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FGF signal transduction in PC12 cells: Comparison of the responses induced by endogenous and chimeric receptors

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Summary Rat phaeochromocytoma (PC12) cells respond to many growth factors and produce different phenotypes, including neurite outgrowth. Receptor tyrosine kinases (RTK), which activate multiple signalling pathways in response to ligand binding, initiate many of these. One such family of receptors, the fibroblast growth factor receptor (FGFR), has four different members and expresses at least three of these in PC12 cells. A chimeric tyrosine kinase receptor, consisting of the extracellular domain of human plasma-derived growth factor receptor-β (hPDGFR-β) and the transmembrane and intracellular region of FGFR1 (designated PFR1), was constructed and was stably transfected into cloned PC12 cell lines. This chimera, which can be activated without stimulating endogenous RTK including other FGFR, induces neurite outgrowth in a PDGF-dependent manner. By altering the protocol for preparing the retroviral vectors, cells with a wide range of expression levels can be obtained. The amount of these chimeric receptors seems to correlate with the time and the intensity of response as observed in neurite outgrowth assays. Analysis of proteins implicated in FGFR1 signalling indicates that upon stimulation, a tyrosine phosphorylated protein designated FRS2 associates with SOS, Grb2 and the receptor. The chimeric receptor appears entirely similar to that observed for the stimulation of native PC12 cells with FGF2. These results support the view that FRS2 is the dominant FGFR1 signalling entity in PC12 cells.

Key words: chimeric receptor, fibroblast growth factor, FRS2, neuronal differentiation, PC12 cells, signal transduction, tyrosine kinase.

Introduction

The PC12 (rat phaeochromocytoma) cell, a rat tumour cell line, is an excellent model for studying the signalling mechanisms involved in growth factor-stimulated differentiation. They can be reversibly differentiated by a number of agents that activate different signalling pathways. Two growth factors, nerve growth factor (NGF) and fibroblast growth factor (FGF), produce this response by the stimulation of receptor tyrosine kinases.2 Although the pathways utilized are not completely defined, they are clearly not identical. However, the cessation of cell division, extension and maintenance of neurites and sustained activation of the ERK (p42 and p44) are characteristic of their responses.3 Interestingly, other growth factors, most notably epidermal growth factor (EGF), only stimulate PC12 cells to divide and are unable to produce a sustained activation of ERK under normal conditions.4

The receptor tyrosine kinases utilized by these factors consist of an N-terminal extracellular ligand binding domain, a single pass transmembrane-spanning region and an intracellular domain mainly composed of a tyrosine kinase effector.5 Upon ligand binding, these receptors become autophosphorylated, producing phosphorylated tyrosine residues at intracellular sites. Intermediate signalling molecules interact with the activated receptor, mainly through phosphorylated tyrosine residues via Src homology (SH2) or phosphotyrosine binding (PTB) domains, to initiate biological effects such as division and differentiation.6 For example, Shc and Grb2, complex with the guanine nucleotide exchange factor SOS, to activate the small guanosine triphosphate (GTP) binding protein, Ras, and thereby link receptor tyrosine kinase activation to a cytosolic protein kinase cascade. A critical downstream event in signal transduction from the receptor is the activation of ERK.

Four distinct genes encode the known FGF receptor (FGFR) family members.7 One feature of some of the FGFR is the enormous repertoire of variants that result from alternative splicing. The FGFR are found in a wide range of tissues and cell lines. During development and in the adult animal, the FGFR1 and FGFR2 genes typically exhibit broad but distinct patterns of expression. On the other hand, the FGFR3 and FGFR4 genes appear to have more restricted patterns of expression. In PC12 cells all FGFR genes are apparently expressed, with FGFR1 being the most abundant. All the FGFR bind FGF1 (acidic FGF) and each receptor, including FGFR1, can bind several of
the FGF. Thus it is difficult to interpret the signalling events mediated by FGF stimulation of native cells.

