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Chimeras of the Native Form or Achondroplasia Mutant (G375C) of Human Fibroblast Growth Factor Receptor 3 Induce Ligand-Dependent Differentiation of PC12 Cells

LESLIE M. THOMPSON, SIMONA RAFFIONI, JOHN J. WASMUTH,† AND RALPH A. BRADSHAW*

Department of Biological Chemistry, College of Medicine, University of California, Irvine, California 92697

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Mutations in the gene for human fibroblast growth factor receptor 3 (hFGFR3) cause a variety of skeletal dysplasias, including the most common genetic form of dwarfism, achondroplasia (ACH). Evidence indicates that these phenotypes are not due to simple haploinsufficiency of FGFR3 but are more likely related to a role in negatively regulating skeletal growth. The effects of one of these mutations on FGFR3 signaling were examined by constructing chimeric receptors composed of the extracellular domain of human platelet-derived growth factor receptor β (hPDGFRβ) and the transmembrane and intracellular domains of hFGFR3 or of an ACH (G375C) mutant. Following stable transfection in PC12 cells, which lack platelet-derived growth factor (PDGF) receptors, all clonal cell lines, with either type of chimera, showed strong neurite outgrowth in the presence of PDGF but not in its absence. Antiphosphotyrosine immunoblots showed ligand-dependent autophosphorylation, and both receptor types stimulated strong phosphorylation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase, an event associated with the differentiative response of these cells. In addition, ligand-dependent phosphorylation of phospholipase Cγ and Shc was also observed. All of these responses were comparable to those observed from ligand activation, such as by nerve growth factor, of the native PC12 cells used to prepare the stable transfectants. The cells with the chimera bearing the ACH mutation were more rapidly responsive to ligand with less sustained MAPK activation, indicative of a preactivated or primed condition and consistent with the view that these mutations weaken ligand control of FGFR3 function. However, the full effect of the mutation likely depends in part on structural features of the extracellular domain. Although FGFR3 has been suggested to act as a negative regulator of long-bone growth in chrondrocytes, it produces differentiative signals similar to those of FGFR1, to which only positive effects have been ascribed, in PC12 cells. Therefore, its regulatory effects on bone growth likely result from cellular contexts and not the induction of a unique FGFR3 signaling pathway.

The fibroblast growth factors (FGFs) are a diverse family of extracellular agents, presently consisting of at least nine members, that variably function in a broad spectrum of regulatory activities, beginning during embryonic development and continuing throughout adult life (5, 12). Similarly, there are several types of receptors and binding proteins that may transmit FGF signals in responsive cells. One family, containing tyrosine kinases, is composed of four main types of receptors (FGFR1 to 4) but is amplified by many additional subforms arising from mRNA splice variants (12, 14). Although the molecular events initiated by these FGF/FGFR complexes have not been fully delineated, they have been associated with mitogenic, angiogenic, neurotrophic, and differentiative responses in a variety of cell types (15). In the case of FGFR1, these complexes are known to activate signaling pathways involving RAS and phospholipase Cγ (PLCγ) (35, 37).

The essential participation of FGF signaling in skeletal development has recently been demonstrated by the association of a number of FGFR gene mutations with a range of human skeletal dysmorphologies (for reviews, see references 4 and 22). Several genetic forms of short-limbed dwarfism, including the most common type, achondroplasia (ACH), result from mutations specifically in FGFR3 (32, 36). ACH is most frequently caused by a glycine-to-arginine substitution at position 380 (G380R) but can also be caused by a glycine-to-cysteine mutation at position 375 (G375C) (13, 39); both mutations are within the transmembrane (TM) domain of the receptor. Mutations causing the two types of thanatophoric dysplasia (TDI and TDII) (33, 40, 41), a neonatal lethal form of dwarfism, and hypochondroplasia (2), a mild form of short-limbed dwarfism, have also been identified in FGFR3. Craniosynostotic syndromes, including Crouzon, Apert, and Pfeiffer syndromes and most recently Beare-Stevenson cutis gyrata syndrome, are caused by a variety of mutations in FGFR1, -2, and -3 (3, 20, 28; for a review, see reference 22). Most of these disorders are characterized by single amino acid substitutions in specific regions of the protein, although splicing mutations have also been identified in FGFR2 (21).

ACH is an autosomal dominant disorder with complete penetrance characterized by rhizomelic shortening of the long bones, lumbar lordosis, and relative macrocephaly with a high sporadic rate of mutation (80 to 90%) (16, 30). Although in the heterozygous state symptoms are predominantly limited to skeletal defects, the homozygous state is lethal in the neonate and resembles TD. There is widespread tissue expression of FGFR3 (6, 27, 42); however, the defect associated with ACH is primarily manifested in the developing long bones (16, 30). Since transgenic mice lacking FGFR3 show overgrowth of long bones and vertebrae (9, 10), FGFR3 may negatively regulate bone growth; consistent with this model, the ACH mutations

* Corresponding author. Present address: 238-D Med. Sci. I, Department of Physiology & Biophysics, College of Medicine, University of California, Irvine, CA 92697. Phone: (714) 824-6236. Fax: (714) 824-8036. E-mail: RABLAB@UCI.EDU.

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(as well as other FGFR3 mutations) may lead to a partial or full activation of the receptor with loss of ligand responsiveness. Transfection experiments showing constitutive activation of Neu or FGFR1 chimeric receptors by in vitro kinase or autophosphorylation assays (24, 45) have reinforced this hypothesis. Thus, the inappropriate activation of FGFR3 in chondrocytes by mutations that cause skeletal dysplasias should result in the reduced growth of long bones.

