Point Mutation in the Fibroblast Growth Factor Receptor Eliminates Phosphatidylinositol Hydrolysis without Affecting Neuronal Differentiation of PC12 Cells*

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Fibroblast growth factors (FGF) stimulate growth arrest and differentiation in rat pheochromocytoma PC12 cells. We examined the role of phosphatidylinositol (PI) hydrolysis in FGF-induced differentiation of PC12 cells by exploring the biological and biochemical activity of a mutant FGF receptor 1 (fg) defective in stimulation of PI hydrolysis. We show that point mutation at Tyr-766 (Y766F) of the FGF receptor prevents tyrosine phosphorylation of phospholipase Cγ and eliminates acidic FGF (aFGF)-induced stimulation of PI hydrolysis in PC12 cells. Treatment of PC12 cells expressing either wild-type or the Y766F mutant with aFGF led to tyrosine phosphorylation of Shc, the association of Shc with GRB2, a shift in the electrophoretic mobility of the Ras guanine nucleotide-releasing factor, Sos (son of sevenless), and enhancement in mitogen-activated protein kinase phosphorylation. Moreover, stimulation with aFGF led to a typical neurite outgrowth of PC12 cells expressing either wild-type or the Y766F FGF receptor mutant. These experiments indicate that PI hydrolysis is not essential for FGF-induced neuronal differentiation of PC12 cells. Moreover, the aFGF-induced Ras signaling pathway, which is essential for PC12 cell differentiation, is not affected by elimination of PI hydrolysis.

Acidic or basic fibroblast growth factors (FGF) and nerve growth factors (NGF) act by binding to cell surface receptors with tyrosine kinase activity (reviewed in Schlessinger and Ullrich, 1992). Ligand binding results in receptor activation leading to a variety of biological responses. Exposure of the rat PC12 pheochromocytoma cell line to acidic FGF (aFGF), basic FGF, or NGF induces growth arrest and a dramatic change in cell morphology. PC12 cells, which are round and have an adenoviral medullary chromaffin-like phenotype, respond to FGFs and NGF by growing long neurite extensions (Greene and Tischler, 1976; Togari et al., 1983, 1985; Rydel and Green, 1987). These cells serve as a useful model system for studying neuronal differentiation and signaling pathways of the FGF and NGF receptors.

Accumulating evidence suggests that Ras (reviewed in Satoh et al., 1992 and Polakis and McCormick, 1993), a 21-kDa GTP-binding protein, is activated by FGF and NGF and plays a crucial role in mediating FGF and NGF-induced differentiation of PC12 cells. Ras acts as a molecular switch (Satoh et al., 1992; Wittinghofer and Pai, 1991), which is active in the GTP-bound form and inactive in the GDP-bound form. The regulation of Ras activation involves guanine nucleotide releasing factors (Sos or GRF) and proteins that stimulate its intrinsic GTPase activity (Ras-GAP or NF-1). Treatment of PC12 cells with either FGF or NGF increases the amount of active, GTP-bound, Ras (Qi and Green, 1991; Li et al., 1992; Nakafuku et al., 1992). FGF- or NGF-induced Ras activation was shown to be obligatory for activation of a kinase cascade including Raf, mitogen-activated protein kinase kinase (MAPKK), and mitogen-activated protein kinase (MAPK) (Wood et al., 1992; Thomas et al., 1992; Robbins et al., 1992), which culminates in the phosphorylation of nuclear transcription factors (Pulverer et al., 1991; Marais et al., 1993; Hunter and Karin, 1992). The central role of Ras in PC12 cell differentiation is demonstrated by the ability of oncogenic Ras mutants to stimulate neurite outgrowth (Noda et al., 1985; Bar-Sagi and Feramisco, 1985; Satoh et al., 1987). Moreover, a dominant negative mutant of Ras or anti-Ras antibodies prevents differentiation induced by either FGF or NGF (Hugag et al., 1986; Szeberenyi et al., 1990).