Several signalling proteins have been identified that respond to FGFR1 stimulation. Only PLCγ has been shown to associate with activated FGFR1. A point mutation at Tyr-766 (Y766F) of the FGF receptor eliminates PLCγ-receptor association, phosphorylation, and phosphatidylinositol hydrolysis, without affecting neuronal differentiation of PC12 cells. In response to FGF stimulation, a protein of ~89 kDa becomes tyrosine phosphorylated and has been proposed to be important for FGFR signal transduction. Recently, a protein termed FRS2 has been cloned and is likely to be synonymous to p89, SNT, and SLP, all designations given to this protein by various groups.

As a means to isolate FGF signalling responses in PC12 cells, chimeric FGFR1 receptors have been constructed from the extracellular domain of PDGFR-β and the transmembrane, juxtamembrane and kinase domain of rat FGFR1. When stably transfected into PC12 cells, this construct, designated PFR1, allows stimulation of FGFR1 kinase activity without activation of any endogenous FGFR (PC12 cells do not have PDGFR). A similar chimera of PDGFR-β and TrkA (PTR) has been used previously to dissect the role of TrkA in signal transduction of PC12 cells. Among other manipulations, it allows the substitution of functionally germane residues in the intracellular domain to assess their role in receptor function.

In the present report we demonstrate that the stimulation of PC12 cells stably transfected with the PFR1 chimera causes the cells to differentiate in a manner proportional to their expression level. Characterization of the activated receptor demonstrates that PFR1 associates directly with the phosphoprotein FRS2 and the phospholipase PLCγ. The responses to the chimera appear to be indistinguishable from those induced by FGF2 in wild-type PC12 cells.

Materials and Methods

Northern blot analysis

Poly (A) mRNA was isolated from PC12 cells with oligo dT beads (InVitrogen, CA, USA) according to manufacturer instructions, resolved on a 2.2 mol/L formaldehyde-1% agarose gel and transferred to GeneScreen (DuPont, NEN, MA, USA) nylon membranes. The blots were hybridized with [α-32P]dCTP-labelled probes corresponding to a portion of the extracellular domain of PC12 FGFR1, FGFR3 and FGFR4 cDNA, obtained by screening PC12 cDNA libraries in izzAP. The blots were stripped and reprobed with a CHOB cDNA.

Cloning strategy for chimeric receptor

The naturally occurring 5’ EcoR1 and 3’ Mse I cDNA fragment of hPDGFR-β encoding the N-terminal extracellular domain was ligated with a 5’ Mse I site introduced by polymerase chain reaction (PCR) at the extracellular–transmembrane junction of the cDNA encoding FGFR1 intracellular domain, and flanked by a 3’ EcoR1 site also introduced by PCR. The cDNA construct was subsequently sequenced. The construct was then subcloned into the EcoR1 site of pCMV for transient expression in 293 cells and pLEN for stable transfection into PC12 cells. A full-length form of rat FGFR1 consisting of two Ig-like domains was isolated from a PC12 cDNA library in izzAP.

Cell culture

The viral packaging cell line GP + e86 was transfected with the pLEN retroviral chimera construct or retroviral vector control by the calcium phosphate method. After 2 days, media from retroviral-producing GP + e86 cells was filtered (0.45 μm) and added to PC12 cells in the presence of polybrene. After 24 h, PC12 cells were subjected to G418 (Gibco BRL, NY, USA) selection until individual colonies could be selected and screened for expression of chimera via Western blot analysis. Clones were cultured in Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 2.5% plasma-derived FCS, 5% plasma-derived horse serum (Cocalico, PA, USA) and 1% penicillin/streptomycin (Gibco BRL).

PC12 differentiation assay

To examine the effect of the growth factor PDGF-BB (Austral, CA, USA) on the morphology of PC12 cells, cells stably transfected with PFR1 or vector control were seeded at a density of 1 × 10^5 cells per well on collagen (Collaborative Research, MA, USA)-coated tissue culture dishes (Falcon, NJ, USA) in DMEM supplemented with 1% plasma-derived horse serum and 1% penicillin/streptomycin. Cells expressing PFR1 or mock transfected were stimulated with 30 ng/mL of PDGF or with media alone as a control. The kinetics and extent of growth factor-induced neurite outgrowth were measured as the percentage of cells with neurites greater than two cell bodies in length at specified times.