PC12 cells, a cell line derived from a rat pheochromocytoma (44), respond in culture to a number of factors, including several of the FGFs, by producing neurites, a morphology consistent with a differentiated neuronal phenotype. Although there is some variation in individual isolates, most of these cell lines express multiple types of FGFRs, as judged by Northern analysis and cDNA cloning, with the dominant species derived from the FGFR1 gene (29). The ligand-induced response of these cells is reversible, and the cells can be stably transfected to produce clonal cell lines with altered receptor profiles (26). The introduction of chimeric receptors, in which the extracellular domain has been replaced by one not expressed in PC12 cells, is a particularly useful means to examine responses without the complications arising from activating endogenous receptors or undefined changes in mutant lines. We have used this approach to test the signaling properties of human FGFR3 (hFGFR3) and an ACH mutant (G375C) in PC12 cells. Both chimeras, constructed from the extracellular domain of human platelet-derived growth factor receptor β (hPDGFRβ) and the TM and intracellular domains of hFGFR3, readily differentiate PC12 cells in a ligand-dependent fashion and activate Shc, mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK), and PLCγ, establishing that FGFR3 functions in a differentiative fashion in these cells. Altering the receptor to contain the TM domain ACH mutation (G375C) results in more rapidly responsive cells that show some ligand-independent characteristics, consistent with the view that these mutations relax ligand control.

MATERIALS AND METHODS

Construction of the hPFR3 chimera. Human PFR3 (hPFR3) was constructed by fusing the extracellular domain of hPDGFRβ to the TM and intracellular domains of hFGFR3 (42). A 1.9-kb *Eco*RI-*Mse*I DNA fragment digested from a PDGFRβ cDNA (8) was ligated in frame to a 1.4-kb *Mse*I-*Eco*RI fragment from the FGFR3 cDNA and cloned into the *Eco*RI site of the pCMV-1 (25) polylinker for transient expression into human embryonic kidney 293 cells. The PDGFRβ-FGFR3 (PFR3) construct was subsequently subcloned into the *Eco*RI site of the retroviral vector pLEN (1, 26) for stable transfection into PC12 cells. The *Eco*RI and *Mse*I sites in the FGFR3 gene were introduced by PCR mutagenesis. The oligonucleotide 5′-GCACCAGCAGCAGGGTGGAATTCTAGGGACCCCT C-3′ was used to create an *Eco*RI site 3′ to the stop codon, and the oligonucleotide 5′-CTGACGAGGCGGTTAATGTGTATGCAGGCATCCTC-3′ was used to create an *Mse*I site just 5′ of the amino acid valine at position 372, which is a putative start of the TM domain of FGFR3, without changing the amino acid sequence. The G375C mutation was introduced by altering the *Mse*I mutagenesis oligonucleotide to introduce the required G-to-T change (5′-CTGACGAGGC GGTTAATGTGTATGCATGCATCCTC-3′). PCR amplification was performed under the following conditions: 30-s cycle times of 94, 60, and 72°C for 30 cycles in ammonium sulfate buffer (67 mM Tris-HCl [pH 8.0], 6.7 mM $MgCl_2$, 16 mM ammonium sulfate, 10 mM 2-mercaptoethanol) containing 1.25 mM deoxynucleoside triphosphates, 10% dimethyl sulfoxide, 0.5 pmol of each primer/μl, and 2 U of *Taq* polymerase/reaction. The PCR products were then ligated directly into the TA cloning vector (Invitrogen), and double-stranded DNA was sequenced by the chain termination DNA sequencing method (34) using Sequenase (Amersham Life Science) with gene-specific primers to verify the fidelity of the amplified products. A full-length hFGFR3 cDNA (17) (kindly provided by M. Hayman) was subcloned into the *Hin*dIII site of the pCMV-1 polylinker. To verify the expression of the cDNA constructs, 293 cells (ATCC CRL 1573) were transiently transfected by the calcium phosphate coprecipitation technique (7).

Generation of stable cell lines. The ecotropic virus packaging cell line GP+E86 (19) was transfected with plasmid pLEN containing the wild-type PFR3 chimera, the PFR3 chimera containing the G375C mutation ($PFR3_{375}$), or the retroviral vector control by the calcium phosphate coprecipitation technique. After 2 days, virus-containing supernatants were filtered (0.45-μm-pore-size filter) and added to PC12 cells in the presence of 8 μg of Polybrene (Sigma)/ml for 16 to 20 h. Infected cells were shifted to Dulbecco's modified Eagle's medium (DMEM; Irvine Scientific) containing 2.5% plasma-derived fetal calf serum and 5% plasma-derived horse serum (Cocalico Biologicals). After 24 h, G418 (Gibco-BRL) was added, and selection was carried out for at least 3 weeks or until individual colonies could be selected and screened for chimeric protein expression.

Cell culture and neurite outgrowth assay. PC12 cells were maintained in culture in 150-cm^2 tissue culture flasks (Costar) in DMEM containing 10% horse serum, 5% fetal calf serum, and 1% Pen-strep solution (Gibco-BRL) (complete medium) at 37°C in a 5% CO_2 humidified atmosphere. GP+E86 and 293 cells were maintained in DMEM containing 10% fetal bovine serum and 1% Pen-strep solution. For neurite outgrowth assay, PC12 cells were plated in collagen-coated six-well plates (Falcon) in plasma-derived complete medium at a density of 10^5 cells/well. After 16 to 24 h, the cells were rinsed and cultured in 1% plasma-derived horse serum in the presence of various concentrations of PDGF-BB (Austral Biologicals). Cells were examined for the presence of neurites at various times. Responsive cells were defined as those bearing neurites at least 2 cell diameters in length, and their response was quantitated as described previously (48).

