MOLECULAR AND CELLULAR BIOLOGY, Feb. 1996, p. 648–656 Vol. 16, No. 2
0270-7306/96/$04.00+0

GTPase-Deficient $G\alpha_{16}$ and $G\alpha_q$ Induce PC12 Cell Differentiation and Persistent Activation of cJun NH_2-Terminal Kinases

LYNN E. HEASLEY,[1]* BROOKE STOREY,[1] GARY R. FANGER,[2] LAURA BUTTERFIELD,[1] JEFFREY ZAMARRIPA,[1] DEBORAH BLUMBERG,[1] AND ROBERT A. MAUE[2,3]

Department of Medicine, Division of Renal Diseases and Hypertension, University of Colorado Health Sciences Center, Denver Colorado 80262,[1] and Departments of Biochemistry[2] and Physiology,[3] Dartmouth Medical School, Hanover, New Hampshire 03755

Received 14 June 1995/Returned for modification 8 August 1995/Accepted 15 November 1995

Persistent stimulation of specific protein kinase pathways has been proposed as a key feature of receptor tyrosine kinases and intracellular oncoproteins that signal neuronal differentiation of rat pheochromocytoma (PC12) cells. Among the protein serine/threonine kinases identified to date, the p42/44 mitogen-activated protein (MAP) kinases have been highlighted for their potential role in signalling PC12 cell differentiation. We report here that retrovirus-mediated expression of GTPase-deficient, constitutively active forms of the heterotrimeric G_q family members, $G\alpha_q$Q209L and $G\alpha_{16}$Q212L, in PC12 cells induces neuronal differentiation as indicated by neurite outgrowth and the increased expression of voltage-dependent sodium channels. Differentiation was not observed after cellular expression of GTPase-deficient forms of α_{i2} or α_o, indicating selectivity for the G_q family of G proteins. As predicted, overexpression of α_qQ209L and α_{16}Q212L constitutively elevated basal phospholipase C activity ~10-fold in PC12 cells. Significantly, little or no p42/44 MAP kinase activity was detected in PC12 cells differentiated with α_{16}Q212L or α_qQ209L, although these proteins were strongly activated following expression of constitutively active cRaf-1. Rather, a persistent threefold activation of the cJun NH_2-terminal kinases (JNKs) was observed in PC12 cells expressing α_qQ209L and α_{16}Q212L. This level of JNK activation was similar to that achieved with nerve growth factor, a strong inducer of PC12 cell differentiation. Supportive of a role for JNK activation in PC12 cell differentiation, retrovirus-mediated overexpression of cJun, a JNK target, in PC12 cells induced neurite outgrowth. The results define a p42/44 MAP kinase-independent mechanism for differentiation of PC12 cells and suggest that persistent activation of the JNK members of the proline-directed protein kinase family by GTPase-deficient $G\alpha_q$ and $G\alpha_{16}$ subunits is sufficient to induce differentiation of PC12 cells.

Signalling through specific receptor tyrosine kinases in PC12 pheochromocytoma cells promotes neuronal differentiation manifested by extension of neurites and increased electrical excitability due to induction of voltage-dependent sodium (Na^+) channels (14, 18, 21, 22, 38). Not all receptor tyrosine kinases direct PC12 cell differentiation (17, 50), an observation that may be related to the ability of receptor tyrosine kinases that promote differentiation to persistently activate key effector enzyme systems (24, 40, 45, 57). Among these effector systems, the Ras-dependent activation of the p42/44 mitogen-activated protein (MAP) kinases (MAPKs) and stimulation of phospholipase C (PLC) have been proposed as the minimal signals required to initiate the morphologic aspects of PC12 cell differentiation (33, 48, 55, 59). Considerable progress has been made in understanding the pathways mediating p42/44 MAP kinase activation (reviewed in references 4 and 5). As a whole, the studies demonstrate that receptor tyrosine kinases facilitate in the activation of Ras, which then cooperates in the activation of at least two protein serine/threonine kinases, Raf-1 and MAPK/ERK kinase (MEK) kinase (MEKK). Activated Raf-1 phosphorylates and activates MEKs, which in turn phosphorylate and activate the p42/44 MAP kinases. Reported findings that constitutive active forms of Ras (32, 36, 47), Raf-1 (61), and MEK (9) promote neurite outgrowth when expressed in PC12 cells support the idea that the MAP kinase pathway is a dominant pathway in PC12 cell differentiation. However, there are indications that other mechanisms also play an important role in the neuronal differentiation of these cells, as evidenced by the induction of Na^+ channel expression, a key response that occurs via mechanisms that are independent of Ras activity and not activated in response to phorbol esters (10, 16, 32).

In addition to receptor tyrosine kinases, many extracellular signals that influence cell growth and differentiation are transduced across the plasma membrane through seven membrane-spanning receptors that couple to heterotrimeric G proteins. Like the receptor tyrosine kinase systems, the G-protein-coupled receptors can stimulate mitogenesis in cultured fibroblasts, and autocrine activation of seven membrane-spanning receptor systems contributes to neoplastic growth of human tumors such as small-cell lung carcinomas (reviewed in reference 13). In addition, GTPase-deficient forms of G_s, G_i, G_o, G_q, and $G_{12/13}$ have been demonstrated to induce cellular transformation when expressed in selected cultured cell lines, and GTPase-deficient, constitutively active forms of G-protein α subunits have been identified in certain human tumors (reviewed in reference 13). Thus, GTPase-deficient forms of G proteins are capable of constitutively activating signalling pathways that integrate into the control of cell growth. In particular, α_{i2}Q205L, the GTPase-deficient form of α_{i2}, transforms Rat1a fibroblasts and constitutively activates the p42/44 MAP kinase pathway (20, 49). In addition, GTPase-deficient α_q constitutively activates PLCβ (52) and has been reported to induce transformed cell growth in NIH 3T3 fibroblasts (31), although

* Corresponding author. Mailing address: Department of Medicine, Division of Renal Diseases and Hypertension, C-281, University of Colorado Health Sciences Center, 4200 E. Ninth Ave., Denver, CO 80262. Phone: (303) 270-6065. Fax: (303) 270-4852. Electronic mail address: Heasley_L@Defiance.hsc.colorado.edu.

profound inhibition of growth has been observed in other cell types following expression of GTPase-deficient $G\alpha_{16}$ (51), a member of the G_q family.

In light of the ability of GTPase-deficient forms of various heterotrimeric G proteins to stimulate cell growth of many different cell types, we hypothesized that specific GTPase-deficient, constitutively active G-protein α subunits would persistently engage key signalling pathways and lead to neuronal differentiation of PC12 cells. In this study, we expressed constitutive active forms of α_{i2}, α_o, α_q, and α_{16} and observed that only the G_q family members induced neuronal differentiation of PC12 cells. In addition to PLC stimulation, the G_q family members caused persistent activation of the recently defined (12, 37) cJun NH_2-terminal kinases (JNKs) or stress-activated protein kinases but elicited little or no activation of the related p42/44 MAP kinases. The results suggest that activation of JNKs or stress-activated protein kinases can induce aspects of neuronal differentiation of PC12 cells independent of p42/44 MAP kinase activation.

MATERIALS AND METHODS

Materials. The $EGFR_{662-681}$ peptide (containing amino acids 662 to 681 of the epidermal growth factor [EGF] receptor [EGFR]) and IP-20 were synthesized and high-pressure liquid chromatography purified by Macromolecular Resources (Fort Collins, Colo.). The polyclonal rabbit antisera to α_{i2}, α_o, and α_{16} were generous gifts from Gary Johnson (National Jewish Center for Immunology and Respiratory Medicine, Denver, Colo.). The anti-ERK1 and anti-ERK2 antibodies directed against the C termini of the p42 and p44 MAP kinases were from Santa Cruz Biotechnology, Santa Cruz, Calif. The recombinant glutathione *S*-transferase (GST)–cJun(1-79) was expressed in bacteria and purified by using glutathione (GSH)-agarose as described previously (28). Sera, powdered growth media, and G418 for cell culture were from Gibco BRL.