Antisera

The antibodies used for immunoprecipitation and Western blot analysis include: anti-FGFR1, PY20, anti-SOS, and anti-PLCγ, all of which were purchased from Santa Cruz Biotechnology Inc. (CA, USA). In addition, the anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology (NY, USA). Antibodies against Grb2 and Sck and the p13 suc-agarose were purchased from Transduction Laboratories (KY, USA) and PDGFR-β monoclonal antibodies used for immunoprecipitation or Western blot analysis were purchased from Genzyme (MA, USA) and Austral (CA, USA), respectively.

Immunoprecipitation

Following stimulation of cells expressing the vector control or PFR1, PC12 cells were scraped from the dishes and pelleted. Culture media were removed and the cell pellet washed in cold PBS containing 1mmol/L Na3VO4. Lysis buffer (10 mmol/L Tris HCl pH7.5, 5 mmol/L EDTA, 1% Triton X-100, 50 mmol/L NaCl, 30 mmol/L sodium pyrophosphate, 50 mmol/L NaF, 1 mmol/L PMFS, 100 μmol/L Na2VO3) was added and cells lysed for 10 min on ice. Cell debris was removed by centrifugation. Supernatants were transferred and the protein was quantified. Antibodies were added to 1 mg protein lysates. The samples were mixed on a rocker for 1.5 h at 4°C prior to addition of Protein A-Sepharose (Pharmacia, NJ, USA) for a final 1.5-h mixing. The precipitates were washed four times in 1× lysis buffer containing 100 μmol/L Na2VO4. The samples were subjected to SDS-PAGE before electroblotting to PVDF membrane.
Immunoblot analysis

The membranes were blocked at room temperature in Tris-buffered saline (TBS) containing 3% BSA or 5% non-fat dry milk for 4 h. Primary antibodies were diluted in TBS containing 3% BSA and incubated with the membranes for 2 h at room temperature. After three washes in TBS the membranes were incubated for 1 h with the appropriate horse radish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Lab Inc., PA, USA or Amersham Life Sciences, OH, USA) diluted in TBS containing 5% non-fat dry milk. After three washes in TBS the bands were visualized by ECL Chemiluminescent detection system (Amersham). Before re-probing the filters they were stripped in 0.2 mol/L glycine-HCl pH 2.5, 0.05% Tween-20 at 80°C.

Results

The expression of endogenous FGFR in PC12 cells was analysed by northern blot. (Fig. 1). Fibroblast growth factor receptor 1 is the most abundant mRNA in PC12 cells, but FGFR3 and FGFR4 are also expressed, although at a much lower level. These results were confirmed by the number of clones isolated by cDNA cloning experiments (data not shown). No FGFR2 cDNA clones were obtained from cDNA library screening. This suggests that in the PC12 cells employed in these studies, this receptor must be present at a very low level, if at all. Thus, FGF ligands can be expected to produce signals from at least three receptor types.

Phaeochromocytoma 12 cells were stably transfected with the PFR1 chimera, and several clones expressing different levels of chimera were isolated for further analysis. As judged by Western blot analyses, these cell clones express a wide range of chimeric protein, with clone C3 expressing the highest level, B3 the lowest and A2 as well as C2 expressing intermediate levels. (Fig. 2a). Fibroblast growth factor receptor 1 is only weakly detected immunologically in total lysates from PC12 cells. Immunoprecipitation of the receptor followed by immunoblotting is necessary to obtain a strong signal (data not shown).

All PFR1 expressing PC12 cell clones differentiate in response to PDGF. The timing and intensity of neurite formation correlates with PFR1 expression levels. Cells from clones A2, C2 and C3 have 80–95% of their cells producing neurites, at least two cell bodies in length, after only 24 h of exposure to PDGF-BB (Fig. 2b). In contrast, native PC12 cells require several days to fully differentiate when stimulated with FGF2 (basic FGF). PFR1-PC12 cell clone C2 was selected for further analysis based on its intermediate level of expression.

