin. Control cells (*C*) were not treated with growth factors; the other lanes show phosphoproteins from NGF-treated (*N*) or EGF-treated cells (*E*). The position of the 60-kD phosphoprotein is indicated by the arrow. Averaged densitometer scans of three autoradiograms indicates that COM treatment alone caused a 4-fold increase in phosphorylation of the 60-kD protein; NGF alone, a 9.6-fold increase; EGF a 5.7-fold increase. In the compactin-treated cells, NGF caused a 4.2-fold increase in phosphorylation of the 60-kD protein and EGF caused a 3.0-fold increase. (*B*) Compactin treatment was with 10 μg/ml compactin for the times indicated. The position of the 60-kD phosphoprotein is indicated by an arrow; that of a 24-kD compactin-induced phosphoprotein by an arrowhead. Increase in density of the 24-kD protein was 2.4-fold at 24 h and 5.9-fold at 48 h with no significant change due to NGF treatment.

phosphorylation of a number of PC12 cell proteins, notably a 60-kD species thought to be tyrosine hydroxylase (TH) (31, 46, 54). The increase in phosphate content of this species as a result of 1 h of NGF or EGF treatment is shown in Fig. 7 A. Prior exposure to 6 μg/ml compactin for 120 h to eliminate membrane-associated p21^{ras} inhibits neither NGF- nor EGF-stimulated phosphorylation of TH (Fig. 7 A). This treatment with compactin does itself reproducibly stimulate phosphorylation of TH, as can be seen by comparing the intensity of the 60-kD species in the lanes from compactin-treated and compactin-untreated cells. Fig. 7 B shows that the compactin induced stimulation of TH phosphorylation is detectable within 24 h of addition of compactin and is prominent within 48 h. Compactin treatment of PC12 cells also induces the appearance of a novel 24-kD protein, apparent within 24 h and prominent by 48 h of treatment (Fig. 7 B). Fig. 7 A shows that this 24-kD phosphoprotein is transient, not visible after 120 h of compactin treatment.

Compactin Blocks NGF- and EGF-induced Rapid Changes in Surface Morphology

NGF and EGF treatment both rapidly induce an increase in PC12 cell surface motility, although by somewhat different mechanisms (15, 54). This motility is most easily observable as the appearance of large lamellipodiae or "ruffles" after NGF or EGF treatment (Fig. 8, B and F). PC12 cells treated with compactin (1 μg/ml for 24 h) completely lack this response to growth factor stimulation (Fig. 8, D and H). This inhibition of membrane motility is general, affecting both EGF- and NGF-induced ruffling, however, this does not imply that p21^{ras} acts as a signal transducer for this response for either growth factor. Inhibition of ruffling occurs after a regime of compactin treatment (1 μg/ml for 24 h) which results in only a partial blockade of isoprenylation of newly synthesized p21^{ras} and does not allow sufficient time for replacement of previously synthesized p21^{ras}. These figures also show compactin-induced cell rounding and loss of cell–cell adhesion consistent with that shown above in cells observed by phase contrast microscopy.

Effects of Compactin on PC12 Cells

While compactin and lovastatin do not significantly reduce the NGF responses we investigated in PC12 cells, these inhibitors of mevalonate biosynthesis are not without effects on these cells. For example, we have observed compactin inhibition of p21^{ras} processing, p21^{ras} membrane localization and function, inhibition of growth factor–induced rapid changes in surface morphology, induction of morphological changes such as rounding and "spiking," a small transient increase in ODCase expression, an increase in tyrosine hydroxylase phosphorylation, and a transient increase in expression of p21^{ras} and a 24-kD phosphoprotein. These compactin-induced changes do not occur if the cells are compactin-treated in the presence of 100 μM mevalonate (not shown). Thus, these changes are a direct result of compactin inhibition of mevalonate biosynthesis. The major mevalonate-derived product in cells is cholesterol; however, it is unlikely that the effects observed in PC12 and UR61J cells as a result of compactin treatment are due to cholesterol deprivation. The culture medium contains ≈130 μg/ml cholesterol derived from the added calf and horse sera (information supplied by Hazleton Biologics, Inc.). Tracer [³H]cholesterol was added to

the culture medium and uptake was assayed as described in Materials and Methods. Compactin-treated PC12 cells took up 15% of the total available cholesterol pool over a 3-d incubation period. This indicates that the supply of cholesterol to the cells is not limiting and PC12 cell responses to compactin are not likely to be a result of cholesterol starvation. Nevertheless, compactin responses are not necessarily a result of inhibition of protein isoprenylation. Mevalonate is used in the biosynthesis of many cellular products and the relevance of all of these has not yet been investigated.

