Effect of Transmembrane and Kinase Domain Mutations on Fibroblast Growth Factor Receptor 3 Chimera Signaling in PC12 Cells

A MODEL FOR THE CONTROL OF RECEPTOR TYROSINE KINASE ACTIVATION*

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The effect of six point mutations causing various human skeletal dysplasias, occurring in the transmembrane (TM) and kinase domains (KD) of fibroblast growth factor receptor 3, were introduced into a chimera composed of the extracellular domain of human platelet-derived growth factor β and the TM and intracellular domains of hFGFR3. Stable transfectants in rat PC12 cells showed distinct differences in the two classes of mutations. The cells containing TM mutants displayed normal expression and activation but higher responsiveness to lower doses of ligand. The KD mutants showed significantly altered expression patterns. Normal amounts of a lower Mr receptor (p130) reflecting incomplete glycosylation, but only greatly decreased amounts of the mature (p170) form, were observed. However, the latter material showed normal ligand-dependent activation. In contrast, the p130 form, which is regularly observed in the expression of both native and chimeric receptors, exhibits strong ligand-independent tyrosine phosphorylation, particularly with the K650E mutation. Expression of two of the KD mutants (K650M and K650E), under control of an inducible metallothionein promoter, indicated that this receptor was sufficiently autoactivated to produce at least partial differentiation and, in the case of the K650E mutation, to induce ligand-independent neurite outgrowth. A model is presented that suggests that the low Mr (p130) KD mutants can, under the right conditions, signal intracellularly, but when they are fully glycosylated and move to the cell surface they adopt a normal, inhibited conformation, in the form of ligand-independent dimers, that neutralizes the effects of the mutations. When ligands bind, these dimeric receptors are activated in a normal manner. This model suggests that unliganded dimers may be a common intermediate in receptor tyrosine kinase signaling.

The fibroblast growth factor receptor (FGFR)1 tyrosine kinase family consists of four (FGFR1–4) members that are implicated in many developmental and regenerative processes, including cell proliferation, differentiation, and angiogenesis (1–3). Signaling by these receptors is mediated by the high affinity binding of the fibroblast growth factors (FGF), a diverse family consisting of at least 10 characterized members, aided by heparan sulfate proteoglycan interactions (4). Further diversity in expression and ligand specificity is achieved by the generation of subforms of the FGFRs mediated by alternative mRNA splicing events (4, 5). The ligand-receptor complexes activate the receptor kinases inducing tyrosine phosphorylation and a cascade of signaling events mediated by target effector proteins including Ras/MAPK and phospholipase Cγ (PLCγ) (6–8).

It is now well known that mutations in three of the four members of the FGFR family (FGFR1–3) cause a variety of autosomal dominant human skeletal dysplasias (for review see Refs. 9 and 10). Among these is the most common genetic form of short-limbed dwarfism, achondroplasia (ACH) (11, 12) caused by a point mutation in FGFR3, which results in a glycine to arginine substitution at amino acid position 380 within the transmembrane (TM) domain of the receptor. An additional mutation (G375C) is found in a limited number of other ACH patients (13). ACH is neonatal lethal in its homozygous form (14, 15).

Mutations that cause disorders thought to be allelic to ACH have been identified in FGFR3 as well. Thanatophoric dysplasia (TD) types I and II (16, 17), which can be distinguished radiographically (16, 18), are neonatal lethal forms, and hypochondroplasia (HCH) (19) is the least severe form of short-limbed dwarfism. TDI is associated with a mutation (K650E) within the tyrosine kinase domain, adjacent to the autophosphorylation site of the receptor (16), whereas TDI is associated with several types of mutations including extracellular domain substitutions of unpaired cysteine residues, stop codon subtitutions, and a K650M mutation, the identical residue altered in TDII (16, 20–23). This latter mutation causes a range of phenotypes from severe ACH to TDI (22). Both point and splice site mutations in FGFR1–3 also underlie several craniosynostosis syndromes, characterized by premature fusion of the cranial sutures (9, 10, 24). One of these, Crouzon syndrome with acanthosis nigricans, is caused by a TM domain mutation in FGFR3 (A391E) in close proximity to the common ACH

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‡ The abbreviations used are: FGFR, fibroblast growth factor receptor; FGF, fibroblast growth factor; RTK, receptor tyrosine kinase; MAPK, mitogen-activated protein kinase; PLCγ, phospholipase Cγ; ERK, extracellular-signal regulated kinase; ACH, achondroplasia; TM, transmembrane; TD, thanatophoric dysplasia; HCH, hypochondroplasia; PDGF, platelet-derived growth factor; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium; NGF, nerve growth factor.
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G380R (25, 26).

