Expression of a Constitutively Active Phosphatidylinositol 3-Kinase Induces Process Formation in Rat PC12 Cells

USE OF Cre/loxP RECOMBINATION SYSTEM*

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It has been shown that inhibition of phosphatidylinositol (PI) 3-kinase blocks neurite outgrowth of PC12 cells stimulated with nerve growth factor. To further assess the role of PI 3-kinase, the active form of PI 3-kinase was expressed in PC12 cells by the adenovirus mediated introduction of a site-specific recombinase, Cre. After expression of the active PI 3-kinase, elevation of the levels of PI 3,4-diphosphate and PI 3,4,5-trisphosphate as well as formation of neurite-like processes was observed. The process formation was inhibited by wortmannin, a selective inhibitor of PI 3-kinase, which suggests that high activity of PI 3-kinase is responsible for the formation of these processes. The processes lacked accumulation of F-actin and GAP43 at the growth cone, which suggests that the processes were incomplete compared with neurites. Instead, the bundling of microtubules was enhanced, which suggests that organization of the microtubules might be driving the process of elongation in the cells expressing the active PI 3-kinase. Induction of active PI 3-kinase resulted in activation of Jun N-terminal kinase but not of mitogen-activated protein kinase or protein kinase B/Rac protein kinase/Akt. These results suggest that PI 3-kinase is involved in neurite outgrowth in PC12 cells and that activation of Jun N-terminal kinase cascade may be involved in the cell response.

The PC12, rat pheochromocytoma cell line provides a useful model system for the differentiation of neuronal cells. They respond to nerve growth factor (NGF1) with growth arrest and exhibit typical characteristics of neuronal cells (1). After stimulation with NGF, a number of signaling pathways are activated, including the Ras-MAP kinase, phospholipase C, and phosphatidylinositol (PI) 3-kinase cascades (2). In the setting of multiple activation of signaling pathways, it has been suggested that sustained activation of MAP kinase in particular is involved in the differentiation (3, 4). Indeed, constitutive activation of MAP kinase by activated Ras or MAP kinase kinase results in full differentiation of the cells (5–7). Besides this, we have shown that PI 3-kinase activity is required for neurite outgrowth in PC12 cells (8). The results suggested that the PI 3-kinase activity was required especially for the neurite elongation. The analysis of the levels of phosphoinositides in NGF-treated PC12 cells revealed that PI 3-kinase was strongly activated immediately after NGF treatment, and this activity declined rapidly. However, even long after the burst of PI 3-kinase activation, levels of the products of PI 3-kinase remained slightly higher than that of unstimulated cells (8). The PI 3-kinase activated by NGF stimulation consists of two subunits, p85 and p110 (9). The regulatory subunit, p85, contains one SH3 domain and two SH2 domains, which may be involved in interaction with other proteins. The p85 subunit binds to p110 through the region between the two SH2 domains of p85 (iSH2) (10, 11). P110 is the catalytic subunit, and p85 binding is necessary to achieve full catalytic activity. The addition of iSH2 to the N-terminal region of p110 through a bridge of glycine residues constitutively activates PI 3-kinase (12).

We constructed a similar expression vector to analyze the effect of expression of the active PI 3-kinase in the cells. We attempted to establish stable cell lines expressing active PI 3-kinase; however, we have been unable to obtain such a cell line, perhaps due to the cell responses following the expression of the active PI 3-kinase. In this paper, we report establishment of cell lines for expression of the active PI 3-kinase by using the “Adex” system (13). In this system, the active PI 3-kinase gene was introduced to PC12 cells as a silent form with a stuffer of the neomycin resistance gene located between the CAG promoter (cytomegalovirus IE enhancer + chicken β-actin promoter + rabbit β-globin poly(A) signal) and the PI 3-kinase gene with loxP sequences in both ends. The stuffer is then cleaved out by a sequence-specific recombinase, Cre, by infection of the cells with an adenovirus coding for the recombinase (AxCANCre) to make the PI 3-kinase gene active. Expression of the PI 3-kinase gene was completely absent to allow the introduction of the toxic gene and recombination of the gene was very efficient, which made it possible to carry out the biochemical experiments.

