Activated G
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α Potentiates Platelet-derived Growth Factor-stimulated Mitogenesis in Confluent Cell Cultures*

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We studied the effects of activation of the G
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α signaling pathway on mitogenesis by expressing a mutant (Q209L), activated α-subunit of G
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α (α*
) in NIH-3T3 cells. A clonal NIH-3T3 cell line expressing α*
 in an inducible manner was isolated. Expression of α*
 is induced with dexamethasone, allowing the use of non-induced cells as controls for the effects of α*
 expression. We found that, by itself, expression of α*
 did not increase either DNA synthesis or mitogen-activated protein (MAP) kinase activity in serum-starved cells. Because α*
 transforms cells grown in the presence of serum (De Vivo M., Chen, J., Codina, J., and Iyengar, R. (1992) J. Biol. Chem. 267, 18263–18266), we tested whether growth factor-stimulated signaling and mitogenesis were affected by expression of α*
. Platelet-derived growth factor (PDGF) stimulated thymidine incorporation modestly (50%) in contact-inhibited, confluent cell cultures. In cells expressing α*
, PDGF-stimulated DNA synthesis up to 3-fold over basal. Concomitant with the potentiation of PDGF-stimulated DNA synthesis, expression of α*
 potentiated PDGF-stimulated p44 MAP kinase activity. PDGF was much more effective in stimulating both DNA synthesis and p44 MAP kinase activity in subconfluent cell cultures and expression of α*
 exerted little or no effect on PDGF-stimulated effects in subconfluent cells. These data show that cooperation between signaling pathways may occur in a cell state-specific fashion. Such cooperation in part may be responsible for the triggering of complex cellular responses such as cell transformation.

Distinct signaling pathways mediate mitogenic signals in cells by different mechanisms. Transduction by receptor tyrosine kinases, such as platelet-derived growth factor (PDGF)
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 and epidermal growth factor receptors, involves tyrosine phosphorylation and signaling through proteins that contain SH2 and SH3 domains (1). Transduction by G protein-coupled receptors, including those for thrombin, lysophosphatidic acid, and serotonin, involves activation of appropriate G proteins (2–4). Recently it has become clear that there is some convergence of signals from receptor tyrosine kinase and G protein-coupled receptor pathways, as evidenced by changes in the activity of mitogen-activated protein kinase (MAP kinase).

Many mitogens increase the activity of MAP kinases (5, 6). Receptor tyrosine kinases such as PDGF stimulate at least two forms of the enzyme, called p42 and p44. G protein-coupled receptors also stimulate MAP kinases. For example, thrombin persistently activates MAPK activity in hamster fibroblasts (4). The pathway leading to activation of MAPK by receptor tyrosine kinases has been elucidated and involves activation of intracellular components Ras, Raf, and MEK (7). Although the specific mechanism whereby MAPK initiates growth in quiescent cells has not been established, it is thought that cytoplasmic MAPK is phosphorylated in response to cell surface signals, translocates to the nucleus, and regulates specific transcription factors leading to cell growth.

We have investigated the role of G
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α in cell growth. Members of the G
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α family mediate activation of phospholipase C-β (8, 9). We constructed a mutant cDNA encoding a putative GTPase-deficient, persistently activated form of the α-subunit of G
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α (α*
) and transfected it into NIH-3T3 cells. We isolated cell lines that expressed α*
, as evidenced by both constitutively increased phospholipase C activity and immunoblotting (10). Expression of α*
 results in cells that exhibit an increase in basal, serum-stimulated, and receptor-stimulated phospholipase C activity (10). Cells expressing mutant, active α*
 become transformed (10, 11), indicating that α*
 influences mitogenic pathways. However, expression of α*
 did not stimulate DNA synthesis under our experimental conditions. Transformation assays are typically conducted in the presence of serum; hence, we determined whether cooperation between the G
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α and other signaling pathways could be manifested as enhanced DNA synthesis. In this study, we analyzed the interaction of the G
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α pathway with the receptor tyrosine kinase pathway. We show that expression of α*
 potentiated PDGF-stimulated mitogenesis in contact-inhibited, confluent fibroblasts.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant PDGF B/B was from Boehringer Mannheim, PGF
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 from Sigma, and [methyl-3H]thymidine, myo-[3H]inositol, and [γ-32P]ATP from Du Pont NEN. Antibodies to phosphoprotein C-21 and phosphoprotein C-23C3 were the kind gift of Dr. Sue Goo Hkee, and antibody C-16 to MAP kinase 1 and 2 was from Santa Cruz Bio-technology Inc. Sources of all other materials have been described (10).