Retrovirus-mediated gene transfer. The cDNAs encoding the GTPase-inhibited forms of $G\alpha_{16}$ (α_{16}Q212L), $G\alpha_{i2}$ (α_{i2}Q205L), and $G\alpha_o$ (α_oQ205L) were ligated into the *Hin*dIII site of pLNCX (43). The cDNA encoding α_qQ209L was inserted at the *Hin*dIII site of pMV7 (34). An *Eco*RI fragment encoding the constitutively active BXB-Raf-1 construct (27) was ligated into the *Eco*RI site of pLXSN (43). The human *cjun* cDNA was inserted into the *Hin*dIII and *Hpa*I sites of pLNCX. The ecotropic retroviral packaging cell line GP+E-86 (39) was transfected with the various retroviral expression vectors as well as the retroviral vector pMV7, lacking a cDNA insert, as a control by using Lipofectin (Gibco BRL) as instructed by the manufacturer. The pMV7-α_qQ209L construct was packaged in PA317 cells (42), as the construct exhibited cytotoxicity in GP+E-86 cells. Following selection in G418, stable GP+E-86 and PA317 clones were isolated and screened for overexpression of the various Gα subunits and BXB-Raf polypeptides. Confluent cultures of the retroviral packaging cell lines were allowed to secrete virus into Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml for 16 to 24 h. The medium was collected, supplemented with 8 μg of Polybrene per ml, filtered through a 0.45-μm-pore-size filter, and incubated for 16 to 24 h with PC12 cells that had been plated on collagen-coated 10-cm-diameter tissue culture dishes. The next day, the virus-containing medium was removed from the PC12 cells and replaced with fresh virus for another 16 to 24 h. Following the second retrovirus exposure, the PC12 cells were cultured in normal growth medium (DMEM containing 5% heat-inactivated horse serum, 2.5% newborn calf serum, 2.5% fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml) supplemented with 0.5 mg of G418 per ml. Studies were performed 2 to 3 weeks later with pooled cultures of G418-resistant PC12 cells.

Inositol phosphate production. G418-resistant cultures of PC12 cells previously infected with pMV7, pMV7-α_qQ209L, or LNCX-α_{16}Q212L were plated in 35-mm-diameter dishes. Two to three days later, the cells were incubated for 24 h in 1 ml of inositol-free DMEM (Gibco BRL) containing 0.1% bovine serum albumin (BSA) and 1 μCi of [^{3}H]inositol (20.5 Ci/mmol; Dupont NEN, Boston, Mass.) per ml. The cells were washed twice with DMEM containing 0.1% BSA and once with the same containing 20 mM LiCl. The cells were then incubated in 1 ml of medium containing 20 mM LiCl for 10 min at 37°C and then for another 20 min in the presence or absence of 100 nM bradykinin. The incubations were quenched with 2 ml of methanol-HCl (100:1), 1 ml of distilled water, and 2 ml of $CHCl_3$, and total inositol phosphates in the aqueous phase were purified on AG 1-X8 resin (Bio-Rad, Richmond, Calif.) and quantified as previously described (19).

p42/44 MAP kinase assays. Cultures of G418-resistant PC12 cells were lysed in mixture containing 0.5% Triton X-100, 50 mM β-glycerophosphate (pH 7.2), 0.1 mM sodium vanadate, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol (DTT), 2 μg of leupeptin per ml, and 4 μg of aprotinin per ml. Following a 5-min centrifugation (10,000 × *g*) to remove nuclei and cell debris, portions of the soluble extracts (0.5 ml, 1 mg of protein) were applied to a Pharmacia HR5/5 Mono Q column equilibrated in 50 mM β-glycerophosphate (pH 7.2)–0.1 mM sodium vanadate–1 mM EGTA–1 mM DTT and eluted with a 30-ml gradient of 0 to 350 mM NaCl in the same buffer. Fractions (1 ml) were collected, and aliquots (20 μl) of the eluted fractions were mixed with 20 μl of a mixture containing 50 mM β-glycerophosphate (pH 7.2), 0.1 mM sodium vanadate, 20 mM $MgCl_2$, 200 μM [γ-^{32}P]ATP (5,000 cpm/pmol; Dupont NEN), 50 μg of IP-20 (TTYADFIASGRTGRRNAIHD) per ml, 1 mM EGTA, and 400 μM $EGFR_{662-681}$ peptide (RRELVEPLTPSGEAPNQALLR) as previously described (24). The kinase reaction mixtures were incubated for 15 min at 30°C, and the reactions were terminated with 10 μl of 25% trichloroacetic acid. $EGFR_{662-681}$ peptide phosphorylation was assessed by binding to P-81 phosphocellulose filters (Whatman) as described previously (24).

In some experiments, p42 and p44 MAP kinases were purified by immunoprecipitation, and protein kinase activity in the immune complexes was assessed. Cell extracts (0.5 mg of protein in 0.5 ml of MAP kinase lysis buffer described above) from the PC12 cell cultures expressing the various G-protein α subunits were incubated for 1 h at 4°C with 1 μg of anti-ERK1 and 1 μg of anti-ERK2 antibodies (Santa Cruz Biotechnology) and then for another hour with 100 μl of a 10% suspension of formalin-fixed *Staphylococcus aureus* (Sigma Chemical Co). The immune complexes were collected by microcentrifugation, washed three times with lysis buffer, and suspended in 70 μl of lysis buffer. Aliquots (20 μl) were assayed in triplicate, using the $EGFR_{662-681}$ peptide kinase assay described above.

JNK activity assay. For analysis of JNK activity, G418-resistant PC12 cell cultures were lysed for 30 min at 4°C in 0.5 ml of 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.7)–20 mM β-glycerophosphate–0.1 mM sodium vanadate–0.1% Triton X-100–0.3 M NaCl–1.5 mM $MgCl_2$–0.2 mM EDTA–0.5 mM DTT–2 μg of leupeptin per ml–4 μg of aprotinin per ml as described previously (28). Following a 5-min microcentrifugation (10,000 × *g*), aliquots of the extracts containing 400 μg of protein were incubated for 2 h at 4°C with GST–cJun(1-79) immobilized to GSH-agarose (10 μl of packed beads per sample containing 10 to 20 μg of protein) as described previously (28). The GSH-agarose beads were washed four times by repetitive centrifugation in 20 mM HEPES (pH 7.7)–50 mM NaCl–2.5 mM $MgCl_2$–0.1 mM EDTA–0.05% Triton X-100 and then incubated for 20 min at 30°C in 40 μl of 50 mM β-glycerophosphate (pH 7.6)–0.1 mM sodium vanadate–10 mM $MgCl_2$–20 μM [γ-^{32}P]ATP (25,000 cpm/pmol). The reactions were terminated with 10 μl of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer, boiled, and submitted to SDS-PAGE (10% polyacrylamide gel). The GST–cJun(1-79) polypeptides were identified in Coomassie blue-stained gels, excised, and counted in a scintillation counter.

Immunoblot analyses. Stable, pooled populations of PC12 cells expressing the various Gα subunits were collected and lysed in 20 mM HEPES (pH 7.4)–2 mM $MgCl_2$–1 mM EGTA–0.5% Triton X-100 containing aprotinin and leupeptin. The extracts were microcentrifuged (5 min, 10,000 × *g*), and solubilized polypeptides were electrophoresed on SDS–10% polyacrylamide gels and transferred to nitrocellulose filters. The filters were blocked in Tris-buffered saline–0.1% Tween 20 (TTBS) containing 3% nonfat milk and then incubated for 16 to 24 h in the same buffer containing a rabbit antiserum specific for α_{i2}, α_o, or α_{16}. The filters were extensively washed in TTBS, incubated for 1 h with horseradish peroxidase-labeled protein A in TTBS containing 3% milk, washed in TTBS, and developed by enhanced chemiluminescence as instructed by the manufacturer (Du Pont NEN).

Isolation and analysis of Na^+ channel α-subunit mRNA. Cells plated at ~10^6/100-mm-diameter tissue culture dish (Falcon) were harvested, and total cellular RNA was isolated by the method of Chirgwin et al. (6). For Northern (RNA) blot analysis, 40-μg RNA samples were size fractionated on 0.8% agarose gels containing 2.0 M formaldehyde and transferred to a nylon membrane (Zetabind; Cuno, Inc., Meriden, Conn.) overnight, and the membrane was baked at 80°C for 2 h. [^{32}P]UTP-labeled cRNA probes specific for Na^+ α-subunit channel mRNA and cylcophilin mRNA were generated and hybridized with the membrane at final activities of 6×10^6 and 5×10^5 cpm/ml, respectively, as described previously (16, 17). After hybridization for 36 h at 65°C, the membrane was washed and exposed to Kodak XAR film at −80°C for 24 h. RNase protection analysis was performed as described previously (16, 17). A commercially available kit (Promega, Madison, Wis.) was used to generate [^{32}P]UTP-labeled probes specific for type II Na^+ channel α-subunit mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. After the probes were hybridized with 20-μg samples of RNA, the mixture was digested with 24 mg of RNase A per ml and 160 U of RNase T_1 (Ambion, Austin, Tex.) for 1 h at 30°C, separated on a 6% acrylamide gel, and exposed to Kodak XAR film for 24 h at −80°C. The NIH IMAGE program was used for densitometric analysis of the autoradiographic signals representing the Na^+ channel, GAPDH, and cyclophilin mRNAs.

