Fibroblast Growth Factor Receptor 3 Induces Gene Expression Primarily through Ras-independent Signal Transduction Pathways

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The fibroblast growth factors (FGFs)1 play roles in development, angiogenesis, wound healing, and tumorigenesis (reviewed in Ref. 1). FGF actions are mediated by activation of FGF receptor (FGFR) tyrosine kinases. FGFRs are a gene family of four members, termed FGFR-1–4. These receptors are widely expressed in many tissues and different cell types, and the temporal expression of the receptors and their ligands is regulated during development (reviewed in Ref. 2). Analysis of naturally occurring mutations in these receptors has indicated that they control the differentiation of specific cell types during development. Point mutations within the genes encoding human FGFR-1, -2, or -3 cause different syndromes that involve bone development (reviewed in Refs. 3 and 4) and some of these syndromes (Apert's and thanatophoric dysplasia) may also manifest effects in the central nervous system. Point mutations in FGFR-3 that cause activation of its tyrosine kinase activity have been shown to be responsible for the commonest form of dwarfism in humans (4–7). Recently FGFR-3 has also been implicated in multiple myeloma, where its abnormal overexpression because of a chromosomal translocation has been detected in ~25% of cases (8, 9). However, it is not clear what role this expression contributes to the phenotype of this disease. Studies analyzing the consequences of null mutations in FGFRs in mice also implicated these receptors as playing a role in development. The knockout of either FGFR-1 or FGFR-2 (10, 11) in mice resulted in embryonic lethality, whereas that of FGFR-3 was nonlethal. The FGFR-3-deficient mice developed an overgrowth of the long bones and abnormal curvature of the spine and tail (12, 13) and were deaf (12).

The FGFRs are very similar in structure. In particular, their tyrosine kinase domains are highly conserved, and overlapping subsets of ligands induce their activation. Regulation appears to take place at two different levels. Temporal control of the expression of both ligands and receptors is an important mechanism for regulating signal transduction during development. In addition, the receptors seem to have differing signaling capabilities. Studies have indicated that FGFR-1 is much better at producing mitogenic signals than either FGFR-3 or FGFR-4 when assayed in BaF3 cells (14–16). We have demonstrated that there is also a difference between FGFR-1 and -3 in their abilities to induce neurite outgrowth in PC12 cells when activated by FGF-1 (17, 18). FGFR-3 can barely induce neurite outgrowth, whereas activation of FGFR-1 induces rapid and robust neurite outgrowth. In the BaF3 and PC12 cell systems it appears that sustained signals that lead to the Ras-dependent activation of the extracellular regulated kinases are necessary for the biological phenotypes observed.

The above results imply that FGFR-3 is not able to induce strong sustained signals. However, in vivo it obviously plays key roles in development. There are two potential nonexclusive hypotheses that could explain these observations. The threshold for signaling in these developmental tissues may be low,
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and sustained signals may not be mandatory. Alternatively, FGFR-3 may induce physiologically relevant and strong signals using signaling pathways that are distinct from the Ras-extra-cellular regulated kinase pathway. In this study we have chosen to examine this latter possibility. The nature of FGFR-3 signaling and the downstream targets are still not well understood and are the subject of intense investigation. To establish the signaling capabilities of FGFR-3 and to determine the nature of FGFR-3 induced signals, we took advantage of the PC12 cell system in which either NGFR or FGFR-1 can induce the elaboration of a neuronal phenotype. Underlying the growth factor-induced acquisition of neuronal phenotype are a variety of signaling pathways and gene expression changes that lead to the specific neuronal traits. Prominent neuronal traits are mediated by both Ras-dependent and Ras-independent signaling pathways. For example, gene expression events leading to morphological differentiation (neurite outgrowth) are predominantly mediated via a Ras-dependent mitogen-activated protein kinase pathway, whereas the establishment of a sodium based action potential is mediated by the expression of voltage-dependent sodium channel genes in a Ras-independent manner. Normal PC12 cells express both FGFR-1 and FGFR-3, and both of these receptors can be activated by FGF-1. This makes it difficult to distinguish pathways activated by FGF-1 from those activated by FGFR-3. However, a variant PC12 cell line, termed fnr-PC12, exists that has lost the expression of functional FGFR-1 but has retained functional FGFR-3 (17, 18). The use of these cells allowed us to assay the ability of FGFR-3 to activate several distinct signal transduction pathways that are important for the development of the neuronal phenotype. In this paper we report that although FGFR-3 is not capable of inducing sustained activation of Ras-dependent pathways, it is as capable of inducing the activation of Ras-independent pathways to levels equivalent to those seen with the activation of FGFR-1.