GRB2 is a small SH2- (src homology) and SH3-containing adaptor protein that links both receptor and nonreceptor tyrosine kinases to the Ras-signaling pathway (Lowenstein et al., 1992). GRB2 binds to tyrosine-phosphorylated epidermal growth factor receptor through its SH2 domain (Lowenstein et al., 1992) and to the guanine nucleotide-releasing factor Sos through its SH3 domains (Li et al., 1993; Egan et al., 1993; Rozakiss-Adcock et al., 1993; Galle et al., 1993; Buday and Downward, 1993; Chardin et al., 1993; Simon et al., 1993; Olivier et al., 1993; Skolnik et al., 1993). GRB2 is also associated with the tyrosine-phosphorylated SH2 domain-containing adaptor protein Shc (Pellici et al., 1992). It has been shown that overexpression of Shc in PC12 cells leads to Ras-dependent neurite outgrowth of PC12 cells (Rozakiss-Adcock et al., 1992), suggesting that the interaction between Shc and GRB2 may be important in regulating Ras activation.

Treatment of target cells with FGF or NGF also stimulates tyrosine phosphorylation of phospholipase Cγ (Burgess et al., 1990; Vetter et al., 1991). Growth factor-induced phosphorylation of phospholipase Cγ on tyrosine residues enhances its catalytic activity (Nishibe et al., 1990) and is required for phospholipase Cγ activation in intact cells (Kim et al., 1991). Phospholipase Cγ hydrolyzes phosphatidylinositol (4, 5) bisphosphate to diacylglycerol, which activates protein kinase C, and inositol trisphosphate, which releases Ca2+ from intracellular stores. The demonstration that phospholipase Cγ is a substrate for the FGF and NGF receptors, and the stimulation of PI hydrolysis by NGF in PC12 cells (Traynor et al., 1982; Contreras and Guroff, 1987; Albin and Bradshaw, 1990; Pessin 14419
et al., 1991), suggests that PI hydrolysis may also be involved in PC12 cell differentiation.

Activation of the Ras-signaling pathway and PI hydrolysis has also been implicated in differentiation and development of *Xenopus laevis* embryos. Moreover, FGF is essential for mesoderm induction during early development of the *Xenopus* embryo (Slack et al., 1987). Microinjection of messenger RNA encoding a dominant inhibitory mutant of Ras into *Xenopus* eggs blocked FGF-induced mesoderm induction (Whitman and Melton, 1992), suggesting that Ras is involved in *Xenopus* mesoderm induction. The normal embryonal development can also be distorted by treatment with Li\(^+\), an inhibitor of PI hydrolysis, which results in deformed dorsal embryos (reviewed in Berridge et al. (1989) and Berridge (1993)). It is thought that the teratogenic effect of Li\(^+\) on developmental processes in *Xenopus* embryos is mediated by inhibition of inositol formation (Busa and Gimlich, 1989; Maslanski et al., 1992), which is required for the regeneration of phosphatidylinositol (4, 5) bisphosphate. Hence, PI hydrolysis may play a role in amphibian embryonic mesoderm induction.

We have previously shown that Tyr-766 in the carboxy-terminal tail of FGFR 1 (flg) functions as a high affinity binding site for the SH2 domain of phospholipase C\(\gamma\) (Mohammadi et al., 1991). The elimination of Tyr-766 by site-directed mutagenesis prevented both the association of phospholipase C\(\gamma\) with FGF and the tyrosine phosphorylation of phospholipase C\(\gamma\), leading to the elimination of FGF-induced PI hydrolysis and Ca\(^{2+}\) release (Mohammadi et al., 1992; Peters et al., 1992). However, the Y766F FGFR mutant was able to stimulate DNA synthesis in transfected L-6 myoblasts, indicating that PI hydrolysis is not essential for FGF-induced mitogenesis. These results are in agreement with others (Valius et al., 1993; Seedorf et al., 1992; Rönström et al., 1992) in demonstrating the lack of requirement for PI hydrolysis in the mitogenic signaling pathways of other growth factor receptors.