Electrophysiological recording and analysis. Na^+ current density in cells maintained in 35-mm-diameter tissue culture dishes (Falcon) was determined by using whole-cell patch clamp measurements of peak Na^+ current amplitude and cell membrane capacitance as previously described (16, 17). To minimize the contribution of other voltage-activated currents during recording, cells were placed in a saline solution (150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM

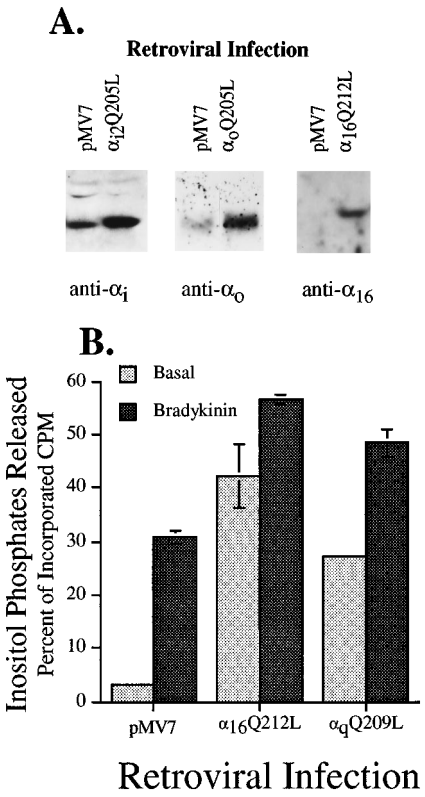

FIG. 1. Expression of GTPase-deficient Gα subunits in PC12 cells. (A) Immunoblot analysis of α-subunit expression in retrovirus-infected PC12 cells. Extracts from the indicated G418-resistant PC12 cell cultures were resolved by SDS-PAGE (10% polyacrylamide gel), transferred electrophoretically to nitrocellulose, and immunoblotted with polyclonal antisera to the indicated α subunit. (B) PLC activation in PC12 cells expressing α_qQ209L and α_{16}Q212L. The indicated PC12 cell cultures were labeled for 16 to 24 h with [^{3}H]inositol, and the accumulation of total inositol phosphates was determined in cells stimulated with or without 100 nM bradykinin as described in Materials and Methods. Data are means and standard errors of the means of triplicate determinations from an experiment that is representative of three independent experiments.

$MgCl_2$, 6 mM HEPES, 6 mM glucose [pH 7.2]), and patch electrodes (3 to 7 mΩ) contained 140 mM CsCl, 10 mM EGTA, and 10 mM HEPES. By using a List EPC-7 patch clamp amplifier (Medical Systems Corp., Greenvale, N.Y.) and an Atari computer-based acquisition system (Instrutech Corp., Elmont, N.Y.), cells were held at −80 mV and every 3 s prepulsed to −120 mV for 40 ms prior to a 20-ms depolarization to a test potential between −60 and +30 mV. Electronic compensation was used to reduce series resistance errors to 5 mV or less, reduce the time constant of membrane charging, and provide measurements of access resistance and cell membrane capacitance. Current recordings were digitally filtered at 2 kHz during analysis, and scaled pulse (P/4) routines were used to remove linear leakage currents and capacity transients.

RESULTS

Retrovirus-mediated expression of GTPase-deficient forms of α_{i2}, α_o, α_q, and α_{16} in PC12 cells. To investigate the abilities of various G-protein α subunits to induce PC12 cell differentiation, cDNAs encoding GTPase-deficient forms of α_{i2}, α_o, α_q, and α_{16} were ligated into retroviral expression vectors and packaged into replication-defective retroviruses, using GP+E-86 and PA317 cells (see Materials and Methods). Infection of PC12 cell cultures with the high-titer retrovirus preparations encoding the mutant α subunits permitted efficient expression of the cDNAs in PC12 cells and rendered 10 to 50% of the cells G418 resistant by the coexpressed Neo^r gene. Figure 1A shows the levels of expression of the various G-protein α subunits as assessed by immunoblotting in pooled populations of retrovirus-infected, G418-resistant PC12 cells. Both α_{i2} and α_o were overexpressed relative to endogenous levels of these α subunits observed in cells infected with a control virus lacking a cDNA insert. Parental PC12 cells lack detectable endogenous α_{16}, as the expression of this Gα subunit is restricted to cells of hematopoietic origin (2). Thus, successful expression of α_{16} was readily detected in the cells infected with the retrovirus encoding α_{16}Q212L. Overexpression of the α_q polypeptide in PC12 cells infected with viruses transducing α_qQ209L was not readily apparent by immunoblotting, although detection of the expected mRNA encoding the pMV7-driven α_q sequences was verified by reverse transcription of total RNA followed by PCR (23).

To define the functional expression of α_{16}Q212L and α_qQ209L in PC12 cells, basal activity of PLC was measured since α_q and α_{16} are known to engage several isoforms of PLCβ (30, 35). The data in Fig. 1B demonstrate that PC12 cells infected with retroviruses transducing either α_{16}Q212L or α_qQ209L resulted in basal PLC activity that was 8- to 12-fold higher than in PC12 cells infected with pMV7 virus lacking a cDNA insert. The level of PLC activation was comparable to the activation achieved with bradykinin, which activates PLC through an endogenous, G-protein-coupled receptor. Despite the marked activation of PLC by α_{16}Q212L and α_qQ209L, neither the content of diacylglycerol nor the activity of protein kinase C (PKC) was significantly higher in α_{16}Q212L- and α_qQ209L-expressing cells than in pMV7-infected cells (23).

Expression of α_{16}Q212L or α_qQ209L in PC12 cells stimulates neurite outgrowth and induction of neuronal sodium channel mRNA and activity. Inspection of PC12 cell cultures infected with the various α-subunit-encoding retroviruses and selected for resistance to G418 revealed marked neurite outgrowth from PC12 cell cultures expressing α_{16}Q212L or α_qQ209L (Fig. 2) as well as cellular hypertrophy (as indicated by increased cellular capacitance; see the legend to Fig. 3) and cessation of cell division, all hallmarks of nerve growth factor (NGF)-induced differentiation (18, 21, 22). These results were consistently observed with multiple isolates of wild-type PC12 cells. At 2 weeks postinfection, the response with α_{16}Q212L and α_qQ209L was equivalent to the neurite outgrowth observed following expression of BXB-Raf-1, a constitutively active form of Raf-1 that has previously been shown to induce robust morphological differentiation in PC12 cells (61). Expression of α_{i2}Q205L or α_oQ205L failed to induce the extension of long, branching neurites (Fig. 2), although some cells occasionally appeared to be more flattened and extended short processes. In addition, PC12 cells expressing α_{i2}Q205L or α_oQ205L continued to divide in a manner similar to that of PC12 cells infected with control pMV7 virus. Thus, constitutively active forms of the G_q family members, α_{16} and α_q, but not α_{i2} or α_o promoted morphologic differentiation of PC12 cells. A previous report (56) failed to observe neuronal differentiation of PC12 cells after transfection of cells with an α_qQ209L expression vector and, in fact, observed modest neurite outgrowth following transfection of several GTPase-deficient α_o constructs. Their failure to observe differentiation with α_qQ209L could be related to a transfection procedure less efficient than the retrovirus-mediated infection used in our study. We cannot currently explain the discrepancy of our study and the previous report (56) regarding α_o action in PC12 cells. We know that our retroviral construct expresses a functional α_oQ205L polypeptide, as evidenced by its ability to induce a transformed phenotype when expressed in a variety of fibroblast cell lines (23).