Discussion

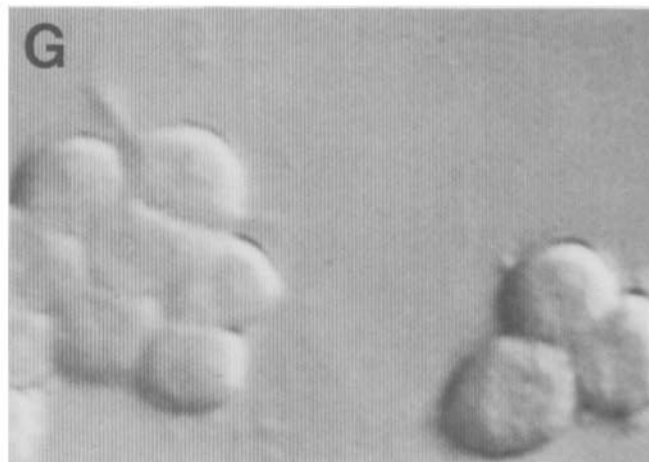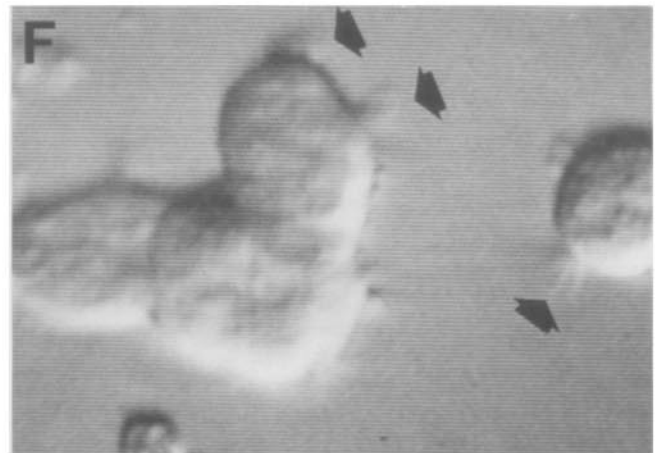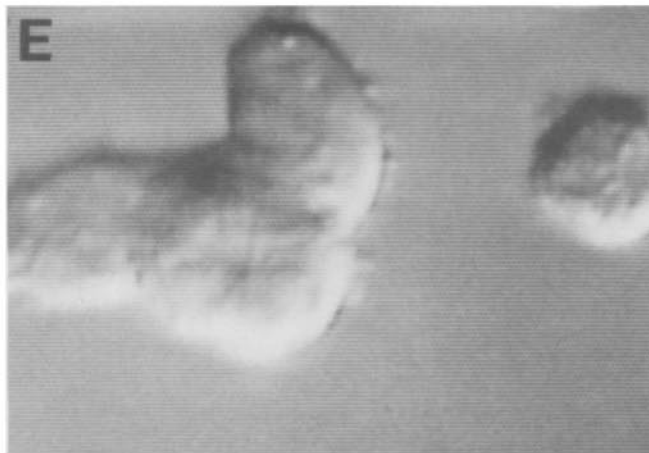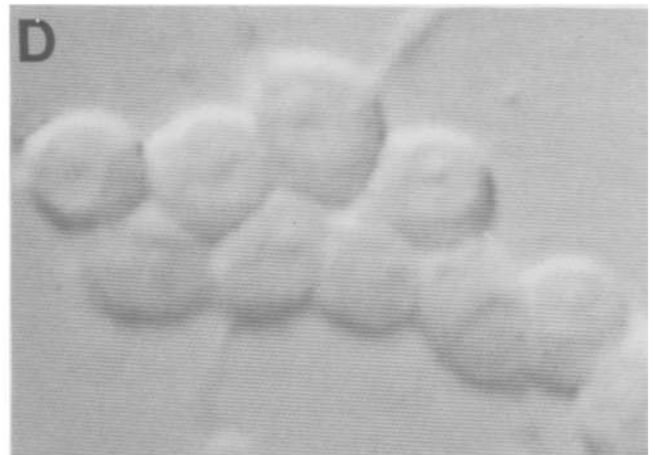### Isoprenylation of p21^{N-ras} Proteins Is Required for their Promotion of Neuronal Differentiation
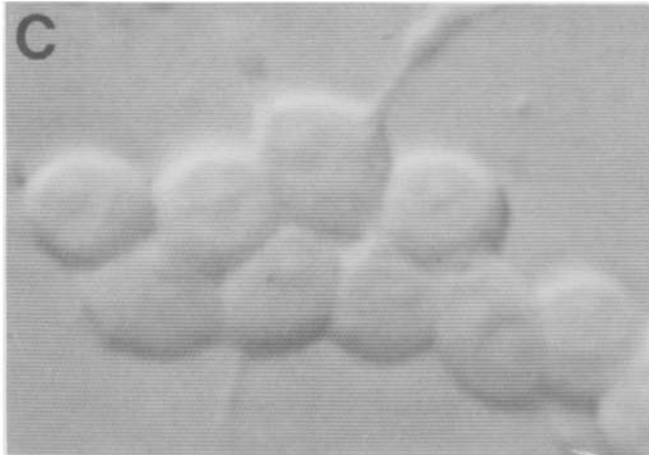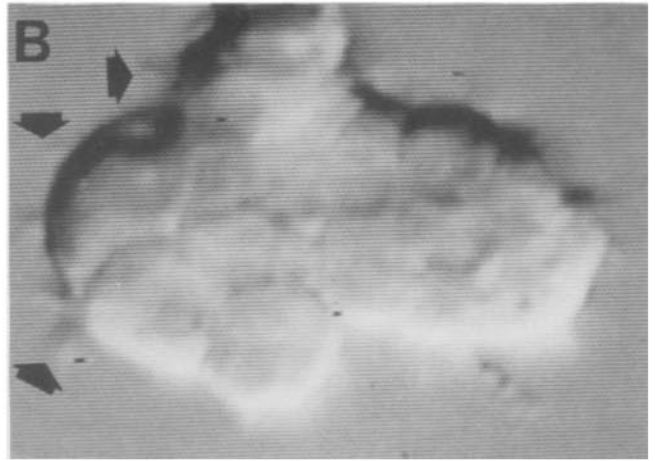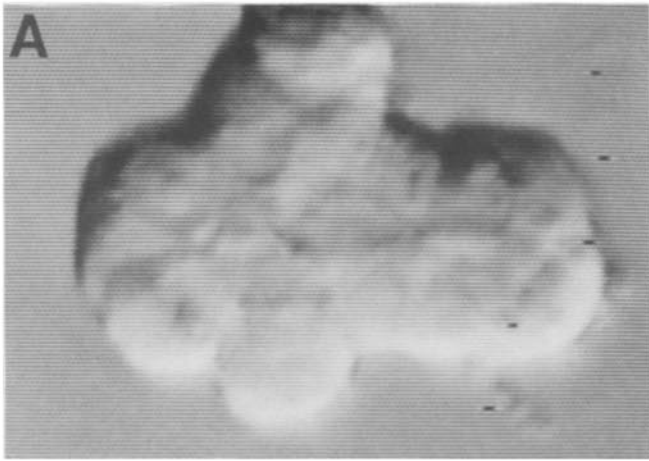
Previous studies, summarized above, have shown that isoprenylation of mammalian p21^{ras} is required for its further posttranslational processing and association with the cell membrane (33, 35, 45, 53). Isoprenylation is likewise necessary for transforming activity of oncogenic p21^{ras} in mammalian cells (35). Inhibition of isoprenylation results in the lack of all subsequent posttranslational processing of ras proteins: carboxyl methylation, removal of the AAX COOH-terminal tripeptide, palmitoylation and localization to the membrane. In view of the observation that mutant oncogenic p21^{H-ras} and p21^{N-ras} proteins that are isoprenylated, but not palmitoylated, exhibit reduced association with the membrane in parallel with reduced transforming activity (33), it is most likely that membrane localization is the process critical for function of ras proteins.