EXPERIMENTAL PROCEDURES

Expression of the Active PI 3-Kinase Gene in PC12 Cells—The BD110 protein has the p110-binding domain of human p85a (amino acids 474–652; “BD” stands for binding domain. The N-terminal end of the sequence was generated by exonuclease reaction to yield minimum fragment capable of binding to p110 (10, 11). The sequence for the Myc

1 The abbreviations used are: NGF, nerve growth factor; MAP, mitogen-activated protein kinase; PI, phosphatidylinositol; PCR, polymerase chain reaction; PIPES, 1,4-piperazinediethylsulfonic acid; JNK, Jun N-terminal kinase; PKB, protein kinase B.
tag with the initiation codon at the 5′ end was conjugated through the linker sequence derived from pUC19 (the C-terminal end corresponds to the PvuII site at 1902 map position) at the N-terminal end of bovine p110 through a bridge of 7 glycines (Fig. 1A). The high activity of the gene product is reported elsewhere. 2 The BD110 gene was cloned into pCALNLw (13). The gene should be kept silent because of the stuffer of the Neo resistant gene. After infection of the cells with AxCANCre (multiplicity of infection = 25) at room temperature, the region between the two loxP sequences is removed by the recombinase Cre to induce the expression of BD110 (Fig. 1B).

Cell Lines—PC12 cells were maintained in Dulbecco’s modified minimal essential medium supplemented with 10% calf serum and 5% horse serum. The expression vector (1 μg) for the BD110 protein (Fig. 1, A and B) was introduced into the cells by electroporation. After G418 selection (200 μg/ml), 24 cell lines were established. The cells were then infected with AxCANCre, and those that expressed the BD110 protein were selected. Two cell lines, PC12Cre12 and PC12Cre20, were obtained.

Detection of Occurrence of the Recombination—The genomic DNA was extracted from the cells incubated for the periods indicated in the figure after infection of the virus. The PCR reaction was carried out with 25 cycles of 95°C for 0.5 min, 60°C for 0.5 min, and 72°C for 1 min using the primers indicated in Fig. 1C to detect the DNA sequence after recombination.

Analysis of the Levels of Phosphoinositides—PC12 cells infected with Adex viruses were labeled with [32P]orthophosphate (1 mCi/ml) for 4 h, and the lipids were extracted, deacylated, and analyzed by strong anion exchange chromatography as described before (14). The radioactivity in the fractions containing each glycerophosphoinositide was counted and normalized against the total counts incorporated into the lipids.

Staining of the Cells for F-actin, Microtubules, and GAP43 Antigen—Cells were infected with AxCANCre and incubated for 48 h to allow the BD110 protein to express in the presence of 100 nM wortmannin to inhibit the PI 3-kinase activity to prevent process formation. At 9 h after removal of wortmannin, cells were fixed with 2% formalin and 0.1% glutaraldehyde/PEM (0.1 M PIPES-NaOH, pH 6.6, 1 mM EGTA, 1 mM MgCl2) at 37°C for 15 min, permeabilized with 0.5% Triton X-100/ phosphate-buffered saline for 3 min, and treated with 50 μM Glycine/phosphate-buffered saline for 30 min. Then the cells were stained with rhodamine-phalloidin for visualization of F-actin. For microtubules and GAP43, cells were incubated with anti-α-tubulin antibody or anti-GAP43 antibody and then with fluorescein isothiocyanate-anti-rabbit IgG or with fluorescein isothiocyanate-anti-mouse IgG.

The Assay of MAP Kinase, JNK, and Akt Activities—JNK assay was carried out as described previously (15, 16) with slight modifications. Cell lysate (30 μg of protein) was resolved on a 10% SDS-polyacrylamide gel, which was polymerized in the presence of the c-Jun N-terminal region (40 μg/ml). After the kinase reaction, protein bands were detected by autoradiography. The activity of MAP kinase and PKB was analyzed as described before (17, 18).