In addition to the morphological changes that occur, the

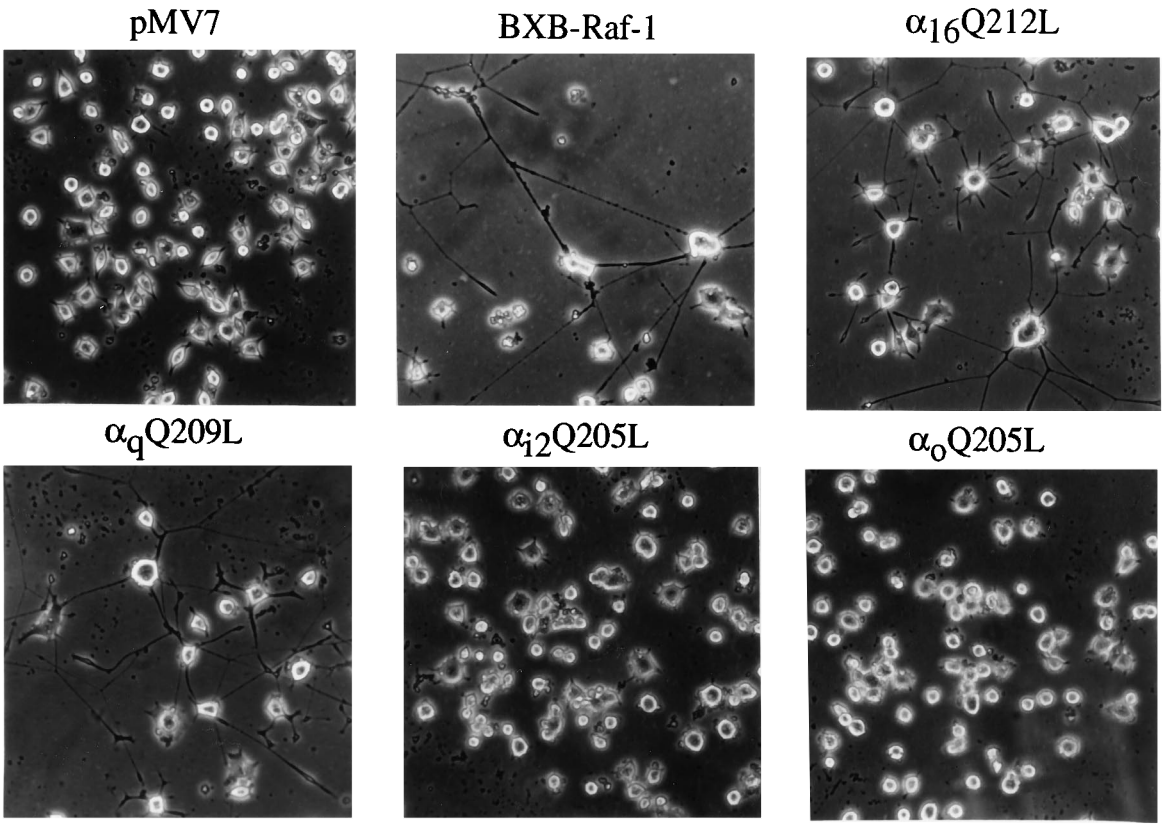

FIG. 2. Neurite outgrowth from PC12 cells infected with α_qQ209L and α_{16}Q212L. PC12 cells were infected with the indicated retroviral constructs and selected in growth medium containing G418. The photographs are of cultures 2 weeks after retroviral infection and are representative of more than 20 different infections and three independent isolates of PC12 cells.

electrical excitability of PC12 cells changes markedly during neuronal differentiation as a result of the increased expression of voltage-dependent Na^+ channels (14, 38, 53). In response to NGF and other growth factors that induce the neuronal differentiation of PC12 cells, there are increases in the expression of neuronal cell-specific genes encoding pore-forming Na^+ channel α subunits (11, 17, 38), including a sustained increase in the expression of the type II Na^+ channel α-subunit gene (17, 38). Coincident with the increase in the level of α-subunit mRNA, there is an increase in the density of functional Na^+ channels in the cell membrane (16, 17, 38). Although the molecular mechanisms underlying the increase in Na^+ channel expression are relatively undefined, it is clear that, unlike many of the NGF-mediated responses examined so far, the induction of Na^+ channel expression during PC12 cell differentiation is independent of Ras activity (10, 16) and as such represents a category of biologically important responses distinct from Ras-dependent responses such as neurite outgrowth. To investigate Na^+ channel induction, Northern blot analysis of total cellular RNA from PC12 cells expressing either α_{16}Q212L or BXB-Raf-1 (Fig. 3A) was performed with a probe corresponding to a highly conserved region in the family Na^+ channel α-subunit genes (38) that is able to detect multiple types of Na^+ channel α-subunit mRNA (7, 54). In both cases, the cells responded, as evidenced by marked neurite outgrowth (Fig. 2). However, in PC12 cells expressing α_{16}Q212L, there was an increase in Na^+ channel α-subunit mRNA (2.7-fold) comparable to the increase (2.5-fold) observed in response to NGF treatment for 7 days (data not shown), while the level remained unchanged in cells expressing BXB-Raf-1 (Fig. 3A). The lack of Na^+ channel α-subunit mRNA induction in response to Raf activity is in agreement with previous studies (10). Consistent with these results, in RNase protection assays using a probe specific for type II Na^+ channel α-subunit mRNA (see Materials and Methods), there were detectable increases in type II Na^+ channel α-subunit mRNA in cells expressing α_{16}Q212L (Fig. 3B). Again, the increase in type II Na^+ channel α-subunit mRNA detected in the cells expressing α_{16}Q212L (2.2-fold) was similar to that observed in the cells treated with NGF (2.2-fold), while there was no detectable increase in the cells expressing BXB-Raf-1 (Fig. 3B). Finally, to determine if there was an accompanying increase in functional Na^+ channel expression in the PC12 cells expressing α_{16}Q212L, whole-cell patch clamp recordings were used to analyze Na^+ currents in these cells. Following expressing of α_{16}Q212L, the proportion of cells (16 of 19) with appreciable (>100 pA) Na^+ currents was higher than that found in control cells (2 of 16). Furthermore, when the approximate 2-fold increase in cell size was taken into account (see the legend to Fig. 3), there was a significant ($P < 0.01$) increase (~5-fold) in Na^+ current density in cells expressing α_{16}Q212L compared with control cells (Fig. 3C). Thus, expression of α_{16}Q212L in PC12 cells induces two key components of neuronal differentiation that are regulated by distinct mechanisms.

Activation of protein kinases in Gα-expressing PC12 cell cultures. To define the signalling pathways potentially involved in G_q-stimulated PC12 cell differentiation, the activities of members of the MAP kinase family were analyzed. It has been previously established that chronic stimulation of endogenous NGF receptors or exogenously overexpressed insulin, EGF

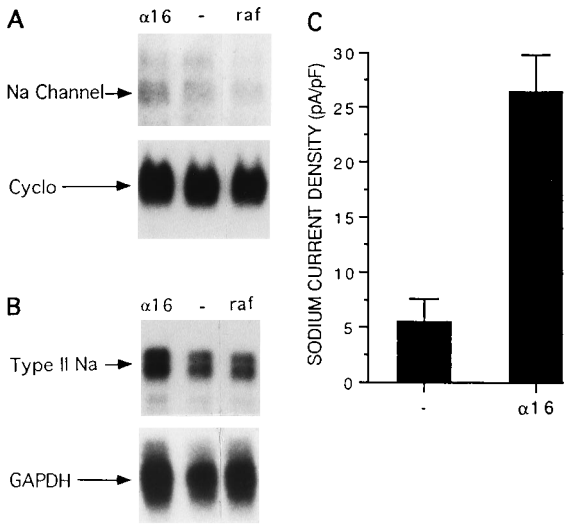

FIG. 3. Increased Na^+ channel expression in PC12 cells expressing α_{16}Q212L. (A) Representative Northern blot of Na^+ channel α-subunit mRNA (Na channel) and the constitutively expressed cyclophilin mRNA (Cyclo) in 40-μg samples of total RNA from PC12 cells 10 days after infection with a control expression vector (−), a vector expressing α_{16}Q212L (α16), or a vector expressing BXB-Raf-1 (raf). The Na^+ channel mRNA signals are shown after 24-h exposure to film, while the cyclophilin mRNA signals are shown after 30-min exposure to film. (B) Representative RNase protection experiment analyzing RNA samples (20 μg) isolated from PC12 cells 7 days after infection with a control expression vector (−), a vector expressing α_{16}Q212L (α16), or a vector expressing BXB-Raf-1 (raf). Samples were hybridized with a probe specific for type II Na^+ channel α-subunit mRNA and a probe specific for the constitutively expressed GAPDH mRNA, with signals representing the GAPDH mRNA used as an internal control for loading variations. Only the signals representing the protected fragments of type II Na^+ channel mRNA (type II Na) and GAPDH mRNA (GAPDH) are shown. The type II Na^+ channel mRNA signals are shown after 24-h exposure to film, while the GAPDH mRNA signals are shown after 30-min exposure to film. (C) Average Na^+ current density in PC12 cells 10 days after infection with a control expression vector (−) or a vector expressing α_{16}Q212L (α16). Data are means ± standard errors of the means (bars). Cell membrane capacitances (means ± standard errors of the means; in picofarads) were 6.6 ± 0.3 for control cells (n = 16) and 11.2 ± 0.51 (n = 19) for cells expressing α_{16}Q212L and are indicative of cellular hypertrophy in the α_{16}Q212L-expressing cells. The increase in Na^+ current density in the cells expressing α_{16}Q212L was statistically significant ($P < 0.001$), as determined by a two-tailed Student t test.