We show here that in PC12 cells, as well, isoprenylation of p21^{ras} is required for its further posttranslational processing and association with the cell membrane. Expression of oncogenic p21^{ras} in PC12 cells results in neuronal differentiation not transformation (5, 26, 46). These observations allow us to use compactin treatment of PC12 cells to determine whether p21^{ras} isoprenylation and membrane association is required for neuronal differentiation as it is for transformation. We find that this is indeed the case, inhibition of isoprenylation and membrane association abolishes PC12 responses to expression of oncogenic p21^{N-ras}. This implies that these two different classes of responses, mitogenesis and differentiation, are both mediated by a mechanism requiring p21^{ras} isoprenylation.

The Role of p21^{ras} in NGF Signal Transduction

Evidence for Involvement of p21^{ras} in NGF Signal Transduction. Responses to oncogenic p21^{ras}, transformation, and differentiation, require isoprenylation. Is this also the case for functions of normal p21^{ras} in signal transduction in mammalian cells? To determine this we chose to examine the effect of compactin treatment on NGF responses in PC12 cells because three lines of evidence suggest that p21^{ras} has a role in NGF signal transduction. Although each observation alone has alternative interpretations, as detailed below, the aggregate, while not conclusive, appears to strongly support this idea.

(a) Expression of mutant transforming *ras* gene products in PC12 cells induces neuronal differentiation similar to that induced by NGF (5, 61, 49). However, it is possible that *ras*-induced neuronal differentiation results from the activa-

tion, by oncogenic p21^{ras}, of an intracellular signal pathway different from the one activated by NGF. In support of this second possibility are observations of differences between NGF-induced responses and ras-induced responses in PC12 cells. For instance, NGF, but not transforming ras, induces expression of c-fos in PC12 cells (27; Qiu, M.-S., A.F. Pitts, and S.H. Green, unpublished observations). Also, down-regulation of kinase C has a stimulatory effect on ras-induced ODCase expression and neurite growth (50) but this same treatment partially inhibits NGF-induced c-fos expression (55) and ODCase expression (Mesner, P., and S.H. Green, unpublished observations). Thus, the observation that transforming ras mimics NGF responses is, by itself, not evidence that it functions as a signal transducer for the NGF receptor. Furthermore, treatment with EGF activates p21^{ras} in PC12 cells to the same extent as does NGF (Qiu, M.-S., and S. H. Green, manuscript in preparation) yet does not induce neuronal differentiation. Thus, expression of oncogenic p21^{N-ras} in PC12 cells is not equivalent to growth factor–mediated activation of the cells' endogenous p21^{ras} and induction of neuronal differentiation by oncogenic p21^{N-ras} does not imply that normal p21^{ras} is involved in NGF signal transduction.

(b) Expression of oncogenic p21^{N-ras} in UR6lJ cells inhibits subsequent c-fos induction by NGF, but not by epidermal growth factor, phorbol-12-myristate-13-acetate (PMA), forskolin, or depolarization with K$^+$ (58). While this result implies an interaction between NGF and oncogenic p21^{ras} intracellular signaling mechanisms, it does not specify the type of interaction and, in particular, does not imply that p21^{ras} is required for NGF signal transduction.

(c) Blockade of p21^{ras} activity by microinjection of Y13-259 antibody (30) or expression of Ha-ras Asn-17, a dominant inhibitory mutant ras gene, (57) inhibits responses to NGF. However, this inhibition may result from a general effect on cell physiology rather than a specific effect on signal transduction. Another difficulty is that in the study of Szeberényi et al., not all cell lines transfected with the inhibitory mutant ras gene were NGF nonresponsive; some lines expressing Ha-ras Asn-17 possessed NGF responses, although most did not (57). Possibly, if both ras-dependent and ras-independent NGF signal transduction pathways exist in PC12 cells, a subset of cells that lose the ras-dependent pathway as a result of a spontaneous mutation lose all ability to respond to NGF if the ras-dependent pathway is subsequently eliminated. Cells retaining the ras-independent pathway would respond to NGF even if expressing Ha-ras Asn-17.

p21^{ras} Membrane Association Is Not Necessary for NGF Signal Transduction. This study further defines the role of p21^{ras} in NGF signal transduction. We examined a variety of responses to NGF: an early transcription-independent response (phosphorylation of tyrosine hydroxylase); an early transcription-dependent response (induction of ODCase);

and late responses, neurite outgrowth and cell survival. Our results indicate that association of p21^{ras} with the membrane is not necessary for responses to NGF. Since currently available evidence, supported by the data presented here, indicates that p21^{ras} must be membrane-associated to be functional, we conclude that it is unlikely that NGF signal transduction is mediated exclusively by a p21^{ras}-dependent mechanism. Rather, we favor a NGF signal transduction mechanism that involves multiple parallel signal pathways with p21^{ras} involved in some but not all of them. However, we have considered four other possible explanations of our data; for the reasons given, we do not believe that these can account for all of our results.