To shed light on the role of PI hydrolysis in the control of PC12 cell differentiation, we have tested the ability of the Y766F FGFR mutant defective in stimulation of PI hydrolysis to elicit the differentiation of PC12 cells in response to aFGF. Here, we show that PC12 cells expressing either wild-type or the Y766F FGF receptors undergo aFGF-dependent neuronal differentiation by displaying typical dense neurite-like processes within 16–20 h. Furthermore, we show that the Ras-signaling pathway is maintained in the absence of PI hydrolysis and that PI hydrolysis is not essential for FGF-induced differentiation of PC12 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—PC12 cells were kindly provided by Dr. M. V. Chao at Cornell Medical Center, New York. *Escherichia coli* expressing recombinant human aFGF, anti-flg 1C, and anti-flg 3B antibodies was previously characterized (Mohammadi et al., 1991; Jaye et al., 1988). Heparim was from Elkins-Sinn, Inc. The following rabbit polyclonal antibodies were generated in our laboratory: anti-phosphotyrosine (anti-P-Tyr) antibodies, anti-phospholipase C\(\gamma\) antibodies raised against a synthetic peptide (residues 1266–1274) from the COOH terminus of phospholipase C\(\gamma\), anti-Grb2 antibodies raised against a glutathione S-transferase fusion protein containing the N-SH3 domain of Grb2, and anti-Sos directed against the catalytic domain of human Sos1. Rabbit polyclonal anti-Shc antibodies are directed against a glutathione S-transferase-SH2 fusion protein (Pelici et al., 1995) (\(^{125}\)I)-carrier-free and \(^{125}\)I-lymophoinositol were purchased from DuPont NEN. The cDNAs and expression vectors encoding wild-type and Y766F FGF receptor mutant (flg) receptors have been described elsewhere (Mohammadi et al., 1991, 1992).

**Generation of Cell Lines**—Parental rat PC12 cells were cotransfected by calcium-phosphate precipitation (Wigler et al., 1979) with 30 pg of cDNA encoding either wild-type human FGF receptor (flg) or the Y766F mutant (Mohammadi et al., 1991, 1992) and 0.5 pg of a selective plasmid carrying a neomycin resistance marker. Clones generated in medium containing Geneticin (G418) were screened for expression of FGF receptor constructs by using two rabbit anti-peptide antibodies. Rabbit anti-flg 3B antibodies directed against the flg kinase insert recognize both rat flg and the transfected human wild-type and mutant receptors. Rabbit anti-flg 1C antibodies directed against the COOH-terminal tail of flg recognize the human but not the rat receptor. Following binding studies of \(^{125}\)I-aFGF (iodinated with chloramine T (Greenwood et al., 1963)) to the cell lines and Scatchard analysis of the binding data, a clone of PC12 cells expressing 2 x 10\(^6\) wild-type FGF receptors/cell and one expressing 1.2 x 10\(^6\) Y766F mutant receptors/cell was chosen for further analysis.

**Immunoprecipitation and Western Blotting Analysis**—Transfected PC12 cells were treated with aFGF, lysed, and subjected to immunoprecipitation and immunoblotting analysis according to published procedures (Mohammadi et al., 1992).

**Phosphatidylinositol Hydrolysis**—Phosphatidylinositol hydrolysis stimulated by FGF in PC12 cells was analyzed according to published procedures (Margolis et al., 1990).

**Differential of PC12 Cells**—Cloned PC12 cells were seeded in 10-cm tissue culture plates at a density of ~2 x 10\(^5\) cells/plate. The cells were incubated in complete medium (Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 10% horse serum, 1% glutamine, and 1% streptomycin and penicillin) to which aFGF (50 ng/ml) and heparin (50 pg/ml) were added. Differentiation was observed within 16–20 h.

**RESULTS AND DISCUSSION**

Parental PC12 cells expressing approximately 8,000–10,000 FGF/cell were transfected with mammalian expression vectors, which direct the synthesis of either wild-type FGF or the Y766F FGF mutant (Mohammadi et al. 1992). Binding studies with \(^{125}\)I-labeled aFGF indicated that the transfected PC12 cells express approximately 2 x 10\(^5\) wild-type receptors or 1.2 x 10\(^5\) Y766F mutant receptors/cell, with a dissociation constant of approximately 0.1 nm (data not shown). The transfected cells were treated with aFGF, solubilized, and subjected to immunoprecipitation with anti-FGF antibodies followed by immunoblotting with either anti-FGF or anti-P-Tyr antibodies. The results presented in Fig. 1 show that both wild-type and the Y766F mutant FGF receptors undergo typical ligand-dependent tyrosine autophosphorylation. Stronger autophosphorylation of wild-type FGF receptor was observed as compared with the mutant receptor due to elimination of Tyr-766, which is a major autophosphorylation site of the FGF receptor (Fig. 1B) (Mohammadi et al., 1991, 1992).