In order to compare the response of chimera bearing and wild-type cells, total cell lysates were examined by Western blot analyses for differences in phosphotyrosine levels following PDGF or FGF2 stimulation. Upon stimulation of the PC12 cells for 5 min with PDGF-BB or FGF2, several tyrosine-phosphorylated proteins can be observed. Bands corresponding to the receptor, p89 (FRS2) and p44 and p42 (ERK) are apparent in both cases. (Fig. 3a,b). In addition, other less predominant phosphotyrosine-containing proteins appear following stimulation. Although there were some differences in the intensities, the patterns were remarkably similar (except that there is much more receptor in the chimera-containing cells).

Chimera and wild-type lysates from cells stimulated for various lengths of time with ligand were also examined by western blot analysis. (Fig. 4a,b). Stimulation of either PFR1 or FGFR1 leads to a sustained tyrosine phosphorylation as well as long-term activation of ERK1 and ERK2 (p44 and p42). The receptor(s) remain phosphorylated for

Figure 1  Expression of fibroblast growth factor receptor FGFR1, 3 and 4 mRNA in rat phaeochromocytoma (PC12) cells. Poly (A)⁺ mRNA (3 µg/lane) was isolated from PC12 cells and subjected to northern blot analysis by probing it with [α-32P]dCTP-labelled cDNA corresponding to a portion of the extracellular domain of PC12 FGFR1 (a), FGFR3 (b) and FGFR4 (c). The blot in (a) was stripped and reprobed with CHOβ to normalize RNA loading differences. The arrows point to the major transcript of each FGFR. An RNA ladder was used for molecular size standard and is shown on the left. The autoradiographs shown were obtained after exposing to X-ray films for 8 h (a) and 48 h (b and c).
several hours following exposure to growth factor. Other proteins including p89 and the ERK have decreased levels of phosphorylation after 4 h of stimulation. Despite differences in the intensity of the response resulting from PFR1 versus FGFR stimulation, the nature of the signal transduction events by FGFR1 and PFR1 are indistinguishable.

To further probe downstream signalling responses, cell lysates were immunoprecipitated with the indicated antibodies to determine functional interactions between various signal transducers following stimulation with growth factor. In response to PDGF-BB, PFR1 is tyrosine phosphorylated and associates with FRS2 as well as PLCγ (Fig. 5a). FRS2 is an adaptor protein which links FGFR1 to the Ras-MAPK pathway. PLCγ is a phospholipase responsible for the formation of IP3 and DAG, secondary messengers involved in signal transduction.

There is also a rapid phosphorylation of Shc, but its association with Grb2 is quite weak and SOS is not readily detected in these complexes (Fig. 5b). The adaptor proteins, Shc and Grb2, interact via contacts between tyrosine phosphorylation sites on Shc and an SH2 domain on Grb2. However, the low levels of Grb2 association raise questions regarding the importance of Shc tyrosine phosphorylation. In contrast, as shown in Fig. 6(a), immunoprecipitation of SOS, which interacts and stabilizes the active (GTP-bound) form of Ras, also brings down FRS2 and Grb2. When p13 succinate agarose, which specifically binds FRS2, is used as precipitant, SOS and Grb2 are also found in the complex. These data suggest a major role of FRS2 in FGFR1 signalling pathways.

Discussion

To date, four distinct FGFR genes have been identified, with several additional isoforms resulting from alternative splicing. The neuronally related PC12 cell line expresses at least three of the FGFR at various levels, with FGFR1 being the most abundant; the stimulation of these cells with FGF ligand induces neuronal-like differentiation. However, the multiple FGFR expressed by PC12 cells have clouded the true contribution of each FGFR to the response. In this report, we have utilized a chimeric receptor to investigate the signalling capacity of the FGFR1 in stably transfected PC12 cells, thus avoiding the activation of endogenous FGFR, and have compared the responses to wild-type cells.