Immunoprecipitation and immunoblot analysis. PC12 clonal cell lines expressing chimeras or mock-infected cells were grown in plasma-derived complete medium in either 100- or 150-mm^2 collagen-coated culture dishes (Falcon) until 60 to 70% confluency was reached. The cells were starved for at least 24 h in DMEM containing 0.2% plasma-derived horse serum and then treated with PDGF-BB (30 ng/ml) for various lengths of time at 37°C as specified. The cells were washed briefly with cold phosphate-buffered saline containing 0.5 mM sodium orthovanadate and lysed in cold lysis buffer (10 mM Tris-HCl [pH 7.5], 50 mM NaCl, 1% Triton X-100, 5 mM EDTA, 0.2 mM sodium orthovanadate, 30 mM sodium pyrophosphate, 50 mM NaF, 10 μg each of aprotinin and leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride) for 15 min on ice. Protein concentration was determined with the Bradford colorimetric assay (Bio-Rad). For immunoprecipitations, lysates (1 mg) were incubated with appropriate antibodies for 2 h at 4°C followed by a 1-h incubation with protein A-Sepharose (Pharmacia). The immunocomplexes were washed four times with lysis buffer and then boiled for 5 min in sodium dodecyl sulfate (SDS) sample buffer. After analysis by SDS-polyacrylamide gel electrophoresis (PAGE), proteins were transferred to Immobilon P membranes (Millipore) by electroblotting at 60 V overnight at 4°C. The membranes were probed with different antibodies, and immunoreactivity was visualized by using appropriate horseradish peroxidase-conjugated secondary antibodies and the ECL detection system (Amersham Life Science). The following antibodies were used throughout these studies: mouse monoclonal anti-hPDGFRβ for immunoprecipitations (Genzyme) and for immunoblotting (Austral Biologicals); rabbit polyclonal anti-FGFR3, anti-FGFR1, and anti-PLCγ1 (Santa Cruz Biotechnology); rabbit polyclonal anti-Shc and mouse monoclonal antiphosphotyrosine (4G10) (Upstate Biotechnology, Inc.); mouse monoclonal anti-Grb2 (Transduction Laboratories); and phospho-specific MAPK antiserum (New England Biolabs).

RESULTS

Replacement of the extracellular domain of FGFR3 with the five-immunoglobulin (Ig)-loop extracellular domain of PDGFRβ, a distinct tyrosine kinase receptor whose ligand specificity is well characterized (47), results in a chimeric receptor (PFR3) that can be specifically activated by PDGF to produce signals through the FGFR3 intracellular domain. Since PC12 cells contain no endogenous PDGF receptors, any signaling stimulated by added PDGF will be solely derived from the transfected chimeric receptor.

To construct the PFR3 chimera (Fig. 1B), an *Eco*RI site was engineered into the FGFR3 gene immediately downstream of the stop codon and an *Mse*I site was engineered into the 5′ end of the putative TM domain by PCR mutagenesis (Fig. 1A). The presence of a single naturally occurring *Mse*I site in the portion of the gene encoding the PDGFRβ extracellular domain allowed the direct ligation of an *Eco*RI-*Mse*I fragment containing this region with the FGFR3 TM and intracellular domains contained within the PCR-generated sites (Fig. 1A). The G375C mutation was substituted into the TM region in a separate PCR using a second primer containing the *Mse*I site. Expression and autophosphorylation of the chimeras were monitored by transiently transfecting human embryonic kidney 293 cells to ensure the integrity of the constructs. A protein of the expected size (170 kDa) was observed on immunoblots containing protein immunoprecipitated by anti-PDGFRβ and

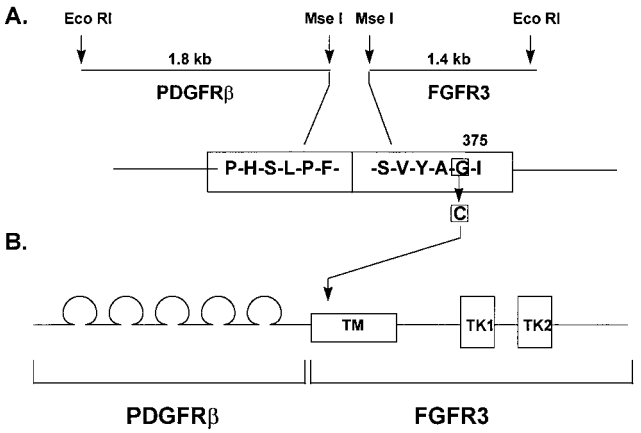

FIG. 1. (A) Schematic representation of engineered PFR3 and $PFR3_{375}$ chimeric receptor cDNAs. Constructs consist of the extracellular domain of hPDGFRβ and the TM and kinase domains of either wild-type hFGFR3 (PFR3) or the ACH G375C mutation ($PFR3_{375}$). For both constructs, the 3′ end of the extracellular domain of PDGFR was fused to the intracellular domain of FGFR3, using an *Mse*I restriction site. While this site is naturally present in PDGFR, it was introduced by PCR at the 5′ end of the TM domain of FGFR3. The single base mutation in the TM domain of FGFR3 was generated by PCR-based mutagenesis. (B) Schematic diagram of PFR3 protein. The extracellular domain contains the five Ig-like domains, and the FGFR3-derived intracellular region is comprised of the TM domain and the split tyrosine kinase domain (TK). The ACH mutation in the TM domain is shown.

detected with anti-FGFR3 antibodies (data not shown). Levels of autophosphorylation of both the normal and G375C mutant receptors were extremely low compared to levels observed with a PDGFRβ-FGFR1 (PFR1) chimera (11), and the G375C mutation was found to be only weakly activating (data not shown). This observation is consistent with the very low levels of autophosphorylation of FGFR3 observed by other groups in transient transfection assays (24, 45) and the weakly activating effect produced by the G380R ACH mutation upon the proliferation of BaF3 cells (24).