FGFR3 is expressed widely in a variety of tissues, including high levels in the brain (27, 28); however, skeletal dysplasia mutations are primarily manifested in the developing bone. Recent studies suggest that FGFR3 is a negative regulator of bone growth (29, 30) and that constitutive activation of the receptor may explain the effect of FGFR3 mutations on shortening endochondral bone growth (10). Consistent with this view, a gradient of ligand-independent activity of chimeric and full-length FGFR2 and FGFR3, corresponding to the severity of the mutation, has been observed (31–36). Other studies have reported Stat I activation for TD mutations (37, 38). Single cell calcium experiments (39), alternatively, support a dosage-dependent dominant-negative effect from specific mutant FGFR3 alleles resulting in a disruption of FGFR signaling. Inherent in several of these studies are complications arising from potential interactions with endogenous FGFR receptors that may alter subsequent signaling.

To clarify further the signaling properties of receptors with these mutations, we have recently described a chimeric receptor (PFR3), consisting of the five immunoglobulin (Ig)-loop extracellular domain of hPDGFR and the TM and intracellular domain of hFGFR3 (40). When stably transduced into PC12 cells, which contain no endogenous PDGFRβ, this chimera can be specifically activated by PDGF to signal through the altered FGFR3 intracellular domain. A mutation that causes ACH, G375C, was introduced by in vitro mutagenesis into the coding region of PFR3, and the biological effects of this mutation on signal transduction in PC12 cells were determined. In this report, we extend these studies to examine the differences in two classes of FGFR3 skeletal dysplasia mutations, those within the TM domain and those within the KD domain.

MATERIALS AND METHODS

Construction of the PFR3 Chimeric Constructs—Construction of hPFR3 (human PDGFRβ-FGFR3) and of PFR3M375C has been described previously (40). I PFR3 cloned into pCMV-1 was used as a template for in vitro mutagenesis (CLONTECH Transformer Mutagenesis Kit) following the manufacturer's instructions. Mutagenic primers to create the relevant mutations are as follows: G380R, 5′-AGCTACAGGGCTTCTCCTGTC-3′; N540K, 5′-CATCTACAACGCTGGGCG-CCTG-3′; K650E, 5′-CCTACAACAGGAC-ACAAACCCG-3′; K650M, 5′-CATCTACAAGGACACAAACCCG-3′; and A391E, 5′-TTTACCTGTTGCTGTCGTTGAC-3′. Each mutation generated twice in cold phosphate-buffered saline and lysed for 20 min in Triton X-100 lysis buffer (1% Triton, 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM EDTA, 100 mM sodium orthovanadate, 1 mM NaF, 0.5 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 0.5 mM sodium orthovanadate, 10 µg/ml each of aprotinin and leupetin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)). Immunoprecipitations were washed twice in cold phosphate-buffered saline and lysed for 20 min in Triton X-100 lysis buffer (1% Triton) for immunoprecipitations (Genzyme) and for immunoblot analysis have been described previously (40). The following antibodies were used throughout these studies: mouse monoclonal antibodies to endogenous FGFRs (GeneTech); rabbit polyclonal antibodies to FGFR3, anti-PDGFR, and anti-SOS (Santa Cruz Biotechnology); rabbit polyclonal anti-Shc and mouse monoclonal anti-phosphotyrosine (4G10) (Upstate Biotechnology, Inc.); mouse monoclonal anti-Grb2 (Transduction Laboratories); and rabbit anti-phospho-MAPK antisemur (Promega). For precipitation studies to detect FRS2 and FRS2-binding proteins, P13K (Onogene Science) was used.

Immune Complex Kinase Assay—PC12 cells were plated in 100-mm dishes and stimulated with 5 min with PDGF-BB. Cells were washed twice in cold phosphate-buffered saline and lysed for 20 min in Triton X-100 lysis buffer (1% Triton, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 40 mM β-glycerophosphate, 200 mM sodium orthovanadate with 1 mM NaF, 20 µg/ml aprotinin, and 20 µg/ml leupetin). Lysates were centrifuged at 13,000 × g for 15 min at 4°C, and the supernatant was added 10 µl of agarose-conjugated anti-Erk1 polyclonal antibody (Santa Cruz Biotechnology) and incubated with gentle rocking at 4°C for 3 h. Agarose beads were washed twice in cold lysis buffer and once in kinase buffer (20 mM Hepes, pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol, 0.1 mM P- nitrophenyl phosphate). Agarose beads were washed twice with 10 mM Tris-HCl buffer, pH 7.5, and 20 µl of kinase buffer containing 2 mM mg/ml myelin basic protein as substrate, 50 µM ATP, and 5 µCi of [γ-32P]ATP. Kinase reactions were performed for 10 min at 30°C and stopped by adding 40 µl of 2X Laemmli sample buffer. Samples were boiled for 5 min and separated on a 12% SDS-PAGE. Gels were stained with Coomassie Blue, dried, and exposed overnight with intensifying screens. Bands were excised from the gel, and radioactivity was counted by liquid scintillation.