RESULTS AND DISCUSSION

The two cell lines, PC12Cre12 and PC12Cre20, were infected with adenoviruses. As shown in Fig. 1C, the BD110 protein was detected at 24 h after infection, whereas the cells infected with AxCANLacZ, a control virus carrying the Neo resistant gene and the stuffer of the plasmid, did not produce the protein. Consistent with this, recombination of the gene to induce the BD110 protein was detected in the PCR analysis (Fig. 1D).

The levels of 3′-phosphorylated phosphoinositides were examined. The levels of PI 3-phosphate, PI 3,4-diphosphate, and PI 3,4,5-triphosphate in AxCANCre-infected cells were 0.47, 0.66, and 0.31% of the total lipids, respectively, at 36 h after infection, whereas those in AxCANLacZ-infected cells were 0.27, 0.27, and 0.09. The elevation of levels of these phospholipids was higher than those in NGF-treated cells (the maximum level of PI 3,4,5-triphosphate in NGF-treated cells was about 0.2% of total phospholipids (8)). These results suggest that PI 3-kinase activity is super-activated in the cells expressing the BD110 protein by Cre/loxP system.

FIG. 1. Expression of the activated PI 3-kinase in PC12 cells. A, the structure of the activated PI 3-kinase (PI3K). BD110. B, the strategy for expression of the gene. The BD110 gene was inserted into the cloning site (SmaI site) of pCALNLow (upper bar) (14). After infection of the cells with AxCANCre, the region between the two loxP sequences is removed by the recombinase cre to induce the expression of BD110. The positions of the primers used in D are indicated by P1 and P2. C, expression of the BD110 protein. The cells were incubated for the periods indicated in the figure after infection of the virus. The BD110 protein was immunoprecipitated (IP) from the cell lysates (200 μg of protein) in 1% Nonidet P-40, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 5 mM EDTA with anti-Myc monoclonal antibody (Mab), 9E10. The sample was separated on the SDS-polyacrylamide gel electrophoresis, and the levels of the BD110 protein were analyzed by Western blotting with 9E10. The arrowheads indicate the position of the BD110 protein. D, PCR analysis of the occurrence of the recombination. At various periods after infection of the viruses, DNA was extracted. PCR was carried out with the primers indicated in B, and the amplified fragment (240 base pair (bp)) was detected (indicated by an arrow in the figure).

Morphological change of PC12Cre12 and PC12Cre20 cells after infection with AxCANCre was observed. After 2–3 days, they extended the processes (Fig. 2, A and B), whereas those infected with AxCANLacZ did not. We found that more than 40% of the cells bore processes and most of the rest cells responded to the AxCANCre infection to change cell shapes with the shorter processes. This indicates that the efficiency of infection was quite high. To confirm that the process formation

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was due to elevation of PI 3-kinase activity, the cells were treated with wortmannin, a selective inhibitor of PI 3-kinase (19, 20). The process formation was completely blocked (Fig. 2A, h and k), indicating that high PI 3-kinase activity is responsible for the process formation. We were able to maintain the cells bearing the processes more than 3 weeks. The BD110 protein was present even after cultivation for 3 weeks, suggesting that the BD110 protein did not affect the cell viability. However, the growth of the cells were somewhat slower after induction of the gene expression. This might explain why we were not able to obtain the BD110 expressing cells. Stimulation of the BD110 expressing cells with NGF resulted in formation of complete neurites longer than those induced without BD110 expression, suggesting that superactivation of PI 3-kinase enhances neurite elongation (data not shown).

The processes induced by active PI 3-kinase were histochemically different from those induced by NGF treatment. It appeared that the tips of the processes were somewhat thinner than those induced by NGF and the growth cones were missing in the former. To view this in greater detail, we analyzed the reorganization of F-actin, microtubules, and GAP43 in the cells (Fig. 3). To synchronize the cell response, cells were treated with wortmannin for 48 h to allow the myc-BD110 protein to express in the presence of 100 nM wortmannin. At 9 h after removal of wortmannin, cells were fixed and stained for F-actin, microtubules, and GAP43 antigen. Cells were infected with AxCANCre and incubated for 48 h to allow the BD110 protein to express in the presence of 100 nM wortmannin. At 9 h after removal of wortmannin, cells were fixed and stained for F-actin, microtubules, and GAP43. For the controls, cells incubated for 36 h after NGF stimulation were also shown.

