Microinjection of activated phosphatidylinositol-3 kinase induces process outgrowth in rat PC12 cells through the Rac-JNK signal transduction pathway

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Accepted 14 January; published on WWW 9 March 1998

SUMMARY

We have previously shown that sustained phosphatidylinositol (PI)-3 kinase activity is necessary for neurite outgrowth of PC12 cells induced by nerve growth factor (NGF). Microinjection of a constitutively active mutant of PI-3 kinase induced process formation suggesting that PI-3 kinase is indeed involved in the neurite outgrowth. However, the processes appeared to be incomplete neurites as they had very poor organization of F-actin and GAP43 antigen. The microtubule network was enhanced in the process-bearing cells and process formation was inhibited by colchicine suggesting that microtubules play an important role in process formation downstream of PI-3 kinase. These cell responses were inhibited by dominant-negative mutants of Rac and Sek1/SAPK but not by a dominant-negative mutant Ras and PD98059, a MAP kinase kinase (MEK) inhibitor, suggesting that not the Ras-MAP kinase pathway but the Rac-Jun N-terminal kinase (JNK) pathway is involved in process formation.

Key words: PI-3 kinase, Neurite, Microtubule, Actin, GAP43

INTRODUCTION

Rat pheochromocytoma PC12 cells are a model system for the differentiation of neuronal cells. After stimulation with nerve growth factor (NGF), neurite-specific genes are induced and neurite outgrowth occurs (Halegoua et al., 1991). In contrast, EGF does not induce differentiation of PC12 cells, but it does induce cell growth. The receptors for these factors are tyrosine kinases and transduce similar signals including activation of the ras protein, MAP kinases, and phosphatidylinositol (PI)-3 kinase (Chao, 1992). The differences between the signals induced by these factors have been examined. It has been proposed that the differential cell responses are due to the sustained activation of Ras and MAP kinases in response to NGF, relative to the transient activation stimulated by EGF (Muroya et al., 1992; Qiu and Green, 1992). Consistent with this is the observation that constitutive activation of Ras or MAP kinase kinase induces neuronal differentiation of PC12 cells (Bar-Sagi and Feramisco, 1985; Cowley et al., 1994; Guerrero et al., 1986; Noda et al., 1985). In addition to these molecules, PI-3 kinase may be also important for neurite outgrowth (Kimura et al., 1994).

The PI-3 kinase activated by receptor tyrosine kinases consists of two subunits, p110 and p85. It catalyzes phosphorylation of the D-3 position of PI, PI-4-P and PI-4,5-P₂ in vitro (Auger et al., 1989). Since the levels of PI-3,4-P₂ and PI-3,4,5-P₃ (PIP₃) are elevated within a few minutes after growth factor stimulation of the cells and they are not substrates of PLCs (Lips et al., 1989; Serunian et al., 1989), it is likely that these polyphosphoinositides may play important roles as second messengers (Auger et al., 1989). For example, atypical protein kinase Cs and Akt protein kinase are activated in vitro by PI-3,4,5-P₃ and PI-3,4-P₂, respectively (Akimoto et al., 1996; Burgering and Coffer, 1995; Franke et al., 1995; Nakanishi et al., 1993).

PI-3 kinase is activated immediately after NGF treatment in PC12 cells (Carter and Downes, 1992; Soltoff et al., 1992). After reaching peak levels at 4 to 6 minutes after treatment with NGF, the levels of PIP₃ decrease to a level that is higher than in unstimulated cells (Kimura et al., 1994). Inhibition of PI-3 kinase by wortmannin revealed that a sustained activation of PI-3 kinase is necessary for neurite outgrowth (Kimura et al., 1994; Okada et al., 1994; Yano et al., 1993). In addition, a dominant-negative mutant of p85 blocks neurite outgrowth (Jackson et al., 1996). The presence of NGF is required throughout the differentiation of PC12 cells (Halegoua et al., 1991). This may reflect the necessity of continuous activation of PI-3 kinase by NGF stimulation at all the phases. In a
previous report, we have shown that the expression of a constitutively active mutant PI-3 kinase using the Cre/loxP recombination system (Kaneyama et al., 1995) results in the process outgrowth of PC12 cells (Kobayashi et al., 1997).

In this study, we further assess the role of PI-3 kinase in PC12 cells. We report that microinjection of an expression vector carrying the active PI-3 kinase gene induces process formation of PC12 cells and that not Ras-MAP kinase cascade but Rac-JNK pathway is involved in the cell response.