RESULTS

Stable Expression of Mutant Chimeras in PC12 Cells—Mutations corresponding to the amino acid substitutions in FGFR3, which cause ACH (G375C, G380R), HCH (N540K), TD types I (K650M) and II (K650E), and Crouzon syndrome (A391E), as well as a kinase-inactive control (K508M), were introduced by site-directed mutagenesis into the PFR3 chimera (Fig. 1). Expression of these mutant receptors was initially monitored by transiently transfecting each construct into human embryonic kidney 293 cells and analyzing receptor protein levels on Western blots. As is normally observed in RTK expression, each PFR3 construct was present as two immunoreactive bands of the expected sizes of 170 (mature) and 130 (immature) kDa, when immunoprecipitated with anti-PDGFFR and detected with anti-FGFR3 antibodies (data not shown). The same PFR3 constructs were also stably transducted into PC12 cells, and individual clones were selected and frozen for future analysis. The experiments described herein utilized pools of transfected cell lines in order to provide a range of receptor expression.
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To determine autophosphorylation levels of the stably transfected receptors in PC12 cells, protein lysates from pools of different transfected lines were immunoprecipitated with anti-hPDGFR and analyzed by anti-phosphotyrosine immunoblotting. As shown in Fig. 2A, the chimeric receptors displayed increased levels of autophosphorylation upon stimulation with ligand, the amount of phosphorylation being dependent upon receptor levels and mutation type (Fig. 2, B and C). PFR3, PFR3G375C, PFR3G380R, and PFR3A391E each showed comparable levels of ligand-responsive tyrosine phosphorylation when normalized for the amount of receptor expressed (Fig. 2, B and C). It has been demonstrated previously for both PFR1 and PFR3 (40, 42) that overexpression of receptor protein leads to independence correlating with the level of receptor expression. This phenomenon was consistently observed with these mutants as well.

In contrast to the TM mutants, all three of the KD mutants, PFR3N540K, PFR3K650E, and PFR3K650M, show low levels of mature receptor expression. For both the chimeric PFR3 constructs, as well as full-length FGFR3 clones (data not shown), there are two immunoreactive receptor bands observed. The upper band (approximately 170 kDa for PFR3), normally the more abundant of the two, represents the species that becomes phosphorylated, whereas the lower band (approximately 130 kDa) is normally not phosphorylated. As reported by others (43, 44), the upper band likely represents the mature, glycosylated form, and the lower is a less glycosylated precursor species. Mutations within the tyrosine kinase domain of the chimeric receptors, N540K, K650E, and K650M, result in a severe decrease in the 170-kDa immunoreactive protein, as judged by anti-PDGFR (Fig. 2B) or anti-FGFR3 (Fig. 2C) Western blotting. In addition, the lower 130-kDa band of PFR3G375C and to a lesser extent PFR3N540K (Fig. 4A shows a darker exposure of Fig. 2A) were autophosphorylated in a ligand-independent manner, a novel modification that may represent a new active form of the receptor. Nonetheless, the low levels of p170 expressed for the KD mutants were apparently terminally differentiated, a condition that precludes their selection with the G418 screen (see below). Ligand-dependent or -independent phosphorylation was not observed in cells containing inactive PFR3K508M.

Effect of Mutations in PFR3 Chimeras on Morphological Responses of PC12 Cells—PC12 cell lines, stably transfected with the various mutant PFR3 constructs, were stimulated with various concentrations of PDGF, and biological responses were monitored at 24 or 48 h following addition of ligand. As shown previously, PDGFR chimeras of TrkA, the nerve growth factor (NGF) receptor (7), and FGFR1 (42), when stably transfected into PC12 cells, produced the same ligand (PDGF)-dependent differentiation, characterized by the growth of neuronal processes, as induced in native PC12 cells by NGF and FGF (45). Neurite outgrowth was observed for all PFR3-containing cell lines, albeit to varying degrees (Fig. 3). At 24 h (data not shown), both ACH mutant-containing lines, PFR3G380R and PFR3G375C, are the most rapidly responsive, with over 70% of the cells producing neurites at 30 ng/ml PDGF. PFR3 (wild type) and the A391E mutant show comparable levels of responsiveness to 30–60% neurite outgrowth, respectively. With PFR3N540K and PFR3K650M-containing cells, 40 and 30% of the cells, respectively, produced neurites at the same time point. PC12 cells containing PFR3K650E showed very little neurite outgrowth (10%) by 24 h. By 48 h, a larger proportion of all cell lines produced neurites (Fig. 3). At this point, the two ACH mutant-containing cells, PFR3N540K and PFR3K650M-containing cells, are almost completely differentiated (90 and 80%, respectively), and PFR3- and PFR3A391E-containing cells show very similar responsiveness to 30 ng/ml ligand (approximately 70%). PFR3N540K (50%) and PFR3K650M (45%), both KD mutants, have decreased maximal ligand responsiveness compared with control and TM domain mutants, whereas PFR3K650E showed the least amount of responsiveness for any of the mutant chimeras (less than 20%). In addition, both PFR3G375C and PFR3G380R, and to some degree PFR3A391E, require less ligand to produce neurites. At 1 ng/ml PDGF, a proportion of both ACH mutant-containing (10%) and, to a lesser extent, the Crouzon mutant-containing (5%) cell lines, are responsive compared with wild type or KD mutant-containing cell lines. It should be noted that the PFR3N540K-containing cell line (40) is clonal, i.e. it was derived from a single PC12 cell, which may explain the slightly higher maximum responsiveness than the pooled cell lines. No neurites were formed by cells containing the kinase-inactive receptor (PFR3K508M).

