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Basic fibroblast growth factor-induced translocation of p21-activated kinase to the membrane is independent of phospholipase C-γ1 in the differentiation of PC12 cells

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Abbreviations: bFGF, basic fibroblast growth factor, FGFR, fibroblast growth factor receptor; PLC-γ1, phospholipase C-γ1; PAK, p21-activated kinase

Abstract

p21-activated kinase (PAK) targeting to the plasma membrane is essential for PC12 cell neurite outgrowth. Phospholipase C-γ1 (PLC-γ1) can mediate the PAK translocation in response to growth factors, since PLC-γ1 binds to both tyrosine-phosphorylated receptor tyrosine kinases and PAK through its SH2 and SH3 domain, respectively. In the present study, we examined a potential role for PLC-γ1 in the basic fibroblast growth factor (bFGF)-induced PAK translocation using stable PC12 cell lines that overexpress in a tetracycline-inducible manner either the wild-type FGFR-1 or the Y766F FGFR-1 mutant. Phosphatidylinositol hydrolysis was increased 6.5-fold in response to bFGF in the wild type cells but negligible in the mutant cells. The recombinant GST-PLC-γ1 SH3 was able to bind to PAK1 but not GST alone. However, examination of PLC-γ1 as an adaptor for translocation of PAK1 in cells showed that both cells transfected with pEGFP-PAK1 was able to differentiate for 24 h, as visualized by laser confocal microscopy. Translocation of PAK1 to growth cones occurs at similar levels in both wild and mutant cells. These results suggest that a protein(s) other than PLC-γ1 is functionally relevant for PAK targeting.

Keywords: PC12 cell, bFGF, PLC-γ1, PAK, translocation

Introduction

Basic fibroblast growth factor (bFGF), a member of FGF family, is a potent differentiation factor in the pheochromocytoma cell model PC12. bFGF exerts its effects through the activation of its high-affinity FGF receptor (FGFR), a receptor tyrosine kinase. One of the target molecules of FGFR is phospholipase C gamma-1 (PLC-γ1), which, upon bFGF stimulation, binds to the receptor and becomes tyrosine phosphorylated and activated. PLC-γ1 catalyzes hydrolysis of PI-4, 5-bisphosphate (PIP2) to the second messengers inositol-1, 4, 5-trisphosphate and diacylglycerol. It has been shown that PLC-γ1-mediated phosphatidylinositol (PI) hydrolysis is not essential for FGF-induced neuronal differentiation of PC12 cells (Spivak-Kroizman *et al*., 1994). Moreover, overexpression of Src homology (SH) domains of PLC-γ1 inhibits nerve growth factor (NGF)-induced differentiation of PC12 cells (Bae *et al*., 1998), suggesting that PLC-γ1 can mediate the NGF-induced signals for proliferation rather than differentiation. Ye *et al*., (2002) demonstrated elegantly that the SH3 domain of PLC-γ1 has a guanine nucleotide exchange factor (GEF) activity for PI3-kinase enhancer, which may account for the mitogenic potential of PLC-γ1. PLC-γ1 binds α1β1 integrin and modulates α1β1-dependent adhesion independent of PLC-γ1 tyrosine phosphory-lation in PC12 cells (Vossmeyer *et al*., 2002). These results suggest that PLC-γ1 can act as a signaling molecule in multiple ways, including protein-protein interaction.

p21-activated kinase (PAK) is a member of serine/threonine kinase family that comprises six members, PAK1-6. PAK is a downstream effector of Rho family small G proteins such as Rac1 and Cdc42 that play a critical role in cytoskeletal reorganization (Hall A., 1998). Evidence indicates that PAK1 targeting to the plasma membrane is sufficient for PC12 neurite outgrowth (Daniels *et al*., 1998). However, it is not clear how PAK is targeted to the plasma membrane in the bFGF signaling pathway. In the present study, we examined whether PLC-γ1 can be a mediator for the PAK recruitment to the plasma membrane, since PLC-γ1 contains a SH3 domain that interacts with PAK. Although the SH3 domain of PLC-γ1 binds to PAK *in vitro*, the PLC-γ1-mediated PAK translocation seems to be dispensable in PC12 cells, suggesting that an alternate route functions for the bFGF-induced PAK translocation.

Materials and Methods

Materials

Glutathione agarose, anti-GST antibody, ECL kit and PVDF membrane were obtained from Amersham Bioscience (Uppsala, Sweden). Human recombinant bFGF, LipofectAMIN 2000, G418 and hygromycin B were obtained from Invitrogen (Carlsbad, CA, USA). The Tet-on system, Tet System approved fetal bovine serum (FBS) and pEGFP-C2 were purchased from Clontech (Palo Alto, CA, USA). The QuikChange Site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA, USA). Anti-His antibody, NI-NTA agarose and pQE-60 vector were obtained from QIAGEN Inc. (Valencia, CA, USA).