(a) UR6lJ cells and PC12 cells may have metabolic differences that cause the former but not the latter to be sensitive to compactin. This is unlikely because (a) compactin blocks p21^{ras} posttranslational processing and membrane association in both PC12 and UR6lJ cells, and (b) neurite outgrowth induced by expression of oncogenic p21^{ras} in PC12 cells is blocked by compactin just as it is in UR6lJ cells.

(b) Another explanation for our results might be that long-term treatment of PC12 cell cultures with compactin results in a selection for a subpopulation of compactin-resistant cells which can function normally with reduced mevalonate levels. This possibility is ruled out by the observations that (a) a 5-d treatment with 3 µg/ml compactin kills only 21% of the cells. Therefore, if there existed a compactin-resistant subpopulation it would encompass ≈80% of the population. If this were so then we would have been unable to observe effects of compactin on p21^{ras} processing. (b) All compactin-treated PC12 cells fail to exhibit growth factor-induced rapid changes in surface morphology, indicating that there are no cells resistant to compactin by this criterion. (c) Compactin-treated UR6lJ cells fail to respond to oncogenic p21^{ras} even after 4–5-d compactin pretreatments comparable to those used for studies of PC12 NGF responses (Pitts, A. F., and S. H. Green, unpublished observations) in spite of a comparable degree of cell death. Thus, there is no selection for compactin resistant UR6lJ cells. (d) Most importantly, p21^{ras} is cytosolic in the cells treated with 3 µg/ml compactin in the long-term (Fig. 2 b). Thus, isoprenylation of p21^{ras} in both PC12 and UR6lJ cells is sensitive to long-term compactin treatments and long-term treatment with this drug is an effective means of blocking membrane-p21^{ras} association.

(c) Compactin treatment results in a nearly complete prevention of association of p21^{ras} with the membrane. Possibility, the remaining component is sufficient to fully maintain NGF signal transduction. However, this possibility is unlikely because these same compactin treatments completely block actions of oncogenic p21^{N-ras}.

(d) Our results show that NGF signal transduction is not inhibited by treatments that block membrane association of

Figure 8. Inhibition by compactin of growth factor-induced membrane movements in PC12 cells. PC12 cells, cultured in 35-mm dishes, were viewed using Hoffman optics and continuously photographed with a video camera. Cultures treated with compactin (C, D, G, and H) received 1 µg/ml compactin for 24 h. Video frames transferred to film and reproduced here depict PC12 cells 1 m or 9 m after addition of growth factor (50 ng/ml NGF or 3 ng/ml EGF). After 1 m of growth factor treatment, PC12 cells have not yet begun to exhibit lamellipodiae visible by Hoffman optics and thus demonstrate the quiescent state, while at 9 m such membrane movements have reached a plateau of intensity. Representative lamellipodiae are indicated by small arrows. (A) 1 m NGF, (B) 9 m NGF, (C) 1 m NGF, (D) 9 m NGF, (E) 1 m EGF, (F) 9 m EGF, (G) 1 m EGF, (H) 9 m EGF.

p21^{ras}. Possibility p21^{ras} function in NGF signal transduction is mediated by a cytoplasmic effector that does not require p21^{ras} to be membrane associated. However, this would imply that the mechanisms of action of oncogenic and normal p21^{ras} are different in that the former, but not the latter, requires association with the membrane to achieve its effects; compactin clearly blocks oncogenic p21^{ras} actions in PC12 cells. Although it has been suggested (10) that normal and oncogenic p21^{ras} may interact with different effectors, there is no evidence that these proteins differ in requirement for membrane association for their effects (47, 62), therefore such an observation would be remarkable. Further study is required to characterize p21^{ras} effector proteins and determine whether normal and oncogenic ras gene products differ in their interactions with these proteins. If it is the case that normal and oncogenic p21^{ras} achieve their effects via different mechanisms then the ability of oncogenic p21^{ras} to mimic the action of NGF can't be interpreted as supporting the idea that p21^{ras} is involved in NGF signal transduction.