Effect of Mutations in PFR3 Chimeras on Signaling Responses of PC12 Cells—Activation of PFR3, like other receptor tyrosine kinases, leads to phosphorylation and stimulation of

Fig. 1. Schematic diagram of PFR3 and the mutant chimeric receptors generated introducing single base mutations in the transmembrane domain of FGFR3 by in vitro mutagenesis. The engineered PFR3 chimeric receptor consists of the extracellular domain with the five Ig-like domains of hPDGFR and the transmembrane domain (TM) and split tyrosine kinase domains (TK) of hFGFR3. The ACH mutations, G375C and G380R, and the Crouzon mutation A391E in the transmembrane domain are shown as well as the HCH N540K, the TDI K650M, and the TDII K650E mutations in the tyrosine kinase domain of FGFR3. The kinase-inactivating K508M mutation is indicated as well.

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![Image](57x339 to 288x490)

**Fig. 2.** Tyrosine phosphorylation of PFR3 and PFR3 mutant chimeric receptors. PC12 cells expressing PFR3, the kinase-inactive chimeric receptor (K508M), PFR3 carrying the ACH mutations G375C and G380R, the Crouzon mutation A391E, the HCH mutation N540K, the TDII mutation K650E, or the TDI mutation K650M were incubated at 37 °C for 10 min without (−) or with (+) PDGF-BB (30 ng/ml). Lysates (2 mg) were incubated with an anti-PDGFR antibody, and immunoprecipitates were separated by SDS-PAGE (7.5% gel) and analyzed by immunoblotting (IB) with anti-phosphotyrosine antibody (A) and with an anti-PDGFR antibody (B) and subsequently with anti-FGFR3 antiserum (C). The two forms of the chimeric receptor which is recognized by all three antibodies are indicated by arrows.

![Image](252x502 to 554x729)

**Fig. 3.** Dose-dependent induction of neurites in PC12 cells expressing PFR3 wild type or PFR3 carrying the ACH mutations G375C and G380R, the Crouzon mutation A391E, the HCH mutation N540K, the TDII mutation K650E, or the TDI mutation K650M. Cells were maintained in DMEM containing 1% plasma-derived horse serum in the presence of various concentrations of PDGF-BB (1, 3 and 30 ng/ml). After 48 h of treatment, cells were examined for the presence of neurites. Values are averages of duplicate determinations from a representative experiment; *error bars* are shown.

downstream target proteins/effectors (40, 44). Of importance to PC12 cell differentiation is PLCγ, Shc, FRS2, Grb2, and Sos, and the MAPKs, ERK1 and -2 (7, 46). PLCγ is an inherent part of the response following activation by receptor tyrosine kinase stimulation and leads to the production of diacylglycerol and inositol trisphosphate, which act as downstream modulators of protein kinase C isozymes and calcium fluxes, respectively. Under circumstances where the direct activation of Ras is reduced, PLCγ has been shown to contribute significantly to differentiation (45); however, when other Ras-activation pathways are normal, the PLCγ signal can be completely abrogated without apparent effect. It has previously been shown that PLCγ becomes phosphorylated in response to PDGF stimulation in cells expressing either PFR3 or PFR3K650E (40). A similar activation was observed for the mutant receptors when PLCγ was co-immunoprecipitated with anti-PDGFR (145-kDa band), including PFR3K650E (Fig. 4A). Binding of PLCγ to mutant receptors was increased upon addition of ligand and subsequent phosphorylation of the receptors (Fig. 4B). Thus, the PLCγ pathway undergoes ligand-induced activation with all of the mutant receptors, even those expressed at low levels.

The activation of the Ras pathway is of major importance to PC12 cell differentiation and represents a major activity of the FGFRs (8, 46). Ras stimulation can be accomplished directly by RTKs through a variety of adaptor proteins, including Shc, Grb2, and FRS2 (47). As reported for other FGFRs, immunoprecipitation of Shc from cells expressing the PFR3 mutants with an anti-Shc antibody showed a low level increase in tyrosine phosphorylation of the 46- and 52-kDa isoforms in response to ligand in all cells, correlating with receptor levels (data not shown). Increased binding of Grb2 to phosphorylated Shc following PDGF stimulation was evident for all the mutant chimeras as well (data not shown). The levels of the guanylyl nucleotide exchange factor, Sos, which links the activated receptor complex to the Ras pathway, were also evaluated. Addition of ligand did not result in increased levels of Sos bound to the immunoprecipitation complex for any of the chimeras, consistent with the developing model that Shc is not the primary link to this pathway in FGFR signaling.