We next compared the ability of the wild-type receptor and the Y766F receptor mutant to stimulate tyrosine phosphorylation of phospholipase C\(\gamma\). Following ligand stimulation PC12 cells were lysed, subjected to immunoprecipitation with anti-phospholipase C\(\gamma\) antibodies, and immunoblotted with either...
FIG. 1. Immunoblots were exposed for 18 h at -70 °C.

Fig. 2. Point mutation of Tyr-766 of the FGF receptor inhibits association with and tyrosine phosphorylation of phospholipase Cγ. PC12 cells expressing wild-type (wt) or mutant FGF receptors were stimulated with aFGF, lysed, and immunoprecipitated as described under "Experimental Procedures." A, anti-phospholipase Cγ (PLC-γ) immunoblotting of anti-phospholipase Cγ immunoprecipitates. B, anti-P-Tyr immunoblotting of anti-phospholipase Cγ immunoprecipitates. C, anti-phospholipase Cγ immunoblotting of anti-FGFR (fig 3B) immunoprecipitates. Immunoblottings were performed as described for Fig. 1. Immunoblots were exposed for 18 h at -70 °C.

Fig. 3. The Y766F mutant is unable to stimulate PI hydrolysis in PC12 cells. Parental PC12 cells and cells transfected with wild-type or mutant Y766F FGF receptors were incubated for 24 h in the presence of [3H]inositol in serum-depleted medium (Dulbecco's modified Eagle's medium). Cells were stimulated with FGF, and inositol phosphate formation of aFGF-stimulated cells over control non-stimulated cells. Error bars mark standard error.

Fig. 4. Tyrosine phosphorylation of Shc, its association with GRB2, and mobility shift of Sos in response to aFGF stimulation of wild-type (wt) or Y766F FGF receptors. Parental and PC12 cells transfected with either wild-type or Y766F FGF receptors were incubated overnight in serum-depleted medium and stimulated with aFGF. Treated (+) and nontreated (-) cells were lysed, immunoprecipitated with anti-GRB2 antibodies, and immunoblotted with anti-Sos antibodies (A), anti-Shc antibodies (B), or immunoprecipitated with anti-She antibodies (B) and blotted with anti-P-Tyr antibodies. Immunoblot of panel A was exposed for 18 h at -70 °C, and panels B and C were exposed for 15 h.

Fig. 5. FGF induces tyrosine phosphorylation of mitogen-activated protein kinases (MAPK) in PC12 cells transfected with either wild-type (wt) or Y766F FGF receptors. Parental and PC12 cells transfected with either wild-type or Y766F FGF receptors were grown overnight in serum-depleted medium. Control starved cells (−) or cells treated with aFGF (+) were lysed and immunoprecipitated with anti-P-Tyr antibodies. The samples were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with polyclonal anti-microtubule-associated protein kinase antibodies, which detected two enzyme isoforms (42- and 44-kDa proteins). Immunoblots were exposed for 16 h at -70 °C.

anti-FGFR or anti-P-Tyr antibodies. The results presented in Fig. 2 show that aFGF stimulation led to association between wild-type FGF receptor and phospholipase Cγ and tyrosine phosphorylation of phospholipase Cγ (Fig. 2, B and C). However, the Y766F FGFR mutant was unable to associate with or tyrosine-phosphorylate phospholipase Cγ (Fig. 2, B and C). Measurements of PI hydrolysis in Y766F-expressing cells indicated that the Y766F mutation abrogated aFGF-induced PI turnover (Fig. 3).

To examine the events that lead to Ras activation upon binding of aFGF to the wild-type receptor or the Y766F mutant, PC12 cells expressing either wild-type or Y766F FGF were stimulated with FGF, immunoprecipitated with anti-Ras antibodies and blotted with either anti-Sos or anti-Shc antibodies, or immunoprecipitated with anti-Shc antibodies and blotted with anti-P-Tyr antibodies. Following aFGF treatment, both wild-type and Y766F FGF receptors induced tyrosine phosphorylation of She (Fig. 4C) and its association with GRB2 (Fig. 4B). However, the FGF could not be detected in the GRB2-Shc immunoprecipitates (data not shown), suggesting that GRB2 and Shc do not associate with the FGF or that the association is very weak. In this regard, FGF, NGF, and EGF receptors exhibit differential interaction with the adaptors proteins GRB2 and Shc. The FGF does not bind either GRB2 or Shc; the NGFR binds Shc (Obermeier et al., 1993) but not GRB2 (Suen et al., 1993), while the epidermal growth factor receptor binds both GRB2 (Lowenstein et al., 1992) and She (Pelicci et al.,...
FIG. 6. FGF-induced PI hydrolysis is not required for differentiation of PC12 cells. Parental PC12 cells (panels 1 and 2) and PC12 cells expressing either wild-type FGF receptors (panels 3 and 4) or the Y766F mutant (panels 5 and 6) were grown overnight in complete growth medium (control, panels 1, 3, and 5) or in medium supplemented with aFGF and heparin (panels 2, 4, and 6). Neurite-like structures were observed within 16–20 h of the induction with aFGF and heparin.