Cell Culture—Clonal NIH-3T3 lines were grown in DMEM with 5% bovine calf serum and penicillin/streptomycin. Transfections were done using the calcium phosphate method (10). The vector used for all transfections was the pMAMneo, an inducible vector containing the mouse metallothionein promoter and the neomycin resistance gene (Clontech). Cells were plated at a density of 1 × 10⁶ cells/100-mm culture dish. For experiments using rapidly growing cultures, cells were allowed to grow for 24–48 h after plating in the presence of serum before they were serum-starved for experiments. Postconfluent cultures were obtained by maintaining the cells for 10–12 days prior to serum starvation and experimentation. Medium was changed every 3rd day.

Insoluble Phosphate Accumulation—Cells were plated at a density of 10⁶ cells/ml into 24-well cell culture dishes (10⁶ cells/well). Cells were labeled with 1 μCi/ml myo-[3H]inositol for 2 or 3 days prior to assay. For expression of α*
, 1 μM dexamethasone was included during the labeling step. At the time of assay, medium was removed and cells washed twice with serum-free DMEM containing 10 mM LiCl, 10 mM glucose, and 0.1% bovine serum albumin. Cells were incubated for 30 min in 0.5 ml of this solution containing the indicated reagents. The assay was stopped by aspiration of the solution and by adding 1 ml of ice-cold 10% TCA.
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Our previous observations indicate that activation of Gq-α on DNA synthesis. We used NIH-3T3 cells transfected with cDNA encoding a mutant, active form of the α-subunit of Gq (Gq-α*). Expression of Gq-α* is under the control of the mouse mammary tumor virus promoter and is induced with dexamethasone. Use of the inducible expression system allowed us to use transfected cells that were not induced with dexamethasone as controls in the cell growth experiments. We also used wild type, non-transfected cells grown in the presence of dexamethasone as a control for the effects of dexamethasone.

We found that expression of Gq-α* by itself did not stimulate thymidine incorporation (not shown). However, since transformation assays are performed in the presence of serum, it is possible that the mitogenic effects of Gq-α* may result from potentiation of serum-stimulated mitogenic pathways. Growth factors are major components of serum. Therefore, we tested the effect of Gq-α* expression on PDGF-stimulated DNA synthesis in confluent cell cultures under conditions where expression of Gq-α* results in transformation.

In contact-inhibited, confluent cell cultures, PDGF stimulated thymidine incorporation 50% over basal incorporation. Expression of Gq-α* resulted in a 3-fold stimulation of PDGF-dependent thymidine incorporation (Fig. 1A). Because PDGF stimulates mitogenesis through the activation of MAP kinases, we determined the effects of Gq-α* on PDGF-stimulated MAP kinase activity. In postconfluent cultures of NIH-3T3 cells, PDGF-stimulated MAP kinase activity migrated as a single peak (Fig. 1B). Although p44 was present in fractions eluting at higher salt concentrations, it was not active in the MAPK assay (Fig. 1B, inset). In cells transfected with Gq-α*, but not induced with dexamethasone, the profile of MAP kinase activity from the Mono-Q column was virtually identical to wild-type NIH-3T3 cells shown in Fig. 1B. Upon expression of Gq-α*, PDGF-stimulated MAPK kinase activities migrated as two distinct peaks (Fig. 1C). Immunoblotting profiles show that the later eluting peak corresponds to an active p44 form of MAP kinase (Fig. 1C, inset). Therefore, expression of Gq-α* allows for PDGF stimulation of p44 MAP kinase activity concomitantly with PDGF-stimulation of mitogenesis in confluent cultures.