MATERIALS AND METHODS

Plasmid constructions

The PI-3 kinase genes were expressed using a vector, pMIKNeo (Takayanagi et al., 1996), pmycBD110 was used for expression of the activated PI-3 kinase, the mycBD110 protein (Kobayashi et al., 1997), which has a structure similar to that of p110α reported by Hu et al. (1995). pmycBDKN codes for the mycBDKN protein, a kinase negative counterpart of the mycBD110 protein with a point mutation in the kinase domain (Akimoto et al., 1996). The human p85α gene was also used (p85). A dominant-negative mutant of Sek1/SAPK was a kind gift from Dr Zon (Sanchez et al., 1994). The expression vectors for dominant-negative Rac and Ras were as described before (Feig and Cooper, 1988; Ridley et al., 1992).

Assay for PI-3 kinase activity

Simian Cos7 cells were cultured in Dulbecco’s modified minimal essential medium (DMEM) with 10% calf serum. They were transfected with the constructs by the DEAE dextran method. After incubation for 2 days, the cells were lysed and one half of the cell lysates were used for the PI-3 kinase assay. The exogenous PI-3 kinase was immunoprecipitated with anti-myc monoclonal antibody, 9E10, and the PI-3 kinase activity in the immunoprecipitates was analyzed as described before (Fukui et al., 1989). Similar immunoprecipitates were made from the remaining half and the PI-3 kinase in the immunoprecipitates was quantitated by western blotting with 9E10.

Antibodies and reagents

Anti-myc antibody, 9E10, was prepared from culture supernatant of a hybridoma purchased from ATCC. Anti-α-tubulin monoclonal antibody, rhodamine-phalloidin conjugate, colchicine and NGF were purchased from Sigma Chemical Company, Molecular Probes Inc., Wako Pure Chemical Industry, Ltd (Tokyo, Japan) and Takara Shuzo (Kyoto, Japan), respectively. Anti-GAP43 polyclonal antibody and MEF inhibitor, PD98059, were kind gifts from Drs Graham P. Wilkin and Alan R. Saltiel, respectively.

Microinjection of various constructs and antibody staining

Rat pheochromocytoma PC12 cells were cultured in DMEM supplemented with 10% calf serum and 5% horse serum on coverslips coated with poly-L-lysine. These cells were microinjected with the expression vector (150-200 ng/μl in total: 150 ng/μl for PI-3 kinase construct and 50 ng/μl for others) dissolved in phosphate buffered saline (PBS) containing rhodamine-dextran (2.5 μg/μl). The injected cells were observed under the microscope. Presence of the exogenous PI-3 kinase was confirmed by staining the protein with an anti-myc monoclonal antibody, 9E10. Cells were fixed for 20 minutes at room temperature by adding formaldehyde into the medium (final 4%) and permeabilized by 0.05% Triton X-100/PBS at room temperature for 3 minutes. Cells were incubated with 9E10 at room temperature for 60 minutes in a blocking buffer (DMEM with 10% serum), and then with FITC-conjugated goat anti-mouse IgG antibody as a second antibody. For GAP43 antigen staining, cells were incubated with anti-GAP43 polyclonal antibody, and then with goat anti-rabbit IgG antibody. For α-tubulin staining, cells were fixed with 2% formaldehyde and 0.1% glutaraldehyde/PEM (0.1 M Pipes-NaOH, pH 6.6, 1 mM EGTA, 1 mM MgCl2) at 37°C for 15 minutes, permeabilized with 0.05% Triton X-100/PBS for 3 minutes, and treated with 50 mM glycine/PBS for 30 minutes. Then the cells were stained with anti-α-tubulin antibody as described above except that FITC-conjugated goat anti-mouse IgG antibody was used as a second antibody. F-actin was stained with rhodamine-phalloidin.

RESULTS

The activities of the mutant PI-3 kinases in vivo were analyzed. Cos7 cells were transfected with myc-tagged mutant p110 and the PI-3 kinase activities in anti-myc-immunoprecipitates were analyzed. As shown in Fig. 1B, the immunoprecipitates from pmycBD110-transfected cells exhibited an extremely high activity compared with that from wild-type p110-transfected cells. The expression of the mycBD110 protein was also higher than the wild-type p110. These results suggest that our mycBD110 protein significantly elevates PI-3 kinase activity in the cells. In contrast, a kinase negative mutant, the
mycBDKN protein, did not exhibit the enzyme activity although the expression of the protein was comparable to the mycBD110 protein (Fig. 1B).