Several previous studies using the PC12 cell system have provided much needed insight into the function of FGFR1 and its role in neuronal differentiation. Early studies often involved the stimulation of native PC12 cells with FGF1 or FGF2 and subsequent assays to examine the morphological and molecular events involved in differentiation. Later, the expression of FGFR1 mutants in PC12 cells provided a better understanding of FGFR1 signalling. In these studies, to avoid endogenous FGFR, variant PC12 cell lines expressing low levels of FGFR were used. However, comparisons between normal PC12 cells and these variants can be precarious because the complete description of other deficits that could affect growth factor responses are unknown. Of course PC12 cells are not the only cell line used to study FGF-mediated signalling. A variety of cell lines have been used to study mitogenesis and in some cases differentiation induced by endogenous FGF receptors. Alternatively a cell line such as L6 myoblasts, or BaF3 cells, which do not express FGFR, can be used to study transfected FGFR1. Unfortunately these cells may lack the complement of factors necessary for normal signal transduction mediated by the ligand of interest. The PFR1 chimera allows one to stimulate FGFR1 kinase activity in PC12 cells, while avoiding the activation of endogenous FGFR, where the cells are otherwise intact and known to be responsive.

Differentiation of PC12 cells entails a complex series of events involving growth arrest, sequential activation of specific genes, and neuronal differentiation that are initiated upon exposure to NGF or FGF. Previous experiments have shown that chimeras of PDGFR and TrkA, the NGF receptor, would function in the same manner as the native receptors. The results presented establish that this is true for FGFR1 chimeras, too.

Several stable transfected PC12 clones have been identified which express a range of PFR1. The level of receptor

![Figure 2](image-url)
Figure 3  Protein tyrosine phosphorylation induced by the activation of PFR1 and fibroblast growth factor receptor FGFR1. Native rat phaeochromocytoma (PC12) cells, or those expressing PFR1, were incubated at 37°C for 5 min in the presence or absence of platelet-derived growth factor (PDGF; 30 ng/mL (a)) or fibroblast growth factor (bFGF; 10 ng/mL (b)). One hundred µg of cell lysate was subjected to 7.5% SDS-PAGE and immunoblotting analysis with an anti-phosphotyrosine antibody (αPY), as indicated. The migration of molecular mass standards (in kDa) is shown on the left. Arrows indicate proteins whose phosphorylation is detected after ligand addition.

Figure 4  Time course of protein tyrosine phosphorylation induced by platelet-derived growth factor (PDGF) in PFR1 cells and fibroblast growth factor (bFGF) in native rat phaeochromocytoma (PC12) cells. (a) PC12 cells expressing PFR1 were incubated at 37°C with 30 ng/mL PDGF for 1 min, 5 min, 30 min, 2 h, 4 h, and 16 h. Twenty-five µg of cell lysate was subjected to 7.5% SDS-PAGE and western blot analysis with anti-phosphotyrosine antibody. (b) PC12 cells were incubated at 37°C with 10 ng/mL bFGF for 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, and 12 h. Twenty-five µg of cell lysate was subjected to 7.5% SDS-PAGE and western blot analysis with anti-phosphotyrosine antibody.
expression correlates with an increase in the rate of neurite expression and intensity of response. In the clones expressing the highest amounts of receptors, up to 80% of the cells have neurites within 8 h. Correspondingly these clones have the highest amount of background neurite formation, presumably due to ligand-independent activation of PFR1. A PC12 cell line overexpressing the TrkA receptor also displays a similar rapid rate of differentiation in response to NGF. In contrast, normal PC12 cells require 2 days or more to form similar levels of neurites in response to FGF2.

The variable levels of PFR1 expression in stably transfected PC12 cells arise from a change in the protocol used to prepare the retroviral vectors. In the new procedure the preparation of helper virus in the PA317 cell line was omitted. Viruses subsequently prepared in the GP+e86 cells that employed this step were of much higher titre and invariably lead to cells with very high levels of expression (in the order of 500,000 copies/cell; RA Bradshaw, unpubl. data). The new protocol produces a range of clones and is considerably more useful for subsequent experiments.