and beta platelet-derived growth factor (βPDGF) receptors induces PC12 cell differentiation (15, 17, 24, 58, 59) and is associated with persistent activation of Ras and p42/44 MAP kinases (15, 24, 45, 57, 58). We tested whether sustained activation of MAP kinases is observed in PC12 cells differentiated following expression of α_{16}Q212L. Extracts from PC12 cell cultures stably expressing the empty vector or α_{16}Q212L were partially purified on DEAE columns (25) and assayed for MAP kinase activity with the $EGFR_{662-681}$ peptide substrate (see Materials and Methods). Basal $EGFR_{662-681}$ peptide kinase activity was increased from 18.4 ± 15.1 pmol/min/mg of protein in control pMV7-infected PC12 cells to 58.5 ± 4.2 pmol/min/mg of protein in extracts from α_{16}Q212L-expressing cells, a 3.2-fold stimulation. A 24-h incubation of α_{16}Q212L-expressing cells with 1 μM tetradecanoyl phorbol acetate (TPA) to down-regulate PKC failed to reduce the basal kinase activity in α_{16}Q212L-expressing cells (64.2 ± 5.3 pmol/min/mg of protein), although the 14-fold stimulation of MAP kinase activity observed in control pMV7-infected PC12 cells treated for 5 min with 100 nM TPA was completely abolished by TPA down-regulation. The data indicate that a significantly elevated $EGFR_{662-681}$ peptide kinase activity is observed in α_{16}Q212L-expressing cells relative to control PC12 cells and that the activation is independent of PKC.

To biochemically characterize the stimulated protein kinase activities present in PC12 cells differentiated in response to expression of constitutive active Gα subunits and Raf-1, extracts from retrovirus-infected PC12 cells were fractionated on Mono Q FPLC and assayed for MAP kinase activity with the $EGFR_{662-681}$ peptide substrate (Fig. 4A). PC12 cells expressing BXB-Raf-1 exhibited markedly elevated $EGFR_{662-681}$ peptide kinase activity, with peak fractions at 13 and 17 ml. Immunoblot analysis of cell extracts similarly fractionated with antiphosphotyrosine antibodies indicated that the peaks of $EGFR_{662-681}$ peptide kinase activity at 13 and 17 ml coelute with p42 and p44 MAP kinase polypeptides, respectively (24). This result is predicted given the evidence that cRaf-1 is an upstream component of the MAP kinase pathway (4, 5). Analysis of $EGFR_{662-681}$ peptide kinase activity in fractionated extracts from α_{16}Q212L-expressing PC12 cells failed to detect a significant peptide kinase activation in fractions 13 and 17, although a distinct peak of activity eluting at fraction 16 was observed (Fig. 4A). Fractionation of extracts from PC12 cells infected with α_qQ209L also revealed a distinct peak of $EGFR_{662-681}$ peptide kinase activity that eluted between the p42 and p44 peaks of MAP kinase strongly activated by the expression of BXB-Raf-1 (Fig. 4B). A modest increase in pep-

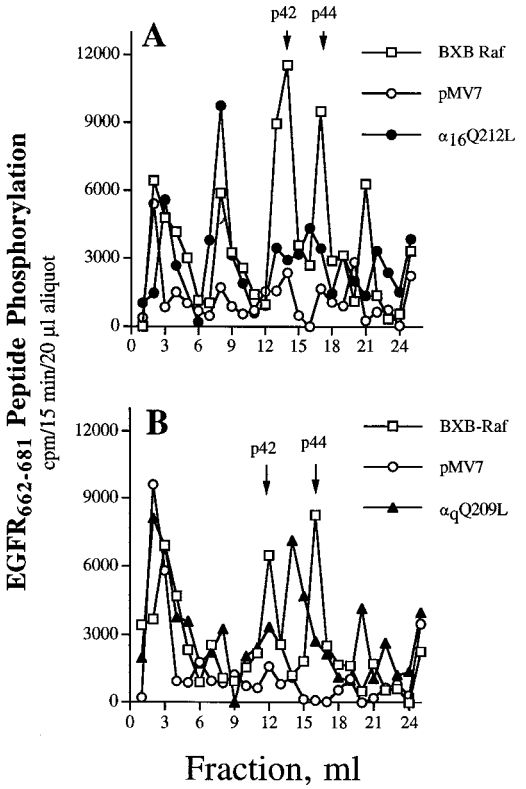

FIG. 4. Mono Q FPLC analysis of $EGFR_{662-681}$ peptide kinase activities in PC12 cells expressing α_qQ209L, α_{16}Q212L, and BXB-Raf-1. (A) Extracts from PC12 cells expressing pMV7, α_{16}Q212L, or BXB-Raf-1 were fractionated on Mono Q FPLC and assayed for kinase activity with the $EGFR_{662-681}$ peptide kinase assay (see Materials and Methods). The elution positions of p42 and p44 MAP kinases activated in extracts from BXB-Raf-1-expressing PC12 cells are indicated. A distinct peak of $EGFR_{662-681}$ peptide kinase activity in extracts from α_{16}Q212L-expressing cells reproducibly eluted between the peaks of p42 and 44 MAP kinases. (B) Extracts from PC12 cells expressing pMV7, α_qQ209L, and BXB-Raf-1 were analyzed similarly to those shown in panel A.

tide kinase activity was consistently observed in fractions 12 and 13 in extracts from α_qQ209L-expressing cells. Thus, the data in Fig. 4 suggest that the p42/44 MAP kinases are not markedly activated in α_qQ209L- and α_{16}Q212L-expressing PC12 cells, although $EGFR_{662-681}$ peptide kinase activities with distinct elution positions from Mono Q FPLC are activated.

To more definitively assess the activity of p42/44 MAP kinases in PC12 cells infected with the various retroviral constructs, soluble extracts were prepared and the p42 and p44 MAP kinases were collected by immunoprecipitation with specific anti-ERK antibodies (see Materials and Methods). Supportive of the data in Fig. 4, analysis of MAP kinase activity in the immune complexes revealed a marked sevenfold elevation of kinase activity in immunoprecipitates from BXB-Raf-expressing cells (Fig. 5A). By contrast, $EGFR_{662-681}$ peptide kinase activity in p42/44 MAP kinase immunoprecipitates from α_{16}Q212L-expressing PC12 cells was not different from the control level. Consistent with a weak activation of p42 MAP kinase by α_qQ209L observed in Fig. 4B, protein kinase activity in p42/44 MAP kinase immunoprecipitates from α_qQ209L-expressing cells was increased 1.8-fold in three independent experiments (Fig. 5A), although this increase was not found to be significant by analysis of variance. Thus, the expression of α_{16}Q212L and α_qQ209L in PC12 cells induces aspects of neuronal differentiation and persistently stimulates several $EGFR_{662-681}$ peptide kinase activities but, in contrast to BXB-Raf, does not lead to marked activation of the p42/44 MAP kinases.

Inspection of the Mono Q FPLC profiles shown in Fig. 4 indicates that α_{16}Q212L and α_qQ209L expression consistently increased two peaks of kinase activity eluting at fractions 7 to 9 and 14 to 16 between the p42/44 MAP kinase peaks. We have previously reported (1) that the $EGFR_{662-681}$ peptide is efficiently phosphorylated by p54 MAP kinase, which has now been identified as a JNK isoform (12, 37). Also, recent reports indicate that the JNKs elute from Mono Q FPLC at lower NaCl concentrations relative to the p42/44 MAP kinases (3). Together, these data implicate the JNKs as protein kinases potentially regulated in response to α_{16}Q212L and α_qQ209L expression. To more directly assess the activity of JNKs in extracts from PC12 cells expressing α_{16}Q212L and α_qQ209L, an assay in which activated JNKs are purified by virtue of their binding to an immobilized GST–cJun(1-79) fusion protein was used. Following extensive washing, the adsorbed JNKs were assayed for the ability to phosphorylate the GST–cJun(1-79) protein resident in the immobilized complex. The data in Fig. 5B show that JNK activity is ~3-fold higher in extracts from PC12 cells expressing α_{16}Q212L or α_qQ209L than in cell extracts from control infections. In comparison, there was a ~7-fold increase in JNK activity (Fig. 5B) achieved with UVC irradiation (192 J/m^2), a strong activator of JNKs in many cells. Thus, α_{16}Q212L and α_qQ209L activated the JNKs to a level approximately 50% of that observed with UV irradiation but induced little or no activation of the p42/44 MAP kinases. Surprisingly, marked JNK activation was also observed following expression of BXB-Raf in PC12 cells (Fig. 5B). cRaf-1 is thought to be restricted to activation of MEK isoforms that lead largely to the phosphorylation and activation of p42/44 MAP kinases (4, 5, 44). The mechanism for this unexpected finding is currently being investigated.