Plasmid and DNA constructs

Rat FGFR-1 cDNA (Kim *et al.*, 1993) was cloned into *Eco*RI and *Xba*I sites of pTRE (Clontech) for inducible expression. To generate mutant cDNAs, mutagenesis was conducted using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's recommendation. Mutagenic PCR primers for the FGFR-1 mutant (Y766F) are as follows: sense, 5'-ACCTCCAACCAGGAGTTTCTGGACCTGTCC-3' and antisense, 5'-GGACAGGTCCAGAAACTCCTGGTTGGAG GT-3'. PAK1 cDNA (a gift from Gary M. Bokoch, Departments of Immunology and Cell Biology, The Scripps Research Institute, La Jolla, CA, USA) was subcloned into *Nco*I and *Bam*HI of pQE-60. pGEX-PIX-SH3 and pGEX-PLC-γ1-SH3 cDNAs were kindly provided by Dr. Park D. (School of Biological Sciences, Seoul National University) and Dr. Suh P. G. (Department of Life science, Pohang University of Science and Biotechnology), respectively.

Cell culture

Cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Tet System approved fetal bovine serum (FBS), 2 mM glutamine, 1X antibiotics (Invitrogen), and 100 mg/ml G418 plus 50 μg/ml hygromycin B at 37°C on 10% CO_2.

Stable transfection

Stable transfection of pTRE-FGFR-1 (wild type or Y766F) was performed with the calcium phosphate transfection reagent (Invitrogen) according to the manufacturer's recommendation. Briefly, transfected cells were grown for 24 h in nonselective culture medium, and then replaced by complete DMEM containing 250 mg/ml hygromycin B. After 14 days, hygromycin B-resistant colonies were isolated and subcloned to obtain populations of single cells. To measure expression levels of FGFR-1, a receptor binding assay was conducted (Shin *et al.*, 1995).

Translocation assay

To analyze translocation of PAK1, cells were induced to differentiate in complete media containing 10 ng/ml bFGF for 48 h, and transfected with pEGFP-PAK1 using LipofectAMINE 2000 (Invitrogen). Transfected cells were incubated in DMEM containing 2% FBS in the presence or absence of 10 ng/ml bFGF for 24-48 h. Cells were washed three times with PBS and fixed in 4% paraformaldehyde/PBS for 15 min. After fixation the coverslips were washed twice in PBS and mounted onto a glass slide with gelvatol. Fluorescence was visualized with a laser confocal microscope (MRC-1024, Bio-Rad Laboratories, Richmond, CA, USA).

PLC-γ1 activity assay

bFGF-stimulated hydrolysis of PIP2 by PLC-γ1 was measured by determining the formation of inositol phosphates according to the procedures (Bae *et al.*, 1998).

Expression and purification of recombinant proteins

GST-PLC-γ1-SH3 and GST-PIX-SH3 fusion proteins were expressed in DH5α cells and purified according to the standard protocol of Amersham Bioscience (GST gene fusion system). His-tagged PAK1 protein was expressed in M15 cells and purified following the standard protocol under native conditions (QIAGEN Inc, QIAexpressionist).

Binding assay

Glutathione-agarose bound GST fusion proteins and 0.5 μg His-tagged PAK1 were incubated in lysis buffer for 1 h at 4°C under constant agitation. Glutathione-agarose beads were washed five times with 1 ml lysis buffer each and twice with 1 ml PBS. Samples were boiled in SDS-sample buffer and separated on SDS-polyacrylamide gel electrophoresis and immuno-blotted with anti-GST or anti-His antibody.

Immunoprecipitation and Western blotting

Immunoprecipitation and Western blotting were performed according to the procedure as described previously (Jin *et al.*, 2001).

Results and Discussion

To determine whether bFGF-induced translocation of PAK involves the tyrosine phosphorylation and the activation of PLC-γ1, we generated stable PC12 cell lines that overexpress in a tetracycline-inducible manner either the wild-type FGFR-1 or the Y766F FGFR-1 mutant, in which Tyr 766 has been replaced by phenylalanine. Cells expressing the wild-type FGFR-1 (PC-FW) or the mutant FGFR-1 (PC-FM) at similar