As an alternative adaptor to Shc, it has been suggested that FGFR3 activates Ras-dependent pathways through Grb2 and Sos via a linker protein of approximately 89 kDa (p89), also known as FRS2 or SNT1 (47–50). To determine whether the mutations in FGFR3 affect signaling via FRS2 (and Grb2 and Sos) in this chimeric system, proteins from total cell lysates of the mutant PFR3-containing cells were precipitated using P13-agarose, which has high affinity for FRS2. As shown in Fig. 5A, upon stimulation with ligand, phosphorylation of FRS2 (p89) was observed for all PFR3-containing cell lines except for PFR3K650E. This identification was confirmed with antisera (kindly provided by J. Schlessinger and I. Lax), raised against the C terminus region of FRS2. Interestingly, the electrophoretic mobility and the intensity of tyrosine phosphorylation of FRS2 varies in the mutants suggesting that different levels of phosphorylation of this protein are possibly linked to different degrees of activity. As shown in Fig. 5C, Grb2 coprecipitated with FRS2 in a ligand-dependent manner in all mutants with the exception of PFR3K650E, where FRS2 phosphorylation is also absent. Under the same conditions, increased amounts and retarded mobility of Sos1 (p170) (Fig. 5B) are observed with the same ligand dependence. As also seen in Fig. 5A, a

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protein of 62 kDa was found to be consistently precipitated with FRS2, which likely corresponds to SHP2, a soluble tyrosine phosphatase. However, its ligand-dependent phosphorylation (and association to FRS2) is more variable than Grb2 and Sos. These results are consistent with FRS2

\[ \text{Frz} \]

Grb2

Sos as a primary modulating complex for PC12 cell differentiation by FGFs, which is utilized by all of the mutants examined, although this conclusion must be viewed as tentative for the residue 650 mutations, and in particular for K650E, because of the lower levels of expression in these cells and the very low amounts of mature (p170) receptor.

To determine the effect of the mutations of the Ras-activated kinase cascade, total cell lysates from cells stably transfected with the PFR3 constructs were immunoblotted and probed with anti-active MAPK antibody that recognizes the dually phosphorylated and active forms of ERK1 (p44) and ERK2 (p42), indicated by the arrows.

\[ \text{FIG. 5. Tyrosine phosphorylation of p89 (FRS2)and its association with Grb2 and Sos.} \]

Cells expressing the PFR3 and PFR3 mutant chimeric constructs were incubated at 37 °C for 10 min without (−) or with (+) PDGF-BB. Lysates (1.8 mg) were incubated with recombinant p13 Suc-agarose; the precipitated proteins were separated by SDS-PAGE (10% gel), and immunoblotting (IB) was performed. A, the middle part of the blot was probed with anti-phosphotyrosine antibody; B, the top part with anti-Sos antibody; and C, the bottom portion with anti-Grb2 antibody, as indicated. Coprecipitating proteins are indicated by arrows. D, activation of MAP kinases (ERK1 and ERK2) by PFR3 and PFR3 mutant chimeric receptors was analyzed in PC12 cells. PC12 cells expressing PFR3 chimeras were incubated at 37 °C for 10 min without (−) or with (+) PDGF-BB. Lysates (50 μg) were separated by SDS-PAGE (7.5% gel) and analyzed by immunoblotting with an anti-active MAPK antibody that recognizes the dually phosphorylated and active forms of ERK1 (p44) and ERK2 (p42), indicated by the arrows.

\[ J. \text{Schlessinger, personal communication.} \]
tion, consistent with the corresponding low levels of mature receptor expression observed for the KD mutations. These levels of ERK activation from cells carrying the K650E mutant are consistent with the lack of ligand-dependent activation of FR52, Grb2, and Sos and the poor induction of neurites.

Stable Expression of PFR3 KD Mutants in PC12 Cells Under the Control of an Inducible Methallothionein Promoter—Stable expression of mutants PFR3K650M and PFR3K650E in PC12 cells resulted in low levels of expression of the mature receptor protein and, in the case of the K650E mutation, strong ligand-independent autophosphorylation of the underglycosylated p130 receptor protein that was expressed in amounts comparable to the other chimeras. During the process of selecting stable PC12 cells with G418 following retroviral transfection with PFR3 constructs containing either KD mutations, cells appeared to become differentiated in the absence of ligand. This suggests that the cells rescued, which were by definition able to continue dividing, represented a subgroup expressing low levels of receptor. In order to verify that this mutation(s) served upon stimulation with PDGF-BB (30 ng/ml) for 48 h. Bar, 100 μm.