To further examine the association between JNK activation observed with α_{16}Q212L and α_qQ209L expression and PC12 cell differentiation, we assessed the regulation of JNKs by factors which signal through receptor tyrosine kinases, not heterotrimeric G proteins. Figure 5C shows that a 15-min incubation with NGF, PDGF-BB, and EGF increased JNK activity ~3- to 4-fold in PC12 cells expressing the human βPDGF receptor (59). Also noted was that a 2-fold JNK activation was maintained after 60 min of incubation with NGF and PDGF-BB, which initiate neuronal differentiation in these cells, but not with EGF (1.2-fold), which fails to stimulate PC12 cell differentiation. This experiment demonstrates that the magnitude of JNK activation achieved with stable expression of GTPase-deficient $G\alpha_q$ proteins is similar to the JNK

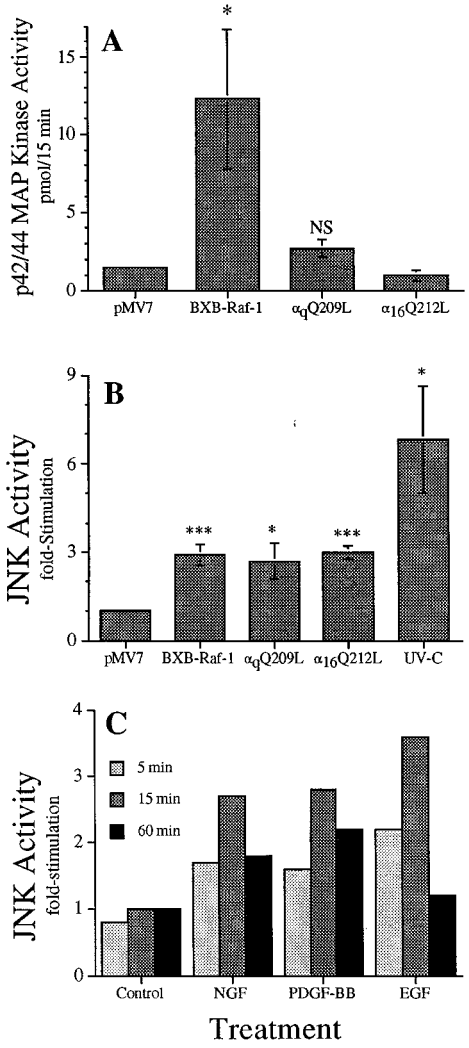

FIG. 5. Regulation of p42/44 MAP kinase and JNK MAP kinase activities by expression of α_qQ209L and α_{16}Q212L and stimulation with growth factors in PC12 cells. (A) Extracts were prepared in MAP kinase lysis buffer as for Fig. 4, and the p42/44 MAP kinases were immunoprecipitated with a mixture of polyclonal ERK1 and ERK2 antibodies (see Materials and Methods). After washing, the immune complexes were suspended in MAP kinase lysis buffer and assayed for $EGFR_{662-681}$ peptide kinase activity. Data are means ± standard errors of the means of three independent experiments. The 1.8-fold increase in p42/44 MAP kinase activity in extracts from α_qQ209L-expressing cells relative to control cells was not significant by analysis of variance. (B) Cell lysates were prepared from the indicated PC12 cell cultures or cells UV irradiated and incubated for 30 min at 37°C. The JNKs were adsorbed and assayed by using GSH-agarose-immobilized GST–cJun(1-79) as described in Materials and Methods. Data are means ± standard errors of the means of four to seven independent experiments. Analysis of variance revealed $P < 0.05$ and $P < 0.001$, as indicated by * and ***, respectively. (C) PC12 cells expressing the human βPDGF receptor (59) were incubated with NGF (100 ng/ml), PDGF-BB (30 ng/ml), or EGF (10 ng/ml) for the indicated times and assayed for JNKs. Data are means of two independent experiments.

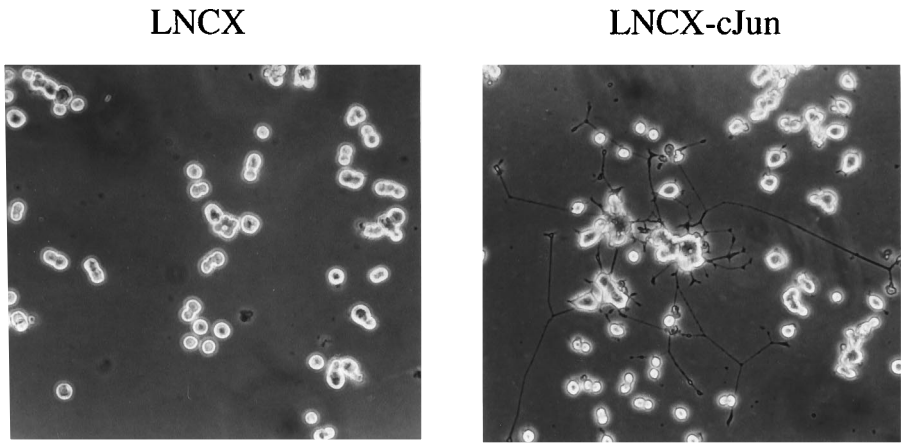

FIG. 6. Retrovirus-mediated expression of cJun in PC12 cells induces neurite outgrowth. PC12 cells were infected with the LNCX retrovirus or a virus expressing LNCX-cJun as described in Materials and Methods. The cells were cultured in growth medium containing G418 and photographed 7 days after infection. The field of cells shown is representative of three independent infections.

activation following stimulation of PC12 cells with NGF or PDGF-BB, which signal through receptor tyrosine kinases. In addition, the data suggest that persistent activation of the JNK pathway may be associated with agents that induce PC12 cell differentiation in a manner similar to that of the previously described regulation of the p42/44 MAP kinases (24, 40, 45, 57).

Overexpression of a JNK target, cJun, induces neurite outgrowth in PC12 cells. The JNKs were identified and purified by virtue of their high affinity and activity toward the transcription factor cJun (12, 28). To further explore the function of the JNK pathway in PC12 cells, we overexpressed cJun by using a retroviral expression vector. Figure 6 shows that overexpression of cJun in PC12 cells induced marked neurite extension observed after 7 days relative to infection with a virus lacking a cDNA insert (LNCX). This observation provides further evidence for a role for JNK and transcription factor targets such as cJun in promotion of PC12 cell differentiation. This finding is especially striking in light of previous reports that overexpression of a related transcription factor, cFos (29), as well as cMyc (41) inhibits rather than stimulates PC12 cell differentiation.

DISCUSSION

The results show that GTPase-deficient G-protein α subunits of the G_q family are competent to signal differentiation of PC12 cells, including the extension of neurites and induction of neuronal sodium channel mRNA and protein. While α_qQ209L has been reported to act as a transforming agent for NIH 3T3 fibroblasts (31), expression of the related Gα protein α_{16}Q212L in Swiss 3T3 cells (51) and small-cell lung carcinomas (26) is associated with marked growth inhibition. Furthermore, similarly to the ability of α_{16}Q212L and α_qQ209L to induce neuronal differentiation of PC12 cells, α_{16}Q212L expression in rat aortic vascular smooth muscle cells inhibits cell growth, induces cellular hypertrophy, and increases smooth muscle α-actin expression, findings indicative of an increased differentiation state of these cells (46). Thus, G_q family members can engage signalling pathways leading to growth inhibition and differentiation in many cellular contexts.

The observation that PC12 cell differentiation induced by expression of α_{16}Q212L and α_qQ209L is associated with threefold activation of the JNK MAP kinases but little or no activation of the p42/44 MAP kinases raises the novel hypothesis that the JNKs signal PC12 cell differentiation in response to expression of GTPase-deficient G_q proteins. Findings that JNKs but not p42/44 MAP kinases are activated in response to stable expression of α_{16}Q212L or α_qQ209L in small-cell lung carcinomas (26), vascular smooth muscle cells (46), and Rat1a (23) and NIH 3T3 (60) fibroblasts argues against the increased JNK activation merely representing an indirect effect of α_{16}Q212L expression in PC12 cells. In fact, NGF and PDGF-BB, which signal PC12 cell differentiation through receptor tyrosine kinases, activated JNKs to a degree similar to that of α_{16}Q212L and α_qQ209L (Fig. 5C). Finally, stimulation of neurite outgrowth following retroviral expression of the JNK target, cJun, provides further support for the potential integration of a JNK pathway into PC12 cell differentiation.