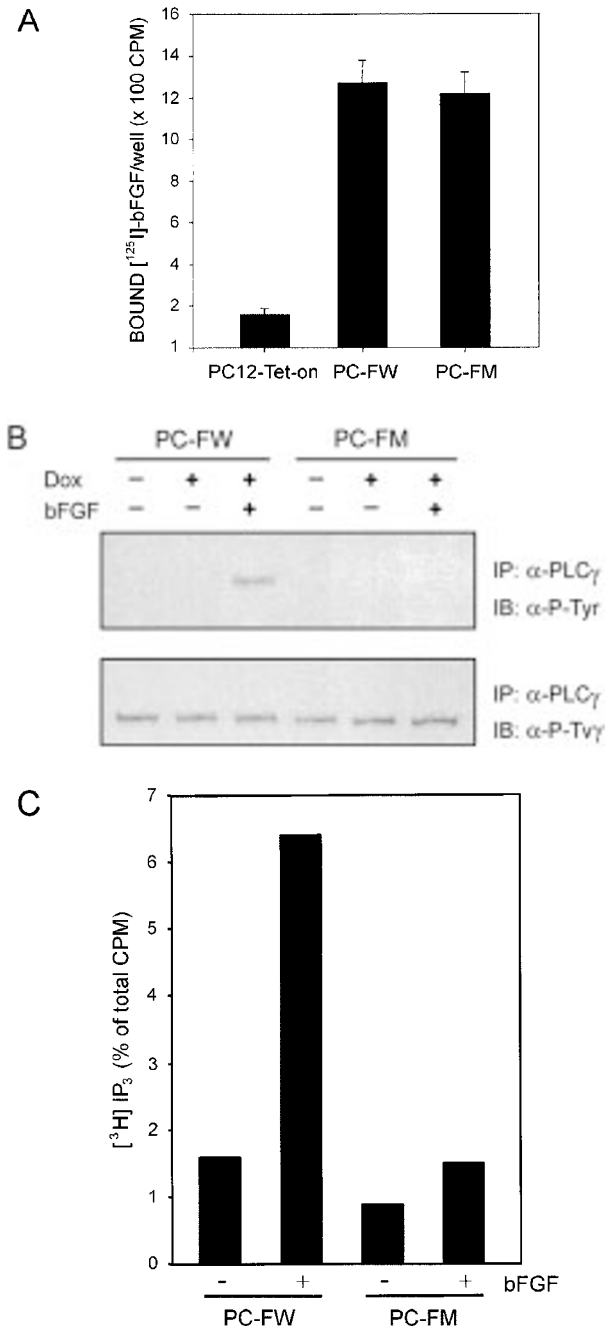

Figure 1. Y766F FGFR-1 mutant is unable to stimulate the tyrosine phosphorylation of PLC-γ1 and PI hydrolysis. A, Parental PC12-tet-on cells, PC-FW cells expressing wild-type FGFR-1 or PC-FM cells expressing mutant FGFR-1 (Y766F) were incubated in the receptor binding assay buffer containing [^{125}I]-bFGF for 3 h at 4°C. Expression levels of FGFR-1 were measured as described previously. B, PC-FW or PC-FM cells were stimulated with bFGF (10 ng/ml) for 10 min, lysed, and subjected to immunoprecipitation followed by immunoblotting with anti-P-Tyr antibodies (top) or anti-PLC-γ1 antibodies for equal loading (bottom). Dox, doxycyline. C, PC-FW or PC-FM cells were labeled with [^{3}H]myoinositol in serum-free medium for 24 h. Cells were then stimulated with bFGF (10 ng/ml) for 2 h, and inositol phosphate formation was measured as described under Materials and Methods. Data represent the percent of the total CPM incorporated into inositol phosphates.

levels approximately 12-fold over the parental PC12 cells were selected as determined by the binding studies with ^{125}I-labeled bFGF (Figure 1A). To characterize these stable cell lines, the cells were treated with bFGF (10 ng/ml) for 10 min, lysed, and subjected to immunoprecipitation with anti-PLC-γ1 antibodies followed by immunoblotting with anti-P-Tyr antibodies. As shown in Figure 1B, PC-FM cells exhibit defect in the tyrosine phosphorylation of PLC-γ1, which is consistent with the previous result (Spivak-Kroizman *et al.*, 1994). We next determined the ability of both PC-FW and PC-FM cells to stimulate PI hydrolysis. Cells were stimulated with bFGF and the formation of inositol phosphates was measured. PC-FW cells show 6.5-fold increase in PI hydrolysis in response to bFGF (Figure 1C). In contrast, bFGF-induced PI hydrolysis is negligible in PC-FM cells, indicating that the elimination of Tyr-766 in FGFR-1 inhibited the PLC-γ1-mediated PI turnover. Taken together, these results indicate that the Y766F FGFR-1 mutant is unable to link the bFGF signal to the tyrosine phosphorylation of PLC-γ1 and the subsequent activation.