To investigate whether PI hydrolysis is required for FGF-induced differentiation of PC12 cells, control, wild-type, and Y766F-expressing PC12 cell lines were stimulated with aFGF, and neurite outgrowth was assessed 16–20 h later. Long and dense neurites were observed upon treatment with aFGF in PC12 clones expressing either wild-type or the Y766F mutant (Fig. 6, panels 1, 2, 4, and 6), whereas no neurites were observed in the parental PC12 cells (Fig. 6, panels 1 and 2). Thus, elimination of aFGF-induced PI hydrolysis does not affect PC12 cell differentiation.

It was proposed that PI hydrolysis plays a role in cell differentiation based on the observation that Lith disrupts both axis determination and mesoderm induction in the developing Xenopus embryo (Busa and Gimlich, 1989; Berridge et al., 1989), an effect that is reversed by addition of exogenous myoinositol (Maslanski et al., 1992). We assume that the difference between these studies and ours, regarding the requirement for PI hydrolysis, reflect the very different models examined. Our study employs PC12 cells, which are a cloned, uniform population of cells that respond to aFGF treatment by extension of neurites. In contrast, the Xenopus embryo contains a variety of cells with different responses to growth factors, including changes in cell shape, and cell mobility. Therefore, the role of PI hydrolysis may not be directly comparable in these two very different systems. Alternatively, it is also possible that Lith has other effects in Xenopus that are not related to PI hydrolysis.

Our results indicate that the Y766F FGFR mutant activates the Ras-signaling pathway in a manner indistinguishable from activation by wild-type FGFR. Thus, the functional significance of activation of phospholipase Cγ and stimulation of PI hydrolysis by wild-type FGFR in PC12 cells is unknown. Both the activated wild-type and Y766F mutant FGF receptors are able to stimulate phosphorylation of Shc on tyrosine residues, the association of Shc with GRB2, and to induce mobility shift of Sos (Fig. 4). Moreover, both receptors are able to activate microtubule-associated protein kinase (Fig. 5), indicating that the Ras-signaling pathway is also activated by the Y766F receptor mutant. In contrast to PI hydrolysis, Ras-dependent signals appear to be essential for FGF- (and NGF-) induced differentiation of PC12 cells (Hagag et al., 1986; Széberenyi et al., 1990). However, other growth factors such as EGF, insulin, and IGF-1 also activate Ras in PC12 cells without inducing differentiation (Nakafuku et al., 1992; Qiu and Green, 1991), indicating that activation of Ras is essential for both mitogenesis and differentiation. Recent studies demonstrate that growth factors that promote neuronal differentiation of PC12 cells (e.g., FGF, NGF, and PDGF) lead to persistent elevation of mitogen-activated protein kinase activity and its translocation to the nucleus, while mitogenic growth factors (e.g., EGF and IGF-1) activate mitogen-activated protein kinase transiently (Chen et al., 1992; Qiu and Green, 1992; Heasley and Johnson, 1992; Nguyen et al., 1993; Lenormand et al., 1993; Gonzalez et al., 1993). Moreover, treatment of PC12 cells overexpressing epidermal growth factor receptor or insulin receptor with either EGF or insulin leads to typical neuronal differentiation. It is therefore thought that receptor number, duration, and amplitude of activation and the cellular localization of target proteins may also influence the cellular response of PC12 cells to a given growth factor. Full understanding of the pathways involved in FGF-induced differentiation of PC12 cells will require a thorough analysis and characterization of additional receptor mutants and their interaction with signaling proteins.

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