FIG. 6. The induction of neurite outgrowth in clonal PC12 cells expressing PFR3, the TDI mutation K650M (4 and 5), or the TDI mutation K650E (6 and 12) under the control of a metallothionein-inducible promoter (MT). Cells were maintained in DMEM containing 1% platelet-poor plasma horse serum with 80 μM ZnSO4 in the presence or absence of PDGF-BB (30 ng/ml) for 48 h. Bar, 100 μm.

In order to determine the tyrosine kinase activity of the MT chimeric receptors, lysates from clones expressing MT-PFR3K650M, MT-PFR3K650E, and mock cells (data not shown), left untreated or exposed to Zn2+ in the presence or absence of PDGF, were subjected to immunoprecipitation with anti-FGFR3 antibodies. As shown in Fig. 7C, immunoblotting with anti-phosphotyrosine antibody revealed that the level of auto-phosphorylation of the induced MT-PFR3K650M receptor forms, p170 and p145, was largely ligand-dependent in both clones. In contrast, the MT-PFR3K650E cells exposed to Zn2+ showed ligand-independent tyrosine phosphorylation of p130 and p145; no corresponding increase in phosphorylation of p130 was observed upon stimulation with PDGF. Only the p170 form of the MT-PFR3K650E receptor remains ligand-dependent, and phosphorylation of this form is only observed upon addition of ligand. Interestingly, all cell lines appear to express the three forms of chimeric receptors, p170, p145, and p130 (Fig. 7D). These results confirm that 1) MT-PFR3K650M clones show ligand-dependent activation of ERK1/2 by the p170 (and p145) form of the receptor, and 2) MT-PFR3K650E clones show ligand-independent activation of ERK1/2 and of the p130 form of the receptor but retain ligand-dependent activation of the p170 form.

DISCUSSION

In a previous study (40), it was shown that PFR3 and the ACH mutant, PFR3G375C, readily induced the differentiation of PC12 cells in a ligand-dependent manner. There were no qualitative differences in the two responses, although there was some suggestion that the mutant-bearing cells were “pre-activated.” Importantly, there were no significant differences between the responses produced by PFR3 and PFR1 (42).3 The G375C substitution occurs close to the extracellular and TM domain interface, and by some alignments is actually the last residue before the TM domain. Therefore, it was potentially not indicative of the TM group of mutations, and two additional TM mutants, G380R and A391E, were substituted into PFR3 and tested. These produced similar morphological and molecular responses as PFR3 and PFR3G375C. PFR3G380R, which contains the most common ACH mutation, particularly resembled PFR3G375C in its ability to induce neurites at low concentrations of PDGF. This was not manifested by any significant differences in the early stimulation of PLC or MAPK, however. Notably, all three TM mutant receptors showed significantly higher responsiveness in inducing differentiation than PFR3, although these differences were negligible at the highest ligand concentration (30 ng/ml). These results support the view

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**Table:**

| Condition          | MT-PFR3 | MT-K650M | MT-K650E |
|--------------------|---------|----------|----------|
| Zn2+               |         |          |          |
| Zn2+ + PDGF        |         |          |          |

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**References:**

3. These results support the view...
that the TM class of FGFR3 mutants acquire some ligand independence when they reach the cell surface and that cells bearing these receptors are partially pre-activated.

The molecular and cellular responses of the chimeric receptors with KD mutations are more varied and generally less robust. However, this is complicated by differences in expression levels of these receptors with the non-inducible constructs and is particularly pronounced in cells containing PFR3K650M, where there is little or no mature receptor detectable by anti-receptor antibodies. Mature receptor becomes visible only following ligand stimulation/phosphorylation. Although significantly reduced in abundance compared with wild type, this receptor chimera is still able to activate the Ras/MAPK pathway and produce neurites in a ligand-dependent manner, albeit less potently. A recent finding that is consistent with these observations is the ligand dependence observed for MAPK activation in cultured chondrocytes derived from TDI fetuses (37). PFR3K650E results in even more profound effects with phosphorylation and activation of downstream targets greatly reduced, presumably because the signaling capacity of this receptor is reduced by this mutation. Whereas proteolytic processing of the p170 protein to yield smaller forms of the receptor has not been ruled out, the recognition by both PDGFR and FGFR3 antibodies as well as the results from previous glycosylation studies (43, 44) makes it much more likely that this decrease of p170 represents a reduction in the amount of mature, glycosylated protein reaching the surface of the cell rather than subsequent destruction of p170.

In addition to the decreased amounts of the 170-kDa form of the KD mutant receptors, a ligand-independent phosphorylation of the 130-kDa form of the receptor in cells containing PFR3K650E (and to a much lesser degree PFR3N540K) was observed. Although these two forms of the receptor are characteristic of many RTKs including native FGFR and PDGFR, phosphorylation of the lower molecular weight form is normally not observed, with or without ligand stimulation, in stably transfected cells. Thus, this phosphorylation is unusual and characteristic of this mutation. Interestingly, it has been described previously for FGFR3 mutant constructs stably transfected into BaF3 cells. Phosphoryrosine was observed in the larger molecular weight species (corresponding to the p170 band reported here) for all the chimeric (FGFR3-FGFR1) and full-length receptors except for the one containing the K650E mutation (35). Thus, the phosphorylation of this lower molecular weight species is not related to the presence of the PDGFR domain.