To determine receptor phosphorylation levels, protein lysates from various PC12 clonal cell lines were immunoprecipitated with an anti-PDGFR monoclonal antibody and immunoblotted with an antiphosphotyrosine antibody. As shown in Fig. 2A, various levels of autophosphorylation of the receptor were observed, corresponding to the number of receptors present (Fig. 2B). Higher levels of receptor autophosphorylation are seen in the PFR3-expressing cell lines B_1, B_6, and C_{13} than in the $PFR3_{375}$-expressing cell lines A_2, B_{6II}, and C_3; there is correspondingly less PFR3 receptor protein expressed for the level of autophosphorylation observed (Fig. 2A and B). In most cases, phosphorylation is completely ligand responsive. In the B_1 and B_6 cell lines, however, there is a low level of ligand-independent phosphorylation that correlates with the level of receptors expressed in a given clonal cell line; the higher the level of expression is, the more receptor autophosphorylation is observed in the absence of ligand. This correlation of autophosphorylation with receptor expression levels has also been observed for clonal lines transfected with a PFR1 chimera (11). As a control for receptor expression, the same protein bands were shown to react with anti-FGFR3 as well as anti-PDGFR antibodies (Fig. 2C). Clonal cell lines expressing PFR3 and $PFR3_{375}$ (Fig. 2A and B) were selected for high receptor expression (B_6 and C_3, respectively) and low receptor expression (C_{13} and B_{6II}), and both types were used in further experiments.

To rule out any transphosphorylation event between endogenous FGFR1, the most abundant receptor of this family, and exogenous chimeric PFR3, the clonal cell line B6 was stimulated with PDGF. Cell lysates were then made and immunoprecipitated with an anti-FGFR1 antibody followed by antiphosphotyrosine immunoblotting. As expected, no phosphorylation of FGFR1 was detected under these conditions, thus excluding the possibility of endogenous FGFR1-mediated signaling events in these cells (data not shown).

When clonal PC12 cell lines expressing high or low levels of PFR3 (B_6 and C_{13}) or $PFR3_{375}$ (C_3 and B_{6II}), respectively, were stimulated with various concentrations of PDGF, neurite outgrowth was observed in all clones examined in a dose- and time-dependent fashion. After 48 h, 60 to 90% of clonal cells displayed a network-like pattern of processes when exposed to PDGF at 30 ng/ml, the maximal concentration used in these studies (Fig. 3A and D). However, as shown in Fig. 3B and C, both clones expressing different levels of the chimera containing the ACH mutation produced neurites with a shorter response time than those expressing the wild-type chimeric receptor. After 8 h, 42 and 19% of C_3 and B_{6II} cells are responsive to 10 ng of PDGF/ml, compared with 6 and 1% of B_6 and C_{13}, respectively. After 24 h, at least 76% of C_3 and B_{6II} cells were responsive, compared with 28 and 47% of B_6 and C_{13} cells, respectively.

The protein phosphorylation patterns of several of the clonal PC12 cell lines expressing PFR3 and $PFR3_{375}$ were examined by Western analysis of whole-cell lysates. Phosphorylation of several cellular proteins following PDGF stimulation was observed (Fig. 4A). There are subtle differences between the PFR3- and $PFR3_{375}$-expressing cells, but the overall phosphorylation profiles of the two are very similar. The identification of the 170-kDa protein as the phosphorylated chimera was confirmed by reprobing the same blot with anti-PDGFR (Fig. 4B). Both anti-PDGFR antibodies in these studies recognize two proteins of approximately 170 and 130 kDa; the upper band most likely represents the mature form, while the lower band

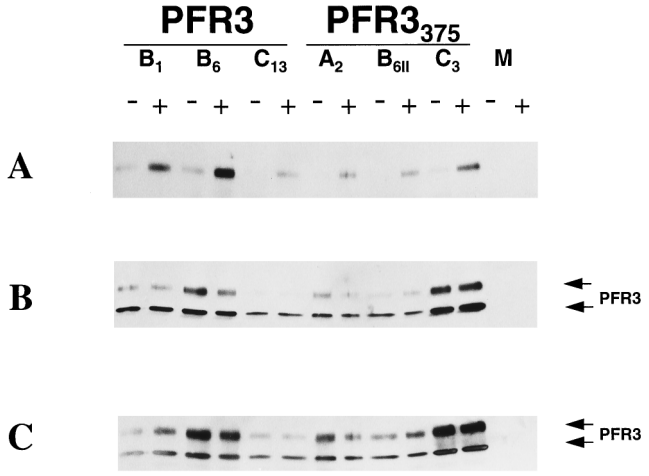

FIG. 2. Tyrosine phosphorylation of PFR3 and $PFR3_{375}$ chimeric receptors. (A) PC12 clonal cell lines expressing PFR3 (B_1, B_6, and C_{13}) and $PFR3_{375}$ (A_2, B_{6II}, and C_3) and mock-infected cells (M) were incubated at 37°C for 5 min without (−) or with (+) PDGF (30 ng/ml). Lysates were incubated with an anti-PDGFR antibody, and immunoprecipitates were separated by SDS-PAGE (7.5% gel) and analyzed by immunoblotting with an antiphosphotyrosine antibody. The antiphosphotyrosine blot was stripped and reprobed first with an anti-PDGFR monoclonal antibody (B) and subsequently with anti-FGFR3 antiserum (C). The antibody recognizes two proteins of approximately 130 and 170 kDa, probably representing precursor and mature forms of the chimeric receptors, as indicated by arrows.