Increased PDGF stimulation of mitogenesis could occur because of an increase in PDGF receptor number. Hence, we measured the amount of PDGF receptors in control and Gq-α*-expressing cells. We found that expression of Gq-α* did not alter the number of PDGF receptors (Fig. 2A). We further checked if the cooperative signaling by Gq-α* and PDGF was manifested in other signaling pathways. PDGF and Gq-α* both stimulate PLCs although of different classes. Therefore we checked if expression of Gq-α* resulted in enhancement of PLC activity. As shown in Fig. 2B, I, we found that expression of Gq-α* enhanced PDGF-stimulated inositol phosphate accumulation. However, when we determined if expression of Gq-α* resulted in an increase in either PLC-β3 or -γ1 protein levels, we did not find an increase (Fig. 2B, II). In fact, expression of Gq-α* reproducibly reduced expression of PLC-β3, suggesting that expression of this molecule is down-regulated in cells with high PLC activity and that expression of PLC-β3 may be a point of regulatory control for the cell. The experiments in Figs. 1 (B and C) and 2 suggest that cooperation between PDGF and Gq-α* in stimulating MAP kinase or phospholipase C activities occurs without increases in the levels of the components studied.

We next determined if the cooperation between PDGF and Gq-α* was restricted to confluent, contact-inhibited cells or would be observable in rapidly growing, subconfluent cells where PDGF might be expected to extensively stimulate DNA synthesis. For this, cells were grown for 2 days, then serum-starved and used for DNA synthesis and MAP kinase assays. As expected, PDGF extensively stimulated DNA synthesis. In the
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Fig. 1. Effects of expression of $\alpha_\ast$ on PDGF-stimulated DNA synthesis and MAP kinase activity in confluent cell cultures. A, confluent NIH-3T3 cells harboring the cDNA encoding $\alpha_\ast$ were grown in the absence (○) or presence (●) of 1 μM dexamethasone for 10 days. Cells were serum-starved for 24 h and then stimulated with the indicated concentrations of PDGF for 16 h. Cells were assayed for $\beta^\text{H}$ thymidine incorporation as described. Averages ± S.E. are shown for three wells. B, wild-type (non-transfected) NIH-3T3 cells were grown to confluence (10 days) in the presence of 1 μM dexamethasone. MAP kinase activity was measured as described. Aliquots (50 μl) of fractions from the Mono-Q column that were used for assaying MAP kinase activity were immunoblotted using a MAP kinase antibody as described. C, NIH-3T3 cells harboring the cDNA encoding $\alpha_\ast$ were grown to confluence (10 days) in the presence of 1 μM dexamethasone. MAP kinase activity was measured as described. Aliquots (50 μl) of fractions from the Mono-Q column that were used for assaying MAP kinase activity were immunoblotted using a MAP kinase antibody as described. In the experiment shown in Fig. 3A, PDGF-stimulated DNA synthesis was 25-fold. Expression of $\alpha_\ast$ modestly increased PDGF stimulation to about 35-fold. In three other experiments, a similar modest increase (30%) in PDGF-stimulated DNA synthesis was observed. We also measured the effect of $\alpha_\ast$ on PDGF-stimulated MAP kinase activity in subconfluent cell cultures. As shown in Fig. 3B, PDGF-stimulated MAP kinase activity was resolved into two peaks on Mono-Q columns. Peak I elutes at lower salt concentrations and corresponds to a mixture of p42 and p44 as evidenced by immunoblots with MAP kinase antibodies (not shown), whereas peak II eluted at higher salt concentrations and was essentially all p44. These results are similar to those seen by other laboratories with either mitogen-
activated NIH-3T3 cells or PDGF stimulation of other cell types (14, 15). Expression of α*5 did not affect PDGF-stimulated MAP kinase activities in either peak (Fig. 3B). Nor did expression of α*5 only restored the PDGF-stimulated MAP kinase profile seen in subconfluent cells, it also increased by several-fold PDGF-stimulated DNA synthesis. Potentiation of growth factor effects in confluent cell cultures may be one of the factors responsible for the transforming properties of α*5 (10, 11). Our observation that α*5 potentiates receptor tyrosine kinase-stimulated proliferation only in confluent cell cultures may explain discrepancies associated with the role of Gq in mitogenesis. Some investigators have found that activation of the Gq family of G proteins has no effect on cell growth (16, 17), whereas others that have measured the effect of Gq (18) or Gq-coupled receptors (19, 20) have come to opposite conclusions. In transformation assays such as focus-formation, cells are grown to confluence in the presence of serum, and then scored for lack of contact inhibition, whereas in most laboratories mitogenesis is measured in serum-starved subconfluent cells, when no effects of α*5 on cell growth would be observable.