The microinjection of the expression vector for the activated PI-3 kinase caused process formation in PC12 cells within 24 hours (Fig. 2A, top panels). About 30% of the pmycBD110-injected cells, and 37.5% of the cells expressing the mycBD110 protein, formed processes without NGF stimulation (Fig. 2B). The pmycBD110-induced processes seemed to be morphologically different from those induced by NGF stimulation. The majority of the processes were thinner and the growth cones were smaller or absent compared with the neurites induced by NGF stimulation. Many of the cells had a few long processes with some branching, and some of them had many short ones from one cell body or forked branches from one process, which were also not found in NGF-treated cells (data not shown). These features may reflect the difference between NGF-induced processes and pmycBD110-induced ones (see Discussion). Therefore, we call the pmycBD110-induced ones ‘processes’ to distinguish them from ‘neurites’ induced by NGF stimulation. A construct carrying another type of activated PI-3 kinase, which had a myristylation signal of lyn protein at the amino terminal end to target the protein to the membrane, gave a similar result (data not shown) (Klippel et al., 1996). In contrast, the kinase negative construct, pmycBDKN, did not cause process outgrowth although the expression of the protein was clearly detected by immunofluorescence analysis with an anti-myc antibody, 9E10 (Fig. 2A, middle panels). The wild-type p110 induced little morphological change suggesting that low level activation of PI-3 kinase is not enough for process formation (data not shown).

The pmycBD110-injected cells were treated with wortmannin, a selective inhibitor for PI-3 kinase. As shown in the bottom panels of Fig. 2A, treatment with wortmannin completely inhibited process formation at a dose of 100 nM, which is sufficient to inhibit the PI-3 kinase activity in vitro (Fig. 2C), suggesting that process formation was due to PI-3 kinase activity introduced by microinjection. Taken together, these data suggest that elevation of PI-3 kinase activity is sufficient for inducing process formation in PC12 cells.

Fig. 2. Effects of the activated PI-3 kinase on process formation of PC12 cells. (A) PC12 cells were microinjected with pmycBD110 (top panels) or pmycBDKN (middle panels). After 24 hours, the cells were fixed, then immunostained with a monoclonal antibody for the myc-tag, 9E10, to confirm the presence of the exogenous PI-3 kinase. In the bottom panels, the cells injected with pmycBD110 were treated with 100 nM wortmannin every 4 hours. Bar, 20 μm. (B) The cells with processes longer than each cell body were scored. Data are expressed as a percentage, based on the rhodamine positive cells (microinjected cells, shown by black bars) or the 9E10-staining positive cells (mycBD110-expressing cells, shown by gray bars). (C) Cos7 cells were transfected with the mycBD110 construct. The mycBD110 protein was immunoprecipitated with a monoclonal antibody, 9E10, and the effect of wortmannin on the PI-3 kinase in the immunoprecipitates was assayed as described in Materials and Methods. The doses of wortmannin are indicated at the bottom of the figure.
Fig. 3. Staining of F-actin, GAP43 antigen and microtubules in mycBD110-injected cells. (A) PC12 cells were microinjected with pmycBD110 together with FITC-IgG as a marker for injection. After 24 hours, the cells were stained with rhodamine-phalloidin for 60 minutes. (B) Cells were microinjected with pmycBD110. After 24 hours, the cells were stained with anti-GAP43 antibody. The cells treated with 20 nM NGF were also analyzed (bottom panels). Effect of the activated PI-3 kinase on microtubule reorganization was analyzed. (C) PC12 cells were microinjected with pmycBD110 (top panels) or pmycBDKN (middle panels), or treated with NGF (bottom panels). After 24 hours, the cells were immunostained with anti-α-tubulin antibody. The pmycBD110-injected cells exhibited stronger staining of microtubules in the extended processes than in the uninjected cells whereas the effect was not seen in pmycBDKN-injected cells. In the bottom panels, the NGF-treated cells exhibited a varied intensity of microtubule staining. The stronger staining was observed in the cells harboring the long neurites (shown by arrowheads) compared with other cells without neurites. Bars, 20 μm (A and B); 50 μm (C).
To characterize pmycBD110-induced processes, we examined two major cytoskeletal proteins present in neurites, tubulin and actin, and a neuron-specific protein, GAP43. As shown in Fig. 3A, the staining of F-actin was rather faint in PC12 cells expressing mycBD110 indicating that reorganization of F-actin was poor, whereas that in NGF-treated PC12 cells was clearly detected. Typically, F-actin was found in the center of growth cones but it was not accumulated at the periphery of growth cones. Consistent with this, the growth cones lacked typical filopodia seen in the cells stimulated with NGF, which normally accumulate F-actin. The bodies of the process-bearing cells looked a little shrunken, probably due to the weak attachment to the substratum. These results suggest that sustained elevation of PI-3 kinase activity does not stimulate actin reorganization or may stimulate depolymerization of F-actin. Similar results were obtained in the expression system by the Cre/loxP recombination system (Kobayashi et al., 1997).