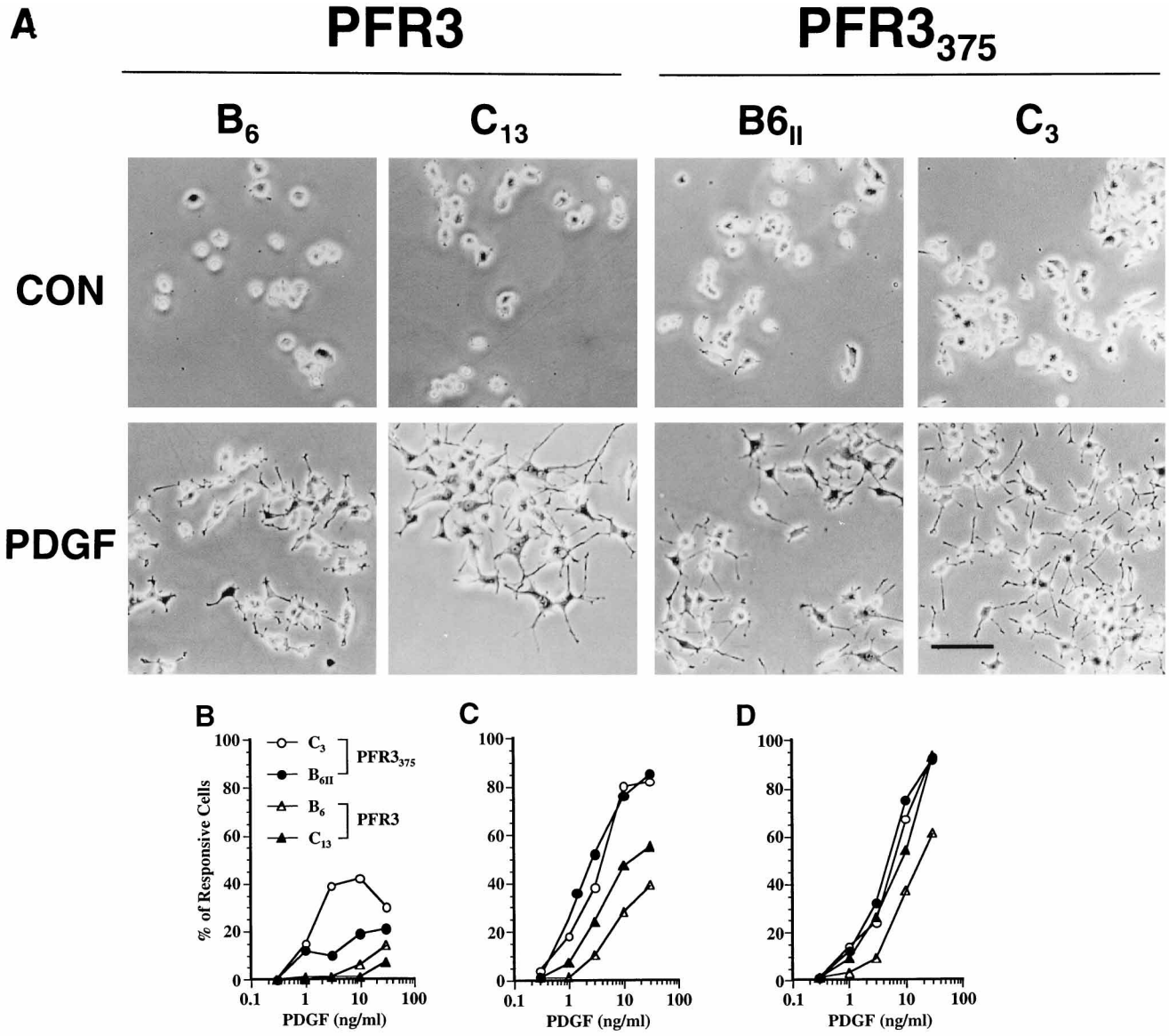

FIG. 3. (A) The induction of neurite outgrowth in PC12 clonal cell lines expressing PFR3 (B_6 and C_{13}) and $PFR3_{375}$ (B_{6II} and C_3). Cells were maintained in DMEM containing 1% plasma-derived horse serum in the presence of medium alone (CON) or PDGF (30 ng/ml) for 24 h. Bar, 100 μm. (B) Dose-dependent induction of neurites in PC12 clonal cell lines expressing PFR3 and $PFR3_{375}$ by PDGF. The four different clones (B_6, C_{13}, C_3, and B_{6II}) were cultured in the presence of various concentrations of PDGF (0.3, 1, 3, 10, and 30 ng/ml). After 8, 24, and 48 h of treatment, cells were examined for the presence of neurites. Values are averages of quadruplicate determinations.

likely represents the precursor form of the receptor (31). In addition, upon ligand stimulation, the phosphorylation of MAPK/ERK was also established with a specific antibody that recognizes the $p44^{ERK1}$ and $p42^{ERK2}$ phosphorylated forms (Fig. 4C).

Cellular target proteins, such as PLCγ, as well as adapter proteins, including Grb2 and Shc, have been shown to bind tyrosine autophosphorylation sites in the cytoplasmic domains of many receptor tyrosine kinases. FGFR1 contains a PLCγ binding site (14) and signals through mechanisms involving PLCγ, Grb2, and Shc (37). The migration patterns of proteins phosphorylated in response to stimulation with PDGF in this chimeric system (Fig. 4A) support the possibility that these proteins are also involved in signaling by FGFR3. Cellular proteins from PC12 clonal cell lines were immunoprecipitated with anti-PLCγ antibody, immunoblotted, and probed with an antiphosphotyrosine antibody. In all PFR3- and $PFR3_{375}$-expressing cells analyzed, PLCγ, migrating at approximately 145 kDa, was found to be phosphorylated upon stimulation with PDGF in a ligand-dependent manner (Fig. 5A). No phosphorylated PLCγ was detected in cells containing the stably transfected vector only, indicating that phosphorylation is derived from the activated chimeric receptors. Finally, no differences in PLCγ activation were observed between normal and mutant receptor cell lines when levels of phosphorylated protein were compared to anti-PLCγ immunoreactive protein levels (Fig. 5B). Although PLCγ is activated by PFR3 autophosphorylation, coimmunoprecipitation with PFR3 was not observed (data not shown).