PLC activity is strongly and persistently elevated in PC12 cells expressing α_{16}Q212L and α_qQ209L (Fig. 1B), yet there is little or no detectable increase in the activity of PKC or the cellular content of diglyceride, the endogenous activator of PKC (23). A recent study demonstrates that the M1 muscarinic receptor, which couples to G_q and PLCβ, markedly activates JNKs in NIH 3T3 cells in a PKC-independent manner (8). Likewise, the increase in $EGFR_{662-681}$ peptide kinase activity in extracts from α_{16}Q212L-expressing PC12 cells is not inhibited following down-regulation of PKC (see Results). Finally, phorbol esters, which directly activate PKC and lead to markedly increased p42/44 MAP kinase activity, only weakly stimulate JNK activity in PC12 cells (23). Thus, the data suggest that G_q proteins may engage effector systems distinct from PLCβ and PKC that lead to the activation of JNKs.

The proximal effectors and activators involved in mediating the stimulation of JNKs in PC12 cells by α_{16}Q212L and α_qQ209L as well as by receptor tyrosine kinases remain largely undefined. NGF-stimulated activation of both the JNKs and the p42/44 MAP kinases in PC12 cells is inhibited by expression of inhibitory N17 Ras (44). Likewise, GTPase-deficient forms of $G\alpha_{12}$ and $G\alpha_{13}$ fail to activate JNKs in the presence of N17 Ras (60). Finally, MEKK-1, which is a protein serine/threonine kinase that selectively phosphorylates JNK kinases and leads to the activation of JNKs relative to the p42/44 MAP kinases, may also be Ras regulated (44). Combined, the findings indicate the involvement of low-molecular-weight G proteins of the Ras family as well as target protein kinases such as MEKK in the activation of JNKs by diverse stimuli.

PC12 cell neuronal differentiation in response to NGF is transduced to a large extent, if not completely, through a receptor tyrosine kinase, not G_q proteins. Yet expression levels of α_qQ209L and α_{16}Q212L are similar to that of NGF in stimulating both neurite outgrowth and Na^+ channel expression. These findings suggest either that a common set of necessary signals are activated in response to receptor tyrosine kinases and GTPase-deficient G_q proteins or that functionally redundant parallel pathways are activated. Indeed, the existence of functionally redundant signalling pathways that control PC12 cell differentiation has been invoked as a result of molecular dissection of signalling through Trk and βPDGF receptors in PC12 cells (33, 48, 55, 59). For Trk signalling, a minimal involvement of SHC, which leads to regulation of Ras, and PLCγ was defined (33, 48, 55). A similar definition of the minimal signals for PC12 cell differentiation mediated by exogenous βPDGF receptors (59) indicated the requirement of the Ras/Raf/MEK/p42/44 MAP kinase pathway and either a PLCγ-derived signal or a Src pathway. With respect to these findings, it is interesting that α_qQ209L and α_{16}Q212L selectively signal through the JNK MAP kinases, strongly activate PLCβ, and induce PC12 cell differentiation, raising the intriguing possibility that the JNKs represent one of several signalling pathways that can mediate PC12 cell differentiation.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants GM48826 and DK19928 to L.E.H. and NS 28767 to R.A.M. from the National Institutes of Health.

REFERENCES

1. **Abdel-Hafiz, H. A.-M., L. E. Heasley, J. M. Kyriakis, J. Avruch, D. J. Kroll, G. L. Johnson, and J. P. Hoeffler.** 1992. Activating transcription factor-2 DNA-binding activity is stimulated by phosphorylation catalyzed by p42 and p54 microtubule-associated protein kinases. Mol. Endocrinol. **6:**2079–2089.
2. **Amatruda, T. T., III, D. A. Steele, V. Z. Slepak, and M. I. Simon.** 1991. $G\alpha_{16}$, a G protein α subunit specifically expressed in hematopoietic cells. Proc. Natl. Acad. Sci. USA **88:**5587–5591.
3. **Bird, T. A., J. M. Kyriakis, L. Tyshler, M. Gayle, A. Milne, and G. D. Virca.** 1994. Interleukin-1 activates p54 mitogen-activated protein (MAP) kinase/stress-activated protein kinase by a pathway that is independent of $p21^{ras}$, Raf-1, and MAP kinase kinase. J. Biol. Chem. **269:**31836–31844.
4. **Blumer, K. J., and G. L. Johnson.** 1994. Diversity in function and regulation of MAP kinase pathways. Trends Biochem. Sci. **19:**236–240.
5. **Cano, E., and L. C. Mahadevan.** 1995. Parallel signal processing among mammalian MAPKs. Trends Biochem. Sci. **20:**117–122.
6. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acids from sources enriched in ribonuclease. Biochemistry **18:**5294–5299.
7. **Cooperman, S. S., S. A. Grubman, R. L. Barchi, R. H. Goodman, and G. Mandel.** 1987. Modulation of sodium channel mRNA levels in rat skeletal muscle. Proc. Natl. Acad. Sci. USA **84:**8721–8725.
8. **Coso, O. A., M. Chiariello, G. Kalinec, J. M. Kyriakis, J. Woodgett, and J. S. Gutkind.** 1995. Transforming G protein-coupled receptors potently activate JNK (SAPK). J. Biol. Chem. **270:**5620–5624.
9. **Cowley, S., H. Paterson, P. Kemp, and C. J. Marshall.** 1994. Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell **77:**841–852.
10. **D'Arcangelo, G., and S. Halegoua.** 1993. A branched signaling pathway for nerve growth factor is revealed by *src*-, *ras*-, and *raf*-mediated gene inductions. Mol. Cell. Biol. **13:**3146–3155.
11. **D'Arcangelo, G., K. Paradiso, D. Shepard, P. Brehm, S. Haleguoa, and G. Mandel.** 1993. Neuronal growth factor regulation of two different sodium channel types through distinct signal transduction pathways. J. Cell Biol. **122:**915–921.
12. **Derijard, B., M. Hibi, I.-H. Wu, T. Barrett, B. Su, T. Deng, M. Karin, and R. J. Davis.** 1994. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell **76:**1025–1037.
13. **Dhanasekaran, N., L. E. Heasley, and G. L. Johnson.** 1995. G protein-coupled receptor systems involved in cell growth and oncogenesis. Endocrine Rev. **16:**259–270.
14. **Dichter, M. A., A. S. Tischler, and L. A. Greene.** 1977. Nerve growth factor-induced increase in electrical excitability and acetylcholine sensitivity of a rat pheochromocytoma cell line. Nature (London) **268:**501–504.
15. **Dikic, I., J. Schlessinger, and I. Lax.** 1994. PC12 cells overexpressing the insulin receptor undergo insulin-dependent neuronal differentiation. Curr. Biol. **4:**702–708.
16. **Fanger, G. R., P. Erhardt, G. M. Cooper, and R. A. Maue.** 1993. Ras-independent induction of rat brain type II sodium channel expression in nerve growth factor-treated PC12 cells. J. Neurochem. **61:**1977–1980.
17. **Fanger, G. R., J. R. Jones, and R. A. Maue.** 1995. Differential regulation of neuronal sodium channel expression by endogenous and exogenous tyrosine kinase receptors expressed in rat pheochromocytoma cells. J. Neurosci. **15:**202–213.
18. **Greene, L. A., and A. S. Tischler.** 1982. PC12 pheochromocytoma cells in neurobiology research. Adv. Cell. Neurobiol. **3:**373–414.
19. **Gupta, S. K., E. Diez, L. E. Heasley, S. Osawa, and G. L. Johnson.** 1990. A G protein mutant that inhibits thrombin and purinergic receptor activation of phospholipase A2. Science **249:**662–666.
20. **Gupta, S. K., C. Gallego, G. L. Johnson, and L. E. Heasley.** 1992. MAP kinase is constitutively activated in *gip2* and *src* transformed Rat1a fibroblasts. J. Biol. Chem. **267:**7987–7990.
21. **Guroff, G.** 1985. PC12 cells as a model of neuronal differentiation, p. 245–272. *In* J. E. Bottenstein and G. Sato (ed.), Cell culture in the neurosciences. Plenum, New York.
22. **Halegoua, S., R. C. Armstrong, and N. E. Kremer.** 1991. Dissecting the mode of action of a neuronal growth factor. Curr. Top. Microbiol. Immunol. **165:**95–118.
23. **Heasley, L. E.** Unpublished data.
24. **Heasley, L. E., and G. L. Johnson.** 1992. The β-PDGF receptor induces neuronal differentiation of PC12 cells. Mol. Biol. Cell **3:**545–553.
25. **Heasley, L. E., S. I. Senkfor, S. Winitz, A. Strasheim, I. Teitelbaum, and T. Berl.** 1994. Hormonal regulation of MAP kinase in cultured rat inner medullary collecting tubule cells. Am. J. Physiol. **267**(Renal Fluid Electrolyte Physiol. 36)**:**F366–F373.
26. **Heasley, L. E., J. Zamarripa, B. Storey, B. Helfrich, F. M. Mitchell, P. A. Bunn, Jr., and G. L. Johnson.** Discordant signal transduction and growth inhibition of small cell lung carcinomas induced by expression of GTPase-deficient Gα16. J. Biol. Chem., in press.
27. **Heidecker, G., M. Huleihel, J. L. Cleveland, W. Kolch, T. W. Beck, P. Lloyd, T. Pawson, and U. R. Rapp.** 1990. Mutational activation of c-Raf-1 and definition of the minimal transforming sequence. Mol. Cell. Biol. **10:**2503–2512.
28. **Hibi, M., A. Lin, T. Smeal, A. Minden, and M. Karin.** 1993. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev. **7:**2135–2148.
29. **Ito, E., J. L. Sonnenberg, and R. Narayanan.** 1989. Nerve growth factor-induced differentiation in PC12 cells is blocked by *fos* oncogene. Oncogene **4:**1193–1199.
30. **Jiang, H., D. Wu, and M. I. Simon.** 1994. Activation of phospholipase C β4 by heterotrimeric GTP-binding proteins. J. Biol. Chem. **269:**7593–7596.
31. **Kalinec, G., A. J. Nazarali, S. Hermouet, N. Zu, and J. S. Gutkind.** 1992. Mutated α subunit of the Gq protein induces malignant transformation in NIH 3T3 cells. Mol. Cell. Biol. **12:**4687–4693.
32. **Kalman, D. B. Wong, A. E. Horvai, M. J. Cline, and P. H. O'Lague.** 1990. Nerve growth factor acts through cAMP-dependent protein kinase to increase the number of sodium channels in PC12 cells. Neuron **2:**355–366.
33. **Kaplan, D. R., and R. M. Stephens.** 1994. Neurotrophin signal transduction by the trk receptor. J. Neurobiol. **25:**1404–1417.
34. **Kirschmeier, P. T., G. M. Housey, M. D. Johnson, A. S. Perkins, and I. B. Weinstein.** 1988. Construction and characterization of a retroviral vector demonstrating efficient expression of cloned cDNA sequences. DNA **7:**219–225.
35. **Kozasa, T., J. R. Hepler, A. V. Smrcka, M. I. Simon, S. G. Rhee, P. C. Sternweis, and A. G. Gilman.** 1993. Purification and characterization of recombinant $G_{16\alpha}$ from Sf9 cells: activation of purified phospholipase C isozymes by G-protein α subunits. Proc. Natl. Acad. Sci. USA **90:**9176–9180.
36. **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.
37. **Kyriakis, J. M., P. Banerjee, E. Nikolakaki, T. Dai, E. A. Rubie, M. F. Ahmad, J. Avruch, and J. R. Woodgett.** 1994. The stress-activated protein kinase subfamily of c-Jun kinases. Nature (London) **369:**156–160.
38. **Mandel, G., S. S. Cooperman, R. A. Maue, R. H. Goodman, and P. Brehm.** 1988. Selective induction of brain type II Na^+ channels by nerve growth factor. Proc. Natl. Acad. Sci. USA **85:**924–928.
39. **Markowitz, D., S. Goff, and A. Bank.** 1988. A safe packaging line for gene transfer: separating viral genes on two different plasmids. J. Virol. **62:**1120–1124.
40. **Marshall, C. J.** 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell **80:**179–185.
41. **Maruyama, K., S. C. Schiavi, W. Huse, G. L. Johnson, and H. E. Ruley.** 1987. Myc and E1A oncogenes alter the responses of PC12 cells to nerve growth