MATERIALS AND METHODS

Cell Culture—PC12, 17N-2 PC12, fnr-PC12 cells, and fnr-PC12-derived transfectant lines have been described previously (17, 18). Cells were grown on tissue culture dishes in Dulbecco’s modified Eagle’s medium supplemented with 10% donor horse serum, 5% fetal bovine serum, and 1% penicillin/streptomycin in an atmosphere of 10% CO2 at 37 °C. Recombinant human NGF was used at a final concentration of 50–100 ng/ml together with heparin (50 mU/ml). The blots were washed twice in 2× SSC-0.1% SDS at 68 °C and twice in 0.2% SSC, 0.1% SDS for 20 min at 68 °C. Levels of radiolabeled probe bound to the blot were determined by PhosphorImager (Molecular Dynamics) analysis, and all values were normalized to the level corresponding to cyclophilin mRNA.

Sodium Current Recordings—Whole cell recordings of PC12 sodium current were made by means of an Axopatch 200A amplifier (Axon Instruments Inc., Burlingame, CA). The recording solution contained 140 mM NaMES, 0.2 mM MgCl2, 0.2 mM CaCl2, 10 mM NaHEPES, pH 7.2. The pipette solution contained 140 mM CsMES, 10 mM CsEGTA, 10 mM CsCl, 2 mM HEPES, pH 7.2. To record sodium currents the cells were held at -100mV and stepped to positive potentials for 20 ms. PC12 cell sodium currents were digitized at 50 kHz and analyzed off-line using HEKA Pulse & Pulse Fit software (Instrutech, Great Neck, NY). Capacitive transients were compensated using a combination of manual compensation on the amplifier and further processing using either a P/4 or P/10 leak subtraction protocol.

RESULTS

In this study we wanted to compare the abilities of FGFR3 to activate various signal transduction pathways in PC12 cells. We were particularly interested in comparing pathways that had been shown previously to be either activated in a Ras-de-
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Receptor allowed us to eliminate differences in signaling that could be attributed to FGF-1 binding because both of the transfected receptors have the same FGF-binding domains. We have shown previously that these two transfected cell lines overexpress equivalent amounts of receptors (17). The use of these cells eliminates the possibility that any differences in signaling can be attributed to receptor number. By assaying for FGF-1-induced signals in the fnr-PC12 cells, we will be able to identify signaling pathways that FGFR-3 can activate. Then, by comparing FGF-1-induced signals in the two transfected cell lines, we can compare the efficiency with which FGFR-1 and FGFR-3 can activate these pathways.

We chose to look primarily at the induction of gene expression because our previous analysis had revealed that the initiation of signaling by FGFR-1 and -3 was similar (17, 18). However, the downstream consequences that require robust gene expression were dramatically different. To initiate these studies, we chose to look at the induction of the gene transin by FGF-1. Transin is induced via a Ras-dependent signaling pathway (20). Therefore, its induction would serve as a control to establish that using induction of a Ras-dependent gene I would also reveal the differing signaling capabilities of FGFR-1 and -3. Fig. 2 shows analysis of the induction of transin mRNA using Northern blot analysis. In the fnr-PC12 cells there was a barely detectable induction of transin mRNA by FGF-1. In contrast, treatment of the fnr-PC12 cells with NGF did induce transin mRNA expression (data not shown), indicating that the signaling pathway to transin mRNA induction is intact in these cells. In the FGFR-3b cell line that overexpresses FGFR-3, transin mRNA induction was now detected after 72 h of treatment with FGF-1. However, analysis of the FGFR-3b cell line revealed that this receptor could induce much higher levels of transin mRNA after 72 h (Fig. 2). Quantitation of the amount of mRNA present under the different conditions demonstrated that the level of induction of transin mRNA at the 72 h time point was ~15-fold higher in the cells that expressed the FGFR-3b chimeric receptor in comparison with the FGFR-3b cells. As a loading control the levels of cyclophilin mRNA was also measured, and this demonstrated that similar amounts of RNA were loaded in each lane (Fig. 2). Together with the lack of induction seen in the fnr-PC12 cells (Fig. 3), these results indicate that FGFR-3 is much less efficient than FGFR-1 at inducing this Ras-dependent pathway. These observations confirm our previous results and validate this approach to show potential differences between signaling by FGFR-1 and -3.