The adapter protein Shc is also phosphorylated in response

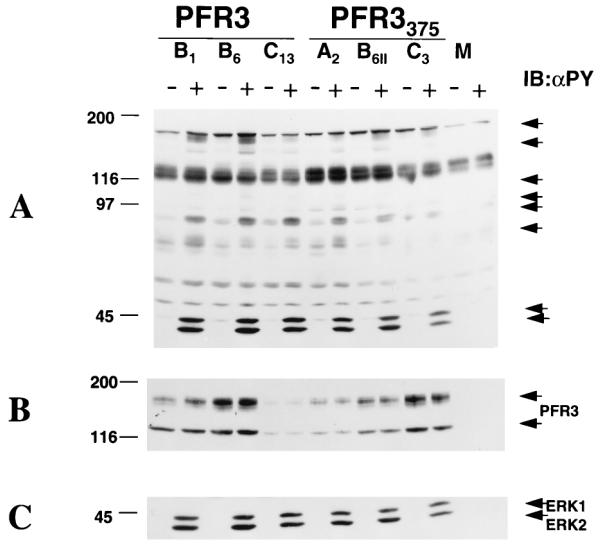

FIG. 4. Stimulation of protein tyrosine phosphorylation in PC12 clonal cell lines expressing PFR3 (B_1, B_6, and C_{13}) and $PFR3_{375}$ (A_2, B_{6II}, and C_3). (A) Cells were incubated at 37°C for 5 min without (−) or with (+) PDGF. Lysates were separated by SDS-PAGE (7.5% gel), transferred to Immobilon P membranes, and immunoblotted with an antiphosphotyrosine antibody. The positions of polypeptides that appear phosphorylated following PDGF addition are indicated by arrows. The migration of molecular mass standards (in kilodaltons) is shown on the left. The antiphosphotyrosine blot was stripped, and the top part was reprobed with a monoclonal anti-PDGFR antibody (B). The arrows point to the two forms of the chimeric receptors. (C) The bottom part was reprobed with an anti-phospho-MAPK antiserum. The antiserum recognizes the phosphorylated forms of both $p44^{ERK1}$ and $p42^{ERK2}$, as indicated by arrows.

to PDGF stimulation in PC12 cell lines containing either PFR3 or $PFR3_{375}$. In cell lines expressing either high (B_6 and C_3) or low (C_{13} and B_{6II}) amounts of chimeric receptor (Fig. 4), immunoprecipitation of lysates with an anti-Shc antibody followed by immunoblotting with an antiphosphotyrosine antibody showed the 52-kDa isoform of Shc to be phosphorylated to a greater degree in both PFR3- and $PFR3_{375}$-expressing cell lines upon PDGF addition (Fig. 6A); the 46-kDa isoform of Shc is obscured by a cross-reacting band (this protein is observed even in lysis buffer [data not shown]). However, Shc is

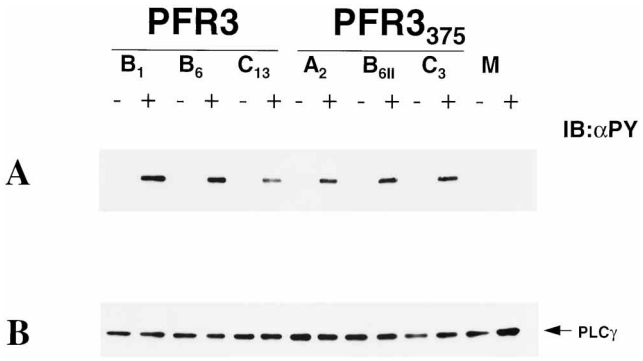

FIG. 5. Tyrosine phosphorylation of PLCγ in PC12 clonal cell lines expressing PFR3 (B_1, B_6, and C_{13}) and $PFR3_{375}$ (A_2, B_{6II}, and C_3) and in mock-infected cells (M). Cells were incubated at 37°C for 5 min without (−) or with (+) PDGF. (A) Lysates were incubated with a polyclonal anti-PLCγ antibody; the immunoprecipitates were separated by SDS-PAGE (7.5% gel) and analyzed by immunoblotting with an antiphosphotyrosine antibody and, after stripping and reprobing, with an anti-PLCγ antiserum (B). The arrow points to the 145-kDa form of PLCγ.

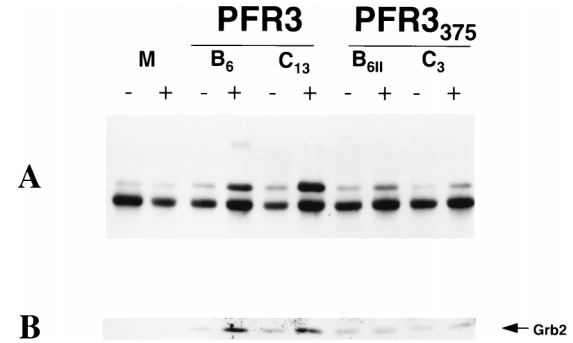

FIG. 6. Tyrosine phosphorylation of Shc and its association with Grb2 in PC12 clonal cell lines expressing PFR3 (B_6 and C_{13}) and $PFR3_{375}$ (B_{6II} and C_3) and in mock-infected cells (M). Cells were incubated at 37°C for 5 min without (−) or with (+) PDGF. Lysates were prepared and incubated with an anti-Shc antiserum. (A) Immunoprecipitates were separated by SDS-PAGE (10% gel) and analyzed by immunoblotting with an antiphosphotyrosine antibody or with a mouse anti-Grb2 antibody (B). In the antiphosphotyrosine blot, the detection of the 46-kDa isoform of Shc is obscured by cross-reaction of the horseradish peroxidase-conjugated anti-mouse IgG with Ig heavy chains in the Shc immunoprecipitates.

less phosphorylated in cell lines containing $PFR3_{375}$ than in those containing PFR3. Higher levels of the adapter protein Grb2 were coimmunoprecipitated with Shc following PDGF stimulation as well (Fig. 6B); however, there are lower levels of Grb2 and Shc association in the $PFR3_{375}$-containing lines than the PFR3-containing lines.