The functionality of the PFR1 chimera helps to clarify two points. First, the ability of PFR1 to efficiently differentiate PC12 cells indicates that heparin sulphates are not necessary for internal signalling events. Fibroblast growth factor requires heparin sulphates to act as accessory molecules to facilitate ligand-receptor binding. In PC12 cells, the addition of heparin sulphates increases the ability of a given amount of FGF to cause differentiation. Second, it is not necessary for FGF1 or FGF2 to be internalized to cause this response, although it cannot be ruled out that receptor internalization is necessary. The use of PDGF-BB as ligand precludes the necessity for FGF in FGFR1 signalling.

Phaeochromocytoma 12 cells characteristically undergo one round of division prior to cell cycle arrest and initiation of subsequent morphological changes which define the differentiated state. Interestingly, in PFR1 overexpressing cells, the time course of ERK activation remains similar to normal PC12 cells, but the quantity of phosphorylated ERK is higher. Indeed, in normal PC12 cells, phosphorylation of ERK and its translocation to the nucleus precedes morphological differentiation. In PC12 cells overexpressing PFR1, a significant percentage of cells have differentiated even before ERK phosphorylation subsides. While it is not entirely clear why PFR1 can accelerate the process of dif-

Figure 5 Activation and association of signal transducers to platelet-derived growth factor (PDGF) in response to PDGF stimulation. (a) Rat phaeochromocytoma (PC12) cells expressing PFR1 were incubated at 37°C with 30 ng/mL PDGF for 5 min. One thousand µg of cell lysate was incubated with PDGFR antibody, collected by Protein A-Sepharose and subjected to 7.5% SDS-PAGE and western blot analysis. After probing, the blot was stripped and reprobed as indicated. Panel 1, blot probed with anti-phosphotyrosine antibody; panel 2, blot probed with PDGFR antibody; panel 3, blot probed with PLCγ antibody. (b) PC12 cells expressing PFR1 were incubated at 37°C with 30 ng/mL PDGF for 5 min. One thousand µg of cell lysate was incubated with Shc antisera, collected by Protein A-Sepharose and subjected to 10% SDS-PAGE and western blot analysis. Panel 1, blot probed with anti-phosphotyrosine antibody; panel 2, blot probed with Grb2 antibody.
Stimulation of the chimeric receptor results in the autophosphorylation of tyrosine residues which can act as binding sites for signal transduction proteins which have SH2 or PTB domains. Several tyrosine residues on the human FGFR1 have been identified both in vitro and in vivo to be phosphorylated. Some of these residues are required for catalytic activity while others provide docking sites for signalling proteins. The crystal structure of human FGFR1 kinase domain reveals a novel auto-inhibitory mechanism and illustrates the role of specific amino acids in catalysis. The phosphorylation of tyrosine residues 653 and 654, located near the catalytic pocket, is essential for kinase activity. Phosphorylation of tyrosine 766 is necessary for PLCγ docking. The Y766F mutant FGFR1 is able to mediate a mitogenic signal and allows neurite outgrowth when expressed in PC12 cells.

Until recently, other than PLCγ, no direct interaction between FGFR1 and a signal transduction molecule had been identified. The adaptor protein FRS2 becomes strongly tyrosine phosphorylated in response to FGF in a variety of cell lines, associates with FGFR1 and is essential for signalling. The demonstration of the PFR1/FRS2/Grb2/SOS complex provides evidence of a direct link between the receptor and the activation of the Ras/MAPK pathway. (Fig. 7). The PFR1 chimera has proven to be an extremely useful tool in elucidating the signalling events necessary for PC12 differentiation. The sequence similarity of the FGF receptors and the fact that several FGFR are expressed in PC12 cells underscores the importance of using chimeras in this cell system. The stably transfected clones expressing various levels of chimera will allow us to in-
vestigate the qualitative and quantitative differences in signal transduction events necessary for timely and efficient neurite formation.

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References