The ability of FGFR-3 to activate pathways that are known to be Ras-independent was examined next. The activation of the Type II sodium channel gene has been shown to occur through a pathway that is independent of Ras. Fig. 3 shows the

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**Fig. 1.** FGF-1 induced signal transduction in PC12 cells expressing a dominant-interfering mutant of Ras. A. 17N-2 PC12 cells were treated with 50 ng/ml FGF-1 for 48 h. Total cellular RNA (10 μg) was electrophoresed through 0.8% agarose gels and transferred onto a nylon membrane. The blot was hybridized with a probe specific for type II sodium channel (top panel) or a probe specific for transin (middle panel) and with a probe specific for the internal control cyclophilin (bottom panel). B. 17N-2 PC12 cell lysates treated for 0, 24, or 72 h with FGF-1 were electrophoresed through a 7.5% SDS-polyacrylamide gel and transferred onto a nitrocellulose filter membrane. Blots were probed with anti-intermediate filament protein antibody and exposed to x-ray film as described under “Experimental Procedures.”

**Fig. 2.** Transin induction mediated by FGFR-1 and FGFR-3 receptors. Cells from the Fn-P12, FGFR-3b, or FGFR-3b cell lines were treated with 50 ng/ml of FGF-1 for 72 h. Total cellular RNA (10 μg) was electrophoresed through 0.8% agarose gels and transferred onto a nylon membrane. The blot was hybridized with a probe specific for transin (upper panel) and with a probe specific for the internal control (CON) cyclophilin (lower panel).
induction of mRNA encoding the type II sodium channel as measured by Northern blot analysis. Analysis of the induction of the Type II sodium channel in the fnr-PC12 cells after 60 h of treatment by FGF-1 demonstrates that FGF-1 could induce the increased expression of the type II sodium channel. As a control we also measured transin induction after 60 h of treatment with FGF-1 (Fig. 3). The data indicated that the endogenous FGFR-3 in the fnr-PC12 cells can induce type II sodium channel, albeit weakly. We compared the abilities of the overexpressed receptors to induced type II sodium channel mRNA. As can be seen in Fig. 3, FGFR-3 is as efficient as FGFR-1 in inducing the increased expression of type II sodium channel mRNA, whereas there is a major difference between these receptors in their abilities to induce the Ras-dependent transin mRNA.

We also measured the induction of functional channel expression by these receptors and the expression of the channels within the cells. Fig. 4A documents that the increase in mRNA levels of the type II sodium channel correlates well with an increase in channel protein levels. Immunofluorescence localization studies revealed a staining pattern indicative of localization of the channel proteins to the surface of the differentiated PC12 cells. To determine that the immunofluorescence corresponded to functional sodium channels, whole cell patch clamp recordings were performed. Recordings from FGF-treated FGFR-3b cells indicated large inward sodium currents when the cells were depolarized to positive membrane potentials (Fig. 4B). In recordings from eight FGF-treated FGFR-3b cells, all exhibited inward current, the overall average corresponding to 357pA. By contrast, in recordings from 11 control cells, only 4 exhibited inward sodium current. All sodium current in treated and nontreated cells was inhibited by addition of 1 μM tetrodotoxin, an inhibitor of voltage-dependent sodium channels.

We next looked at the induction of the protein peripherin. The expression of this protein is controlled through another Ras-independent pathway that in this case involves phospholipase Cy (PLCγ) activation (26).2 Analysis of mRNA levels in the fnr-PC12 cells by Northern blot demonstrated that both FGFR-1 and NGF induced peripherin to similar levels (data not shown). Comparison of the induction of peripherin in the cells overexpressing the two FGFRs showed that in both cases there was induction of mRNA to similar levels (Fig. 5A). Quantitation of the levels of induction indicated that FGFR-3b activation gave rise to a 5-fold increase in mRNA levels after 72 h, whereas the FGFR31b receptor induction was 4-fold. This indicates that FGFR-3 can activate this pathway as efficiently as FGFR-1. We also looked at the induction of the expression of peripherin at the protein level by Western blotting. Fig. 5B shows that FGF-1 can induce peripherin in the fnr-PC12 cells and that in the cells overexpressing the FGFR-3 receptor the induction of peripherin levels is increased significantly. This

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indicates that FGFR-1 and FGFR-3 are equally efficient at the induction of this PLCγ-dependent pathway.