**Fig. 2.** The morphology of the PC12 cells infected with the adenoviruses. PC12 cells were infected with AxCANCre or AxCANLacZ, a control virus carrying lacZ gene instead of the recombinase, cre (multiplicity of infection = 25). Morphology of the cells is shown. A, the morphological change of PC12Cre12 and PC12Cre20 cells after virus infection. PC12Cre12 (a–d and i) and PC12Cre20 (e–h, j, and k) cells were infected with AxCANCre (b–d, f–h, and k) or with AxCANLacZ (i and j). The incubation periods were 1 (b and f), 2 (c and g), and 3 days (d and h–k). h, wortmannin (100 nM) was added every 4 h to inhibit the activity of PI 3-kinase. a and e, uninfected cells. B, the number of the cells with processes longer than their cell body was counted and plotted on the graph. The processes formed by the induction of the BD110 protein did not show the accumulation of F-actin, suggesting that filopodia were not formed. In cultured neurons, GAP43 is localized in axons and growth cones (21–24). GAP43 was co-localized with F-actin at growth cones in NGF-stimulated PC12 cells. In contrast, GAP43 did not accumulate at the growth cone in BD110-expressing cells (Fig. 3, lower part). Instead, GAP43 was found in the cell body as granules. Microtubules stained more densely in BD110-expressing cells than in NGF-treated cells (Fig. 3, upper part). Treatment with colchicine, an inhibitor of polymerization of tubulin, inhibited the process formation, suggesting that microtubule organization may be involved in this cell response (data not shown). It has been shown that microtubule depolymerization causes neurite retraction (25, 26) and inhibition of actin polymerization leads to tubulin expansion to lead abnormal outgrowth of neurites (26–28). Our finding is consistent with these observations. It is possible that PI 3-kinase controls process elongation by regulating the organization of the cytoskeleton. The processes did not have the GAP43 antigen at the tips, suggesting that they were incomplete as neurites. In addition to the PI 3-kinase cascade, activation of other pathways such as MAP kinase and phospholipase C cascades, which are also activated by NGF stimulation, may be required for formation of complete neurites.

The signal transduction events following activation of PI 3-kinase were assessed. It has been suggested that PKB and JNK function downstream of PI 3-kinase (29–32). MAP kinase was also a candidate that might be activated by PI 3-kinase, because activation of MAP kinase by NGF stimulation was partially inhibited by wortmannin (8). We examined activity of these three enzymes after expression of the BD110 protein. We
detected activation JNK by NGF stimulation. The JNK activity was also higher in BD110-expressing cells than in the cells infected with the control virus, confirming that JNK is downstream of PI 3-kinase (Fig. 4A). In contrast, MAP kinase activity was not elevated in BD110-expressing cells, although activation of MAP kinase was clearly seen after NGF stimulation (Fig. 4B). This result suggests that the activity of MAP kinase is not controlled by the PI 3-kinase pathway. We also tested PKB. PKB was immunoprecipitated with anti-PKB antibody, and the kinase activity was analyzed using core histone as a substrate. Basal activity was readily detectable, but no further activation was seen after NGF stimulation. Consistent with this finding, no activation of the PKB was observed in BD110-expressing cells (Fig. 4C). PKB might be regulated by the mechanism different from PI 3-kinase cascade such as phosphorylation in PC12 cells (33–35).

We have explored the effect of microinjection of the expression vector for the BD110 protein. Microinjected PC12 cells also extended processes with a rich network of microtubules. In this system, inhibition of MAP kinase and PKB cascades did not block process formation, whereas dominant negative mutants of Rac and SEK1/SAPK did, suggesting that the pathway to JNK through Rac is involved in the process formation. These findings are consistent with the result that JNK, not MAP kinase and PKB, is activated by the induction of the BD110 protein in PC12 cells.

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