In cultured neurons, GAP43 protein is highly localized in growth cones and axons but not in dendrites (Goslin et al., 1988, 1990; Meiri et al., 1988; Skene et al., 1986). The growing axons harbor more GAP43 protein than mature and stable neurites (Skene and Willard, 1981). Thus, GAP43 may play an important role in pathfinding of axons. GAP43 protein was colocalized with F-actin at the plasma membrane and growth cones in NGF-stimulated PC12 cells (Nielander et al., 1993, and Fig. 3B). In contrast, accumulation of the GAP43 protein was not seen in pmycBD110-injected cells. The GAP43 protein did not accumulate at the growth cone in pmycBD110-injected cells whereas that in NGF-treated cells was clearly detected (Fig. 3B). The lack of F-actin localization may cause the lack of GAP43 localization in pmycBD110-injected cells or vice versa. These results suggest that pmycBD110-induced processes are not complete neurites.

In contrast to the F-actin and GAP43 staining, we observed a stronger staining of microtubule networks in the extended processes in pmycBD110-injected cells than in the uninjected cells (Fig. 3C, top panels), whereas pmycBDKN-injection did not induce this effect (Fig. 3C, middle panels). This observation was consistent with the previous results (Kobayashi et al., 1997). When NGF-stimulated cells were tested, stronger staining was observed in growing neurites than in other parts of the cells or in the cells without neurites (Fig. 3C, bottom panels). Microinjection of the expression vector without the active PI-3 kinase gene did not cause any difference in
microtubule reorganization (data not shown). These results suggest that reorganization of microtubules was indeed induced by NGF treatment and that the active PI-3 kinase mimics the effect. Treatment with colchicine completely blocked the process outgrowth, suggesting that tubulin polymerization induced by the mycBD110 protein was required for outgrowth (data not shown). These results suggest that the elevation of PI-3 kinase activity in the cells causes microtubule reorganization, reproducing the results of expression of the protein by the Cre/loxP recombination system.

Using this microinjection system, we explored the involvement of signaling molecules in process formation of PC12 cells. It has been proposed that Ras can activate PI-3 kinase. However, the fact that the time courses of activation of PI-3 kinase and Ras after NGF treatment of PC12 cells are nearly identical, and that the binding of the phosphotyrosine-containing proteins with PI-3 kinase is detected immediately after NGF treatment, argues against this hypothesis. On the other hand, treatment of PC12 cells with wortmannin partially inhibits the activation of MAP kinase by the NGF treatment (Kimura et al., 1994), implying that the Ras-MAP kinase cascade might work downstream of PI-3 kinase in this case. We, therefore, tested the involvement of the Ras-MAP kinase cascade in process formation by the expression of the active PI-3 kinase. As shown in Fig. 4B, co-injection of the expression vector for the dominant-negative mutant of Ras (RasN17) did not inhibit the process formation induced by the pmycBD110 injection. Microinjection of the same dose of the construct resulted in complete inhibition of the neurite outgrowth after NGF treatment, suggesting that the dominant-negative Ras is effective in blocking the endogenous Ras pathway (data not shown). Consistently, PD98059, an inhibitor of MAP kinase kinase (MEK) (Alessi et al., 1995), did not suppress the process formation induced by injection of pmycBD110 (Fig. 4C) under the conditions in which the neurite outgrowth induced by NGF treatment was clearly inhibited (data not shown). These results suggest that the Ras-MAP kinase cascade is not downstream of PI-3 kinase and that the Ras-MAP kinase cascade is not required for process formation.