The Thy-1 protein is a cell surface glycoprotein whose expression is induced by FGFR-1 treatment of PC12 cells via another distinct Ras-independent pathway that involves a Src-dependent branch point (Ref. 20 and below). Analysis of the induction of mRNA encoding for Thy-1 in the fpr-PC12 cells revealed that at the 72-h time point FGF-1 could induce a 3-fold increase in Thy-1 mRNA levels (Fig. 6A), and this was a similar level to that seen with NGF (data not shown). A time course of the induction of the expression of Thy-1 mRNA in the FGFR-3b and FGFR-3b overexpressing fpr-PC12 cells revealed that both of these receptors induced equivalent levels of Thy-1 mRNA (Fig. 6A). By 72 h FGFR-3b had induced a 7-fold increase and FGFR-3b had induced a 10-fold increase. Analysis of Thy-1 protein levels in the fpr-PC12 cells and the FGFR-3b overexpressing cells also demonstrated that FGFR-1 could induce Thy-1 protein expression and that this was greatly increased in the cells expressing more FGFR-3b (Fig. 6B). These data demonstrate that FGFR-3b can induce Thy-1 expression with similar efficiencies to FGFR-3b. To demonstrate that the induction of Thy-1 by FGFR-3b involves a Src family member we used the inhibitor, PP2. This class of inhibitor preferentially inhibits Src family member tyrosine kinases (27) and at the concentration used has very little effect on FGFR-3b kinase activation (data not shown). Fpr-PC12 cells expressing FGFR-3b were activated by FGF-1 either in the presence or absence of PP2 and the induction of Thy-1 monitored after 48 h by Western blotting. As can be seen in Fig. 6C, in comparison with no treatment or 10 min treatment Thy-1 protein levels after 48 h incubation were increased by the addition of FGFR-1 (lane 3). However, if the cells were treated with PP2 and stimulated with FGF-1, there was no induction of Thy-1 (lane 5). Thus, treatment of the cells with PP2 completely blocks the induction of Thy-1, demonstrating that the induction is via a Src family member-dependent pathway. These data are in agreement with earlier studies that indicated that Thy-1 induction by NGF was via a Src-dependent branch point (7).

Finally, we tested the ability of FGFR-3 to inhibit cell death following withdrawal of serum. As shown in Fig. 7A, withdrawal of serum from fpr-PC12 cells leads to cell death. Stimulation of the cells by NGF allows cell survival. When the cells were stimulated by FGF-1 there was some increase in cell survival, but it was not as efficient as NGF. This indicates that FGFR-3 can activate cell survival pathways but not as efficiently as the NGF receptor. To compare the abilities of FGFR-1 and FGFR-3 cytoplasmic domains to allow cell survival, we compare the fpr-PC12 cells overexpressing these two receptors. As shown in Fig. 7A these two receptors are equally able to induce cell survival when overexpressed. Similar results were also seen when cell survival was measured using annexin V staining (data not shown). Although it is not clear which pathways are important for regulating this effect, the activation of protein kinase B (also known as Akt), via the PI-3 kinase pathway has been shown to inhibit apoptosis in similar systems. Activation of Akt requires phosphorylation, and there are antibodies available that are directed against the phosphoryl-
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DISCUSSION

The cytoplasmic signaling domains of FGF receptors are highly conserved; yet data are accumulating that they do not all signal equivalently. A comparison of the ability of FGFR-1 and FGFR-3 to induce proliferation of the lymphoid cell line BaF3 or neurite outgrowth in PC12 cells indicated that only FGFR-1 was able to mediate these things efficiently (14–18). In these cell systems it appears that sustained and robust signals that lead to the Ras-dependent activation of the extracellular regulated kinases are necessary for the biological phenotype analyzed. This implied that FGFR-3 was not able to induce strong sustained signals. However, in vivo it obviously plays key roles in normal development and in several diseases. This indicated that either the threshold for signaling in these developmental tissues is low so that sustained signals are not mandatory or that FGFR-3 can induce physiologically relevant and strong signals using other signaling pathways. To determine whether the latter possibility was correct, we took advantage of the PC12 cell system in which the elaboration of a neuronal phenotype can be induced by FGF-1. This growth factor mediates changes in gene expression that underlie the induction of various neuronal phenotypic changes through both Ras-dependent and Ras-independent pathways. In this report we demonstrate that the FGFR-3 can in fact induce Ras-independent signaling pathways as efficiently as FGFR-1, and this induction leads to important neuronal traits. Thus FGFR-3 is capable of strong physiologically important signaling via Ras-independent pathways.