In conclusion, we show that Gq and receptor tyrosine kinase pathways cooperate in stimulating mitogenesis in confluent cell cultures. Enhancement of receptor tyrosine kinase-stimulated mitogenesis by expression of α*5 is associated with restoration of stimulator of the p44 MAP kinase activity in contact-inhibited cells. Activation of the Gq pathway and the concomitant enhancement of receptor tyrosine kinase-stimulated mitogenesis in confluent cell cultures may in part contribute to the processes that lead to cell transformation.

REFERENCES

FIG. 3. Effect of expression of α*5 on PDGF-stimulated DNA synthesis and MAP kinase activity in subconfluent cells. A, subconfluent, rapidly growing NIH-3T3 cells transfected with cDNA encoding α*5 were either treated or untreated with 1 μM dexamethasone to induce expression of α*5 as indicated. Cells were incubated with 5 nM PDGF for 15 min, and [3H]thymidine incorporation was measured as described. Data are expressed as n-fold stimulation by PDGF. Basal thymidine incorporation was approximately 10,000 dpm. Means ± S.E. are shown for three wells. B, clonal NIH-3T3 cells harboring the α*5 cDNA were plated at low density (1×10^4 cells/100-mm tissue culture dish) and grown for 2 days in the presence (●) or absence (●) of 1 μM dexamethasone to induce expression of α*5. Cells were serum-starved overnight in DMEM, 0.1% bovine serum albumin, and then stimulated with 5 nM PDGF for 15 min. Cells were lysed, and the cytosolic proteins were separated on a Mono-Q column. Fractions were assayed for MAP kinase activity as described.

DISCUSSION

In its simplest form, a signal transduction pathway functions as a linear readout system for the activity of cell surface receptors. This ensures that an external signal evokes a proportional biochemical or physiological intracellular response. Many acute responses mediated by adenylyl cyclase or phospholipase C pathways fit such a pattern. However, other responses such as stimulation of mitogenesis are not always linear and dependent upon the state of the cell when the signal is received. For example, responses to individual mitogenic signals are suppressed in confluent, slowly growing cell cultures as compared to rapidly growing subconfluent cultures. In addition, simultaneous signals from a second pathway may dampen or potentiate signaling through a primary pathway. We studied interactions between the PDGF and Gq signaling pathways in both rapidly growing and contact-inhibited cell cultures.

As expected, PDGF stimulated DNA synthesis to a much lesser extent in confluent contact-inhibited cell cultures (Fig. 1A) than in rapidly growing subconfluent cultures (Fig. 3A). In conjunction with this reduction in PDGF-stimulated DNA synthesis (from 30-fold to 50%) is a change in the profile of MAP kinase activity eluting from the Mono-Q ion exchange column.