To rule out differences in the duration of cellular protein phosphorylation, cell lines expressing PFR3 (B_6 and C_{13}) and $PFR3_{375}$ (C_3 and B_{6II}) were incubated with PDGF from 5 min to 24 h. As before, subtle differences in phosphorylation patterns were observed (Fig. 7A) between the PFR3- and $PFR3_{375}$-containing cell lines. The most striking difference, however, is the duration of MAPK/ERK activation (Fig. 7C). In both the C_3 and B_{6II} cell lines containing $PFR3_{375}$, phosphorylation of ERK1 and ERK2 falls off dramatically by 6 h; however, phos-

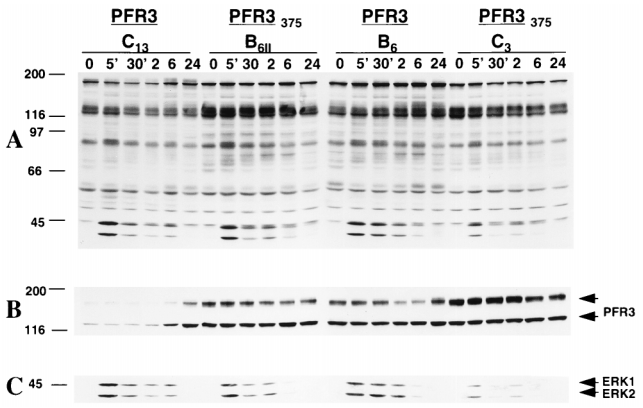

FIG. 7. Time course of protein tyrosine phosphorylation in PC12 clonal cell lines expressing PFR3 (C_{13} and B_6) and $PFR3_{375}$ (B_{6II} and C_3). Cells were incubated at 37°C with PDGF for 0, 5, and 30 min and 2, 6, and 24 h as indicated. (A) Total cell extracts were separated by SDS-PAGE (7.5% gel), transferred to Immobilon P membranes, and immunoblotted with an antiphosphotyrosine antibody. The migration of molecular mass standards (in kilodaltons) is shown on the left. The antiphosphotyrosine blot was stripped, and the top part was reprobed with a monoclonal anti-PDGFR antibody (B). The arrows point to the two forms of the chimeric receptors. (C) The bottom part of the blot was reprobed with an anti-phospho-MAPK antiserum. The antiserum recognizes only the phosphorylated forms of both $p44^{ERK1}$ and $p42^{ERK2}$, as indicated by arrows.

phorylation persists at that time in the PFR3-containing cell lines, B_6 and C_{13}. Despite this difference in time dependence, there was no direct correlation between duration of sustained phosphorylation and receptor levels (Fig. 7B) in these cell lines, suggesting that the decrease in MAPK/ERK phosphorylation is not simply a function of receptor levels. Neurite outgrowth was found to be more robust and rapid in the $PFR3_{375}$-containing cell lines (Fig. 3), in which MAPK/ERK phosphorylation falls off more quickly than in the PFR3-containing cell lines.

DISCUSSION

As judged by molecular cloning experiments and mRNA expression studies of the PC12 cell isolate used in these studies, the most abundant FGFR is FGFR1, and it is expressed predominantly with the three-Ig-loop extracellular domain, although the two-Ig-loop form is also present (29). In addition, significant FGFR4 levels can also be detected. Only small amounts, at best, of FGFR2 and -3 are normally expressed, and it may be presumed that PC12 responses to basic FGF (FGF2) are, in this case, primarily mediated through FGFR1. This is likely true with most PC12 cell lines. In support of this view, the PFR1 chimera containing the extracellular domain of the PDGFR and the TM and intracellular domains of FGFR1, when stably transfected into PC12 cells and activated by adding PDGF, produces molecular and cellular responses that mimic stimulation of native cells by FGF ligands (11), including activation of the RAS-MAPK/ERK pathway followed by characteristic morphological changes (neurite outgrowth). Recent studies (49, 50) have shown that native PC12 cell differentiation apparently requires mitotic suppressor activity and a transcription-independent activation step, but it is unclear what the role of MAPK/ERK in either stage is. Thus, the observations reported here that similar hPFR3 chimeras produce essentially the same molecular and morphological responses as the PFR1 chimera indicate that FGFR1 and FGFR3 function, in this context, in highly similar manners. Thus, although FGFR3 probably does not contribute greatly to FGF2 responses because of low abundance, it is nonetheless capable of doing so.

These results contrast to some degree with recent published data (18) showing that only stable transfection of FGFR1 or of an FGFR3-FGFR1 chimera in a PC12 subline (fnr-PC12), which is defective in neurite outgrowth in response to acidic FGF (FGF1) and expresses very low levels of endogenous FGFR1, efficiently restored neurite outgrowth responses to this ligand. FGF1 induction of fnr-PC12 cells stably transfected with FGFR3 resulted in much less efficient neurite outgrowth and tyrosine phosphorylation of cellular proteins, including a less persistent activation of MAPK/ERK, leading these authors to conclude that PC12 cell responses are mediated mainly via FGFR1. The essentially equivalent responses of stably transfected PFR1 (11) and PFR3 at similar expression levels (either low or high) described here establish that FGFR3 is a germane signal-transducing entity in PC12 cells and is potentially capable of contributing to responses induced by appropriate ligands. While there is not a certain explanation for the differences in the observations described here and those by Lin et al. (18), they are likely due in part to the two types of PC12 cells used. The wild-type PC12 cells used in these studies are considerably more responsive than the fnr-PC12 line, which showed ~5% responsive cells in the same time period that our wild-type cells gave 100% responsive cells when stimulated with nerve growth factor. They were comparably more responsive to FGF ligands as well. Furthermore, these workers (18) saw responses to transfected FGFR3, although the responses were more sluggish than those induced by FGFR1 (as an FGFR3-FGFR1 chimera). This observation may reflect quantitative rather than qualitative differences. Clearly, in the PC12 cell isolate used in our studies, FGFR1 and FGFR3 appear to be equal in their abilities to promote differentiative responses.