The clear distinction in behavior of the two classes of receptors suggests that the changes in function that underlie the human pathologies may result from different effects on receptor structure. The TM group of mutations appears to alter the resting state of the cell, presumably through the acquisition of some ligand-independent character, as has been suggested in other studies (9, 10). However, in the case of PC12 cells, this does not substantially alter the ligand-induced signaling except to lower the apparent amount of ligand required. This may indicate a change in affinity of ligand but more likely suggests that the amount of additional “signal” required to reach the threshold dose is lower. In either case, the effect of the mutation is manifested in the mature (−170 kDa) form. In situ, the mutant receptor can, in theory, form homodimers or heterodimers, the latter with normal FGFR3 (or other FGFR promoters), assuming that they are expressed at the same developmental time point in the same cells. In the PC12 cell model, the chimeras can only form homodimers. Thus, this more closely represents the conditions of the homozygous form of achondroplasia, which is neonatal-lethal (11, 15). It could be expected, therefore, that the signaling defects observed in the PC12 cell/chimera system would be highly pronounced.
As described, the KD mutant cells differ most radically from those of the TM group primarily in expression of the higher molecular weight form (Fig. 2). Initially, it was assumed that this resulted from the constitutive activation of these receptors thereby inducing differentiation of the transfected cells and preventing selection by the G418 screen. However, the induction of expression of PFR3\textsuperscript{K650M} mutants under the control of the ovine MT promoter do not, in the absence of ligand, show significant neurite proliferation, in contrast to K650E containing cell lines which produce neurites in the absence of ligand. It may be that there is sufficient auto-activation of the PFR3\textsuperscript{K650M} (and PFR3\textsuperscript{N540K}) mutants that they can force the cells to exit the cell cycle but have insufficient signal to produce neurite formation. This level of activation may be below what can be readily measured in phosphotyrosine blots. For each KD mutant, there are “normal” amounts of the 130-kDa protein, and the inducible promoter constructs yield substantive amounts of the p170 form. Significantly, all of the p170 forms (from either the normal or inducible constructs) behave in a ligand-dependent fashion, in terms of both signaling responses and neurite proliferation. Even PFR3\textsuperscript{K650E}, which shows substantial activity when induced by metal alone, is considerably further activated by the addition of PDGF (Figs. 6 and 7).

A simple model that accounts for these observations is shown in Fig. 8. It is based primarily on the behavior of the PFR3\textsuperscript{K650E} mutant but likely applies to the other KD mutants as well. Some aspects may apply generally to all FGFRs and possibly to RTKs as a class. As depicted, the receptor first forms a monomeric protomer, identical for purposes of this discussion to the p130 protein, that is widely and commonly observed for most, if not all, RTKs. Normally, this species is not activated or phosphorylated. In the case of the K650E mutant, this species is clearly phosphorylated in a ligand-independent manner (with or without the inducible promoter). This suggests that the K650E mutant may be truly autocatalytic, i.e. the protomer phosphorylates itself. However, intermolecular modifications, through transient dimers, is certainly also possible. This species would then be potentially capable of signaling through the same pathways that are activated from the plasma membrane or through novel ones. If, as is thought to be the case, this species does not exist on the cell surface, it would mean that these would arise from within an intracellular environment.

Why does this mutation then not manifest itself similarly in the p170 form? The second feature of the model accounts for this apparent “loss” of autoactivation. It suggests that a stable dimer is formed, composed of p170 protomers, and that this induces a conformational change in the KDs that neutralizes the effect of the mutation. The interactions that stabilize this dimer would presumably result from the modifications, i.e. glycosylation, that accompany the molecular mass conversion, and, likely the completion of its transit to the cell surface. The corresponding changes in the KD would actually result in a stabilized “inhibited” state (of the unoccupied receptor) and would then, through a change in the orientation of the protomers, form an “activated” dimer when ligand was bound. The new kinase conformation, which would be stabilized by ligand binding and subsequent phosphorylation events, represents the signaling entity. Recent studies have shown that, in at least the case of the EGFR, this is retained even after the ligand dissociates (52). A model in which the activity of FGFR is modulated by conformation has been recently proposed by McKeehan et al. (4) and supports the idea of ligand-independent dimerization of receptor tyrosine kinases. In this case, unoccupied FGFR is a dimer rigidly anchored via divalent cations to a heparan sulfate chain. Upon ligand binding, a transient active state is stabilized. By extrapolation, the FGFR3 species with the mutations occurring in the TM domain would adopt, in the unliganded state, an orientation of receptor protomers closer to the liganded condition.