Akt, Rac and JNK are also candidates for factors downstream of PI-3 kinase (Burgering and Coffer, 1995; Franke et al., 1995; Minden et al., 1995; Nobes et al., 1995; Olson et al., 1995; Wennstrom et al., 1994b). Expression of Akt was extremely high in our PC12 cells exhibiting the high basal activity. Akt was not further activated by the NGF treatment or by the expression of the active PI-3 kinase using the site-specific recombination system mediated by the Cre/loxP system (Kobayashi et al., 1997). On the other hand, JNK activity was elevated in the cells expressing the active PI-3 kinase as well as in the NGF-treated cells, suggesting that JNK might be involved in process formation (Kobayashi et al., 1997). To see whether or not these proteins were involved in process formation, the effects of dominant-negative constructs that can block these pathways were tested. Co-injection of the expression vector for the dominant-negative mutant of Rac (RacN17) completely inhibited the process formation induced by pmycBD110-injection (Fig. 5A,C). The dominant-negative mutant of Sek1, a JNK kinase, (Sek1KR) also significantly inhibited process formation, suggesting that the Rac-JNK pathway is required for process formation (Fig. 5B,C). These dominant-negative constructs also blocked NGF-induced neurite outgrowth, suggesting that the same pathway is working in neurite outgrowth (data not shown). Consistent with the results

Fig. 4. Ras-MAP kinase cascade is not involved in process formation. PC12 cells were injected with pmycBD110 alone (A), with pmycBD110 and the expression vector for the dominant-negative mutant of Ras (RasN17) (B). (C) Cells were treated with MEK inhibitor, PD98059, at the dose of 10 µM. (B and C) Neither RasN17 nor PD98059 inhibited the process formation induced by pmycBD110-injection (see also Fig. 5C). Bar, 50 µm.
that activation of Akt was not detected after the NGF treatment or expression of the active PI-3 kinase in PC12 cells, the dominant-negative Akt did not inhibit process formation (data not shown), suggesting that Akt is not involved in process formation. These results suggest that the Rac-JNK pathway is important in process formation.

**DISCUSSION**

PI-3 kinase has been reported to be involved in various cellular responses, such as membrane ruffling, receptor internalization, chemotaxis and neurite outgrowth, which all require cytoskeletal reorganization (Derman et al., 1995; Kimura et al., 1994; Wennstrom et al., 1994a,b). However, many of these reports depend on the use of an inhibitor, wortmannin, to show the involvement of PI-3 kinase activity on the cell responses. Recently, wortmannin was shown to inhibit some other enzymes at a similar concentration to that required for inhibition of PI-3 kinase (Bonser et al., 1991; Brunn et al., 1996; Cross et al., 1995; Nakanishi et al., 1995). Therefore, we examined the effect of activated PI-3 kinase in PC12 cells in two systems: microinjection of the pmycBD110 expression vector and the Cre/loxP system (Kanegae et al., 1995). Utilizing these systems, we confirmed that the increased PI-3 kinase activity is sufficient for ‘process formation’ in PC12 cells (Kobayashi et al., 1997). After induction of the mycBD110 protein, PC12Cre cells exhibited higher levels of PI-3,4-P2 and PIP3 than the control cells (7.5-fold and 5.0-fold, respectively), whereas no elevation of PI-3-P was detected. This result suggests that either PI-3,4-P2 or PIP3 may play a role in process formation.

The results of staining of the cytoskeleton suggest that activation of PI-3 kinase causes process formation probably through reorganization of microtubules. Enrichment of microtubule networks was observed in neurites induced by NGF treatment, which caused continuous activation of PI-3 kinase. Also, inhibition of PI-3 kinase results in failure of neurite extension (Kimura et al., 1994). Therefore, it is likely that PI-3 kinase is involved in neurite elongation through microtubule

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**Fig. 5.** Rac and Sek1 are involved in the signal transduction of process formation induced by expression of the mycBD110 protein. PC12 cells were co-injected with pmycBD110 and the plasmids carrying the genes for the dominant-negative mutants of Rac and Sek1 together with rhodamine-dextran as a marker for injection (lower panels). (A) The cells were co-injected with pmycBD110 and the expression vector for the dominant-negative mutant of Rac (RacN17) or its control vector (pEFBOS-HA). (B) The cells were co-injected with pmycBD110 and the expression vector for the dominant-negative (Sek1KR) or wild-type (Sek1) Sek1. Dominant negative mutants, RacN17 and Sek1KR, both inhibited the process formation induced by pmycBD110-injection. Bars, 50 μm. (C) Summary of the effects of reagents analyzed. Cells with processes longer than each cell body were counted. Data are expressed as the means of 3-5 experiments, each with more than 50 injected cells. Error bars represent standard deviations. *P<0.001 compared with pmycBD110 control (chi square analysis).
reorganization. The reorganization of microtubules could arise from increased expression of tubulin or from assembly of free tubulin already present in the cells. Western blotting analysis revealed that the protein level of tubulin is not elevated by expression of the mycBD110 protein, suggesting that the polymerization of tubulin might be accelerated in these cells. In contrast to microtubules, pmycBD110-induced processes lack F-actin accumulation and GAP43 antigen at the growth cone, which is typical of NGF-induced neurites. In growing axons, F-actin accumulates at the periphery of growth cones and plasma membrane as a major component of filopodia and lamellipodia, which mediate axon pathfinding. These results suggest that other signaling cascades besides PI-3 kinase are also required for full differentiation of PC12 cells as nerve cells.