Previous analysis of activation of Ras-dependent pathways in PC12 cells indicated that FGFR-3 was unable to induce efficient gene expression through the Ras-dependent pathways (17, 18). Analysis of the ability of FGFR-3 to induce the Ras-dependent gene transin in this report confirms that FGFR-3 is very poor at inducing Ras-dependent genes. However, as we demonstrate in this report, FGFR-3 was clearly capable of inducing all the Ras-independent pathways that we analyzed equally as well as FGFR-1. These included the induction of the expression of the protein peripherin. The expression of this gene is induced through a pathway that requires PLCγ. The activation of PLCγ is accomplished via interaction with a tyrosine autophosphorylation site within the carboxyl terminus of the FGFRs. This site and its surrounding amino acid sequence are conserved between FGFR-1 and FGFR-3; therefore, it is perhaps not so surprising that both the receptors can induce the expression of genes via PLCγ-dependent pathways. Recent experiments using chimeric receptors that could be activated by platelet-derived growth factor also demonstrated that PLCγ was equally well phosphorylated by both FGFR-1 and -3 (28). These data agree with ours that the difference in signaling capabilities between these two receptors cannot simply be explained by differences in kinase activities.

The two other genes we analyzed for induction by FGFR-3, namely the type II sodium channel and Thy-1, are known to be induced via Ras-independent pathways. We found that FGFR-3 was as good as FGFR-1 in its ability to induce these two genes. The activation of cyclic AMP-dependent pathways has been implicated in sodium channel induction. Similarly, Thy-1 is activated in a Ras-independent manner by NGF, and this involves a novel branch point off a Src-dependent pathway. Our studies using the Src family member kinase inhibitor PP2 indicate that FGFR-3 activates Thy-1 expression via a Src-dependent branch point. We also compared the abilities of FGFR-1 and FGFR-3 to inhibit cell death and to activate the protein tyrosine kinase Akt. Again we found that FGFR-3 was equally efficient as FGFR-1 in inhibiting cell death and activation Akt. Therefore, in clear contrast to its inability to activate Ras-dependent pathways, it is clear that FGFR-3 can activate all of the Ras-independent pathways we analyzed equally as well as FGFR-1.

In our analysis we did not identify an FGFR-3-specific signal transduction pathway, in that FGFR-1 was found to also be capable of activating all of the pathways we analyzed. Recently, FGFR-3 has been shown to be able to activate the protein Stat-1, and this is a candidate pathway that may be specific to FGFR-3 among the FGFR family (29, 30). This activation appeared to be cell type-specific because it was only clearly shown in chondrocytes. PC12 cells express high levels of Stat-1; however, we and others were unable to demonstrate activation of Stat-1 by either FGFR-1 or FGFR-3 in the overexpressing cells (30). This indicates that the activation of Stat-1 by FGFR-3

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may involve cell type-specific adaptor proteins that are absent from PC12 cells. Thus, signaling through FGFR-3, in addition to occurring primarily via Ras-independent pathways, may also involve the use of specific proteins to expand the repertoire of the signaling cascades. The unique ability to signal primarily through Ras-independent pathways may explain why activating mutations in FGFR-3 only have penetrance in certain tissues. This may involve the activation of specific pathways that are not easily duplicated by other receptors and may also involve additional cellular proteins that are only expressed in the tissues affected. The unique signaling ability may also explain why only FGFR-3 of the four family members is overexpressed in multiple myeloma cells.

In summary, we have demonstrated that FGFR-3 can induce the equivalent activation of various Ras-independent pathways to FGFR-1. In contrast, as shown previously FGFR-3 could not induce sustained robust activation of Ras-dependent pathways. This indicates that FGFR-3 induces signal transduction primarily through strong activation of Ras-independent pathways in the absence of a robust Ras-dependent signal.

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