PAK3 has been shown to bind to the SH3 domain of PLC-γ1 (Bagrodia *et al.*, 1995). PAK targeting to the plasma membrane is essential for its cellular effects. It seems therefore plausible that PLC-γ1 plays an adaptor role in PAK1 targeting, since PLC-γ1 is recruited to FGFR-1 upon bFGF stimulation. We examined the ability of PLC-γ1 to bind to PAK1. To this end, the SH3 domain of PLC-γ1 and PAK1 were expressed as recombinant GST fusion proteins and His-tagged proteins in E. coli, respectively. As illustrated in Figure 2, the GST-PLC-γ1 SH3 but not GST alone binds to PAK1, which is comparable to that of the SH3 domain of PAK-

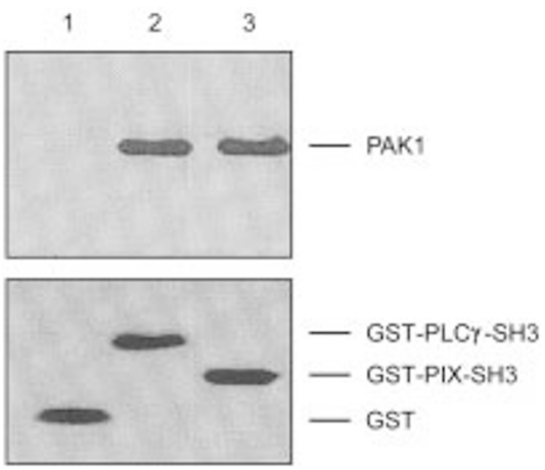

Figure 2. The SH3 domain of PLC-γ1 binds to PAK1. GST and GST-SH3 fusion proteins were expressed and purified with Glutathione-affinity beads. The beads were then incubated with bacterially expressed His-tagged PAK1 and washed extensively. The immobilized proteins were subjected to SDS-PAGE and immunoblotting with anti-His (top) or anti-GST (bottom) antibodies.

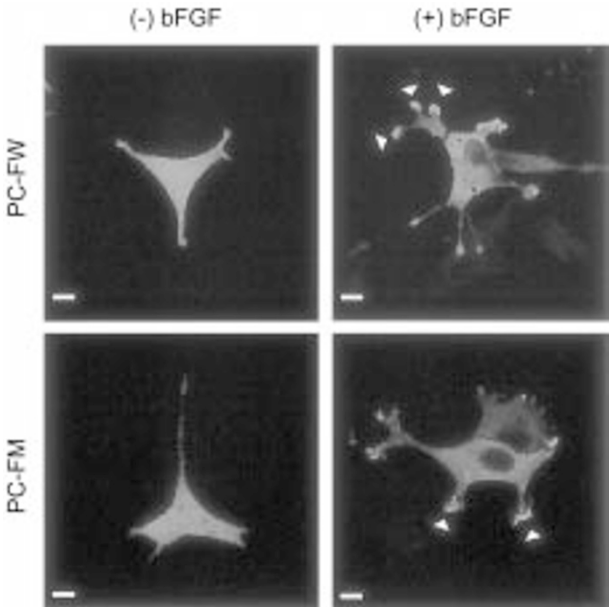

Figure 3. PAK1 translocation in PC-FW and PC-FM cells. Cells went differentiation for 24 h, and transfected with pEGFP-PAK1. Cells were cultured in the absence of serum for 20 h and then stimulated with bFGF for 24 h. PAK1 translocation to growth cones was analyzed by laser confocal microscopy. Representative images for PAK1 concentrated in growth cones are shown (arrowheads). The scale bar, 10 μm.

interacting exchange factor (PIX) (Manser *et al*., 1998). Using PC-FW and PC-FM cells, we next examined the ability of PLC-γ1 as an adaptor for translocation of PAK1. Cells were transfected with pEGFP-PAK1, induced to differentiate for 24 h, and visualized by laser confocal microscopy. Translocation of PAK1 to growth cones occurs at similar levels in both PC-FW and PC-FM cells (Figure 3). Nck, an adaptor protein that possesses a SH2 and three SH3 domains, binds to PAK1 through its second SH3 domain (Bagrodia *et al*., 1995). Following the growth factor stimulation, Nck also binds to the tyrosine-phosphorylated receptor kinase through its SH2 domain (Meisenhelder *et al*., 1992. Park and Rhee, 1992). Therefore, it is reasonable to assume that Nck can mediate PAK targeting to the plasma membrane. However, the PC12 cells that overexpress Nck does not exhibit the morphological changes for neurite outgrowth (Rockow *et al*., 1996), suggesting that Nck is not a potential adaptor for PAK. PIX is another PAK binding protein, as we have shown in Figure 2 (Manser *et al*., 1998). PIX co-localizes with PAK in focal complexes and membrane ruffles. PIX functions upstream of PAK and regulates the PAK kinase activity by stimulating Rac1/Cdc42 or inducing conformational changes (Bagrodia *et al*., 1998; Daniels *et al*., 1999). Therefore, it seems highly likely that bFGF-induced PAK translocation might be mediated by PIX. This possibility is currently under investigation.

Acknowledgements

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