Microtubule assembly provides a force for neurite outgrowth against a retractive force caused by peripheral F-actin network. The imbalance of these two forces causes morphological changes of the neurites: depolymerization of the microtubules leads to neurite retraction (Heidemann et al., 1985; Joshi et al., 1985) and that of F-actin leads to tubulin expansion and abnormal outgrowth of neurites (Bentley and Toroian-Raymond, 1986; Forscher and Smith, 1988; Joshi et al., 1985). In pmycBD110-injected cells, the lack of F-actin organization contrasts to the rich networks of microtubules. This unbalanced cytoskeletal reorganization might cause the several features induced by injection of pmycBD110, such as very long processes, many short processes or forked branches. These observations can be explained either by depolymerization of F-actin or by polymerization of tubulin. Although our results suggest that reorganization of microtubules is enhanced by activated PI-3 kinase, the possibility that the regulation of the actin cytoskeleton by PI-3 kinase is involved in parallel in process formation still remains. It is possible that PI-3 kinase regulates process formation by the balance of actin and microtubule polymerization.

It has been shown that Rac is downstream of PI-3 kinase in the signal transduction of membrane ruffling. In Drosophila embryos, Rac homologue mutants interfere with initiation and elongation of neurite outgrowth (Luo et al., 1994). We found that co-injection of a construct carrying a dominant-negative mutant of Rac with pmycBD110 resulted in inhibition of process formation suggesting that Rac may act downstream of PI-3 kinase. Although accumulation of F-actin is very poor in pmycBD110-injected cells, it is still possible that regulation of actin organization by PI-3 kinase is required for process formation. The Rac signal may be transduced to JNK, which has been suggested to act downstream of Rac (Minden et al., 1995; Olson et al., 1995; Zhang et al., 1995), since a dominant-negative mutant of Sek1/SAPK, a JNK kinase (Sanchez et al., 1994; Yan et al., 1994), also inhibited process formation. In PC12Cre cells, activation of MAP kinase and JNK is readily detectable after NGF treatment, however, Akt was expressed fairly abundant and exhibited high basal activity, and no further activation was detected after NGF treatment. When the mycBD110 gene was introduced, activation of JNK was detected but MAP kinase and Akt were not activated. These results suggest that activation of PI-3 kinase induces activation of only a proportion of the signaling molecules that are activated after NGF treatment. JNK is one of these enzymes. The fact that dominant-negative constructs for the Rac-JNK pathway block the process formation induced by pmycBD110-injection agrees with these observations.

Ras is also known to be activated throughout the neurite outgrowth. PC12 cells expressing activated Ras differentiate exhibiting neuron-specific properties such as elevation of acetylcholine esterase activity and membrane excitability as well as neurite outgrowth. Accumulation of F-actin as well as GAP43 at the growth cone is observed in these cells, suggesting that Ras can induce more complete neurites than the activated PI-3 kinase. Co-injection of a construct for a dominant-negative Ras with pmycBD110 did not inhibit process formation, whereas neurite outgrowth of NGF-stimulated cells was completely inhibited. In addition, inhibition of PI-3 kinase by wortmannin blocked neurite outgrowth in the cells expressing activated Ras (K. D. Kimura et al., unpublished data). It is possible that constitutive activation of Ras can work upstream of PI-3 kinase in the differentiation of PC12 cells.

Altogether, we suggest that PI-3 kinase regulates the neurite outgrowth induced by NGF stimulation through reorganization of the cytoskeleton. This may be due to activation of the Rac-JNK pathway independent of the Ras-MAP kinase pathway.

We thank Drs Gary Ruvkun and Scott Ogg for critical reading of this manuscript. We thank Drs Alan R. Saltiel and Graham P. Wilkin for the gifts of a MEK inhibitor, PD98059 and anti-GAP43 polyclonal antibody. K.D.K. is supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists. This work was supported by Grants-in-Aid no. 08275210 and no. 07456046 for Y.F. from the Ministry of Education, Science, Sports and Culture of Japan.

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