Ras-dependent and ras-independent NGF Signal Transduction. The data presented here indicate that ras is not necessary for NGF signal transduction although p21^{ras} may be involved in NGF signal transduction, as suggested by the studies summarized above. We hypothesize that p21^{ras} function is not necessary for NGF signal transduction because the NGF receptor is associated with multiple intracellular signaling pathways including ras-independent pathways and possibly one or more ras-dependent pathways. Elimination of ras function by compactin treatment would then result in only a partial loss of the NGF signal transduction mechanism with the remaining ones able to induce the NGF responses observed in this study.

The existence of multiple signaling pathways initiated by receptor tyrosine kinases has been well-documented (10). Activation of p21^{ras} is only one of the pathways involved. NGF also induces tyrosine phosphorylation of multiple substrates (38, 39) and therefore is also likely to activate several intracellular signaling pathways. In favor of the idea that parallel pathways exist in NGF signal transduction is the observation that inhibition of some, but not all, protein-serine/threonine kinases by adenosine analogues abolishes some, but not all, responses to NGF (25, 61), whereas more general inhibition of kinases by K-252a eliminates all PC12 cell responses to NGF (34, 39, 50). Other studies have provided evidence for convergence of separate protein-serine/threonine kinase pathways in NGF-induced tyrosine hydroxylase phosphorylation in PC12 cells (16, 46). Furthermore, we have observed that downregulation of protein kinase C results in a partial inhibition of ODCase induction, and Sigmund et al. (55) have shown a similar effect of kinase C downregulation on induction of c-*fos*. This implies that transcriptional activation of these genes involves multiple signal transduction pathways with protein kinase C being one of them. Then, p21^{ras} may function in one or more parallel intracellular signaling pathways, exclusive of protein kinase C, but in the absence of p21^{ras} function, the remaining pathways are able to induce the NGF responses observed in this study.

Compactin-induced Responses in PC12 Cells

We have also shown a compactin-induced increase in p21^{ras}

protein synthesis in PC12 cells treated for 24 h with compactin. Two additional observations indicate that this effect is specific for p21^{ras} (Qiu, M.-S., and S.H. Green, manuscript in preparation): the overall level of protein synthesis, as determined by [^{35}S]methionine uptake into TCA-precipitable material, is unaffected by a 24-h treatment with compactin, and the synthesis rate of a nonisoprenylated protein, β-tubulin, is not affected by compactin. The observed increase in p21^{ras} synthesis is observed not only for the endogenous cellular ras gene product but also for the product of a ras gene introduced by transfection. Since the introduced ras gene has a viral glucocorticoid-responsive promoter replacing the cellular 5'-flanking genomic sequence, the effect of compactin on ras expression is not likely to be purely a result of transcriptional regulation. We are currently investigating the mechanism by which compactin affects ras mRNA and protein levels (Qiu, M.-S., and S.H. Green, manuscript in preparation).

A rapid change in cell surface morphology is virtually the earliest cellular response to growth factors; with scanning electron microscopy the motility is observable within 30 s (14, 15). Compactin blocks rapid changes in cell surface morphology induced by NGF or EGF without effect on other responses to these factors. This indicates that although the surface motility precedes other responses it is not a prerequisite for them.

We have demonstrated that among the effects of compactin on cell metabolism are some which resemble, or interact with, those of growth factors. These include NGF-inhibited cell death; cell surface morphological changes, particularly the induction of short processes or "spikes," and inhibition of growth factor-induced membrane motility; induction of a novel 24-kD phosphoprotein. Membrane morphological changes and inhibition of proliferation have been observed previously (40, 41, 42, 44). Since these effects are all reversed by addition of mevalonate to the culture medium, they are apparently due directly to compactin inhibition of mevalonate biosynthesis. HMG-CoA reductase, the enzyme inhibited by compactin, has a structure like that of G protein-coupled receptors and may be involved in the regulation of cellular metabolism (20). Possibility, regulation of cellular proliferation and physiology by metabolic demand for mevalonate involves some of the same intracellular signaling mechanisms as do growth factor receptors in their regulation of cell division. That NGF protects PC12 cells from compactin-induced cell death implies that the mechanism by which compactin kills PC12 cells may be more closely related to an interaction with growth factor signaling pathways, and developmental programmed cell death, than to simple cytotoxicity.

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