factor and block differentiation. Oncogene **4:**361–367.
42. **Miller, A. D., and C. Buttimore.** 1986. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol. Cell. Biol. **6:**2895–2902.
43. **Miller, A. D., and G. J. Rosman.** 1989. Improved retroviral vectors for gene transfer and expression. BioTechniques **7:**980–990.
44. **Minden, A., A. Lin, M. McMahon, C. Lange-Carter, B. Derijard, R. J. Davis, G. L. Johnson, and M. Karin.** 1994. Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. Science **266:**1719–1723.
45. **Muroya, K., S. Hattori, and S. Nakamura.** 1992. Nerve growth factor induces rapid accumulation of the GTP-bound form of $p21^{ras}$ in rat pheochromocytoma PC12 cells. Oncogene **7:**277–281.
46. **Nemenoff, R. A.** Personal communication.
47. **Noda, M., M. Ko, A. Ogura, D. G. Liu, T. Amano, T. Takano, and Y. Ikawa.** 1985. Sarcoma viruses carrying ras oncogenes induce differentiation-associated properties in a neuronal cell line. Nature (London) **318:**73–75.
48. **Obermeier, A., R. A. Bradshaw, K. Seedorf, A. Choidas, J. Schlessinger, and A. Ullrich.** 1994. Neuronal differentiation signals are controlled by nerve growth factor receptor/Trk binding sites for SHC and PLCγ. EMBO J. **13:**1585–1590.
49. **Pace, A. M., Y. H. Wong, and H. R. Bourne.** 1991. A mutant α subunit of G_{i2} induces neoplastic transformation of Rat-1 cells. Proc. Natl. Acad. Sci. USA **88:**7031–7035.
50. **Pollock, J. D., M. Krempin, and B. Rudy.** 1990. Differential effects of NGF, FGF, EGF, cAMP, and dexamethasone on neurite outgrowth and sodium channel expression in PC12 cells. J. Neurosci. **10:**2626–2637.
51. **Qian, N.-X., M. Russell, A. M. Buhl, and G. L. Johnson.** 1994. Expression of GTPase-deficient $G\alpha_{16}$ inhibits Swiss 3T3 cell growth. J. Biol. Chem. **269:**17417–17423.
52. **Qian, N.-X., S. Winitz, and G. L. Johnson.** 1993. Epitope-tagged Gq α subunits: expression of GTPase-deficient α subunits persistently stimulates phosphatidylinositol-specific phospholipase C but not mitogen-activated protein kinase activity regulated by the M_1 muscarinic acetylcholine receptor. Proc. Natl. Acad. Sci. USA **90:**4077–4081.
53. **Rudy, S., B. Kirschenbaum, and L. A. Greene.** 1982. Nerve growth factor-induced increase in saxitoxin binding to rat PC12 pheochromocytoma cells. J. Neurosci. **2:**1405–1411.
54. **Sills, M. N., Y. C. Xu, E. Barachini, R. H. Goodman, S. S. Cooperman, G. Mandel, and K. R. Chien.** 1989. Expression of diverse Na channel messenger RNAs in rat myocardium. J. Clin. Invest. **84:**331–336.
55. **Stephens, R. M., D. M. Loeb, T. D. Copeland, T. Pawson, L. A. Greene, and D. R. Kaplan.** 1994. Trk receptors use redundant signal transduction pathways involving SHC and PLC-γ1 to mediate NGF responses. Neuron **12:**691–705.
56. **Strittmatter, S. M., M. C. Fishman, and X.-P. Zhu.** 1994. Activated mutants of the α subunit of G_o promote an increased number of neurites per cell. J. Neurosci. **14:**2327–2338.
57. **Traverse, S., N. Gomez, H. Paterson, C. Marshall, and P. Cohen.** 1992. Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Biochem. J. **288:**351–355.
58. **Traverse, S., K. Seedorf, H. Paterson, C. J. Marshall, P. Cohen, and A. Ullrich.** 1994. EGF triggers neuronal differentiation of PC12 cells that overexpress the EGF receptor. Curr. Biol. **4:**694–701.
59. **Vaillancourt, R. R., L. E. Heasley, J. Zamarripa, B. Storey, M. Valius, A. Kazlauskas, and G. L. Johnson.** 1995. Mitogen-activated protein kinase activation is insufficient for growth factor receptor-mediated PC12 cell differentiation. Mol. Cell. Biol. **15:**3644–3653.
60. **Vara Prasad, M. V. V. S., J. M. Dermott, L. E. Heasley, G. L. Johnson, and N. Dhanasekaran.** 1995. Activation of jun kinase/stress-activated protein kinase by GTPase-deficient mutants of $G\alpha_{12}$ and $G\alpha_{13}$. J. Biol. Chem. **270:**18655–18659.
61. **Wood, K. W., H. Qi, G. D'Arcangelo, R. C. Armstrong, T. M. Roberts, and S. Halegoua.** 1993. The cytoplasmic *raf* oncogene induces a neuronal phenotype in PC12 cells: a potential role for cellular *raf* kinases in neuronal growth factor signal transduction. Proc. Natl. Acad. Sci. USA **90:**5016–5020.