Further support for the formation of unliganded receptor dimers on the cell surface comes from studies with PDGFR that clearly demonstrate receptor-receptor interactions, independent of ligand binding, can occur through the fourth IgG domain of the extracellular domain (53). In this case, it was proposed that the receptor exists in a monomer-dimer equilibrium prior to the addition of ligand. The model deduced from the behavior of the K650E chimera is consistent with this observation and further suggests that the unliganded dimer is likely to be the dominant species, at least under certain conditions. However, it remains to be established that this behavior is also observed.
with receptors containing FGFR extracellular domain. The determination of the three-dimensional structure of the KD of FGFR1 (54) provides some potential insight into how these proposed events might occur at the molecular level. In this structure, the “lip” domain of the kinase, which contains the tyrosines 653 and 654 that become initially phosphorylated (and, coincidentally, the 650 position, corresponding to Tyr-656 in FGFR1), was found to lie outside the catalytic site such that the enzyme in this conformation could directly bind ATP, but access of the peptide substrates is predicted to be partially blocked. This is in contrast to the comparable domain from the insulin receptor, where the same segment was found to be located in the catalytic site, largely blocking access to either nucleotide or substrate. The scheme shown in Fig. 8 suggests that the three-dimensional structure of the FGFR KD, determined experimentally, probably reflects more closely that of the K650E mutant. When the dimer of p170 protomers forms, a conformational shift occurs that moves this sequence into an orientation similar to the insulin receptor KD structure. Ligand binding could then “release” the lip again by inducing the formation of the activated structure and allow intramolecular catalysis to phosphorylate the active site loop tyrosines to produce the stable, activated receptor dimer. Thus, the model predicts that neutralization of the effects of the K650E mutation by dimer formation is caused by a conversion of the FGFR KD to a conformation that is similar to that of the insulin receptor.

There are three important concepts, based on the behavior of the K650E mutation, that the proposed model of FGFR3 activation, shown in Fig. 8, develops. First, mutations in the KD of an RTK may lead to intracellular signaling through intramolecular activation. Second, RTKs may pre-exist on the cell surface in dimer form such that the KDs are held in an inactive conformation, and ligand induces a reorientation of the receptor protomers to allow the KDS to become activated. Although a monomer-dimer equilibrium is not excluded, the dimer would be the dominant species. Finally, the conversion of the putative monomeric (p130) state to the dimeric (p170) state is the result of glycosylation and accompanies the transfer of the receptor from an intracellular environment to the plasma membrane.

One of the PFR3 K650M species observed from inducible promoter clones possessed an apparent molecular mass of 145 kDa. This species, which was identified following FGFR3 immunoprecipitation, was ligand-responsive and induced differentiation in a normal manner. There was no p170 species expressed by this subset of cells. The same p145 species was observed in other K650M cell lines and in K650E clonal lines. In these cases, however, the p170 form was the species that appeared to be the dominant ligand–dependent signaling entity. The nature of the p145 receptor form and how it differs from the p170 form is not presently known.

Mice homozygous for a targeted disruption of fgr3 have shown that FGFR3 appears to regulate endochondral bone development in a negative manner; these FGFR3-deficient mice exhibit overgrowth in the length of the vertebral column and long bones (29, 30). Given that endochondral ossification, the process by which bone is deposited upon a cartilaginous matrix, occurs by an orderly conversion of resting to proliferating to hypertrophic (enlarged, terminal differentiated) chondrocytes, the likelihood that FGFR3 modulates both proliferation and maturation of chondrocytes supports the possibility that mutations in FGFR3 could affect regulation of these two aspects, independently. Recent data (37) supports this view; in these studies an increased number of apoptotic cells in cultured chondrocytes from TD fetuses was observed. At the same time, a normal mitogenic response was found. This suggests that, at least in the case of TDI, the mutation induces differentiation, possibly through Stat I, rather than proliferation of chondrocytes.

Two types of bone growth are affected by mutations in FGFR. The skeletal dysplasias characterized by short-limbed dwarfism and caused exclusively by mutations in FGFR3 affect endochondral bone growth. The craniosynostoses, however, result from mutations primarily in FGFR1 and -2, but also in FGFR3, affect intramembranous bone growth. These involve the formation of the flat bones of the skull and do not require cartilage-forming chondrocytes. Because of the participation of the same receptor, FGFR3, in both types of skeletal dysplasias, some type of common pathway involving FGFR3, but influenced by unique downstream cellular targets, must be involved. It may be that a gradient of receptor activity and/or the ability of other FGFRs to compensate determine whether a mutation in FGFR3 will cause a craniosynostosis or dwarfism phenotype, or both, in the case of TDI (K650E). This suggests that both the physiological processes affected and the severity of the alterations may be related to the behavior of some of these receptors in the intracellular compartments and their rate of transit through them.

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