Although a more detailed characterization of either the PFR1 or PFR3 response will be required to fully define the specific signaling pathways necessary to produce a differentiated phenotype, the studies reported herein provide information regarding the recruitment of various signaling molecules upon PFR3 activation. We have shown that the chimeric receptor (PFR3) is phosphorylated in a ligand-dependent manner and leads to the phosphorylation, also ligand dependent, of cellular proteins such as MAPK/ERK, PLCγ, and Shc, the last coimmunoprecipitating with Grb2. The activation of the RAS-MAPK/ERK pathway and phosphorylation of PLCγ by FGFR3 are consistent with the observed morphological responses (35, 37). Additional analyses will be required to evaluate the bases for the subtle differences in phosphorylation patterns observed and to identify other potential downstream targets in these and other cell lines containing either normal or mutant PFR3 constructs. It is appropriate to note that the responses observed for PFR3 in PC12 cells could not have been entirely predicted. It has been suggested that FGFR3 is a negative regulator of long-bone growth; i.e., the activation of the receptor inhibits the continued development of these structures (9, 10). Transgenic mice lacking FGFR3 show abnormally extensive long-bone growth that reflects the loss of FGFR3 regulatory activity. Although the responses of PFR3 observed in PC12 cells may be cell type specific, our results suggest that FGFR3 should function in chondrocytes to induce MAPK/ERK and PLCγ activation and that these or other responses may be interpreted by the chondrocyte machinery in a negative regulatory fashion.

The hypothesis that FGFR3 controls long-bone development by acting as a negative regulator is supported by two studies that show at least partial ligand-independent responses when mutations characteristic of ACH are introduced in different model contexts (24, 45). However, neither of these studies actually measured signal transduction from the FGFR3 kinase domain. In the experiments described here, stably transfected PC12 cells bearing the PFR3 chimera with one TM mutation that causes ACH did not show substantial ligand independence but did respond more rapidly and robustly. Interestingly, they showed decreased activation of MAPK/ERK and Shc as well as decreased Grb2-Shc association. These findings are consistent with the view that the cells bearing the mutated chimera are already partially activated and therefore require less ligand-induced activation. This loosening of ligand control would support the hypothesis that ACH results from partial autoactivation of FGFR3, thus preventing normal long-bone development. However, these results must be interpreted with care. Autoactivation can result from overexpression of receptors, and indeed, cells expressing high numbers of PFR3 do show increased ligand-independent autophosphorylation. Neurite outgrowth still remains ligand dependent, and the effects with the G375C mutant are seen at both high and low levels of receptor. Importantly, neither expression levels nor the mutation changes the qualitative pattern of tyrosine phosphorylation, although there may be significant quantitative differences. At the same time, there remains an incomplete understanding of the mechanisms of differentiation in PC12 cells, and the exact roles of MAPK/ERK and the other transducers are still to be resolved. Lower levels of activation of MAPK/ERK, in the presence of other signaling molecules, might still

produce a stronger and more robust response than cells that lacked (or had lesser amounts) of these latter entities. Although unlikely in this study, it is formally possible that no stable transfectants with full ligand independence were isolated because such cells would probably undergo irreversible differentiation before selection.

The ACH mutation examined in this study (G375C) is less common than G380R, which also occurs in the TM region. Webster and Donoghue (45) have reported that this latter alteration causes a significant increase in autophosphorylation when the full-length receptor is transiently transfected into 3T3 cells. In addition, the G380R mutation can cause an autoactivation of Neu analogous to that by a previously described (46) TM mutation that converts this receptor to a transforming oncogene when the mutated TM segment of FGFR3 is substituted for the TM segment of Neu. They argue that the position of this ACH mutation is analogous to that of the Val-Glu change found in the *neu* oncogene product and that it may function, as has been proposed in that instance (38), to form an H bond with the backbone of the parallel helix of the second receptor protomer, thus stabilizing the dimer and lessening or eliminating ligand control. In support of this view, they show that other amino acids with side chains capable of forming H bonds can substitute (to some degree) for the arginine to activate the Neu-FGFR3 chimera. The extent to which arginine, which will still bear a positive charge, or a cysteine thiol will function like a protonated carboxyl group is unclear. Furthermore, its position five residues upstream ensures that it will not have the same juxtaposition as the G380R to the other helix, unless they are both turned substantially relative to each other (approximately 120°) around the axis of the helix. It also cannot be certain that residue 375 resides in the membrane bilayer, as it occurs very near the putative extracellular-TM interface. The presence of a sulfhydryl group allows for the possibility of covalent dimers (in the homozygous condition; see below), but no such species was observed with the chimera. Thus, the molecular bases that create the same ACH pathology with these two mutations are very likely different. Importantly, we have also examined the behavior of the G380R mutation in PFR3 in stably transfected PC12 cells and found that it is essentially the same as G375C (43). However, other mutations in the kinase domain that produce stronger phenotypes (and more severe pathologies) show much stronger ligand independence, indicating that this paradigm is capable of demonstrating autoactivation of this receptor (43).

ACH is an autosomal dominant condition, manifested in individuals who are heterozygous for the mutation. Homozygotes show a much more severe pathology reminiscent of the neonatal lethal skeletal dysplasia TD (16, 30). These situations, as they likely occur in chondrocytes, are diagrammed in Fig. 8A and are based on two assumptions: (i) ligand (FGF) induces dimeric structures that are the active signaling units and (ii) FGFR1 and FGFR3 can form heterodimeric structures (via extracellular domain contacts). It is likely that if FGFR2 species are present, they will also form such structures, which would, of course, further complicate the picture. (It should also be noted that multiple splice variants of both FGFR1 and FGFR2 have been reported [12, 14], and to the extent that they occur in these cells, they could also contribute to the spectrum of receptors expressed.) As indicated, there are six potential signaling entities present in heterozygous ACH, the relative contributions of which are presently unknown. If, as has been suggested (10), FGFR3 plays the major role in postnatal bone development as judged by a corresponding loss of *fgfr-1* gene expression, the situation would be reduced to that enclosed by the broken box in Fig. 8A (assuming that FGFR2 is also absent). Thus, the FGFR3 molecules in ACH could be entirely heterodimeric, an equal mixture of native and mutant homodimers, or some combination of the three forms shown. In the homozygous state, only three forms are possible; if FGFR1 is absent, only a single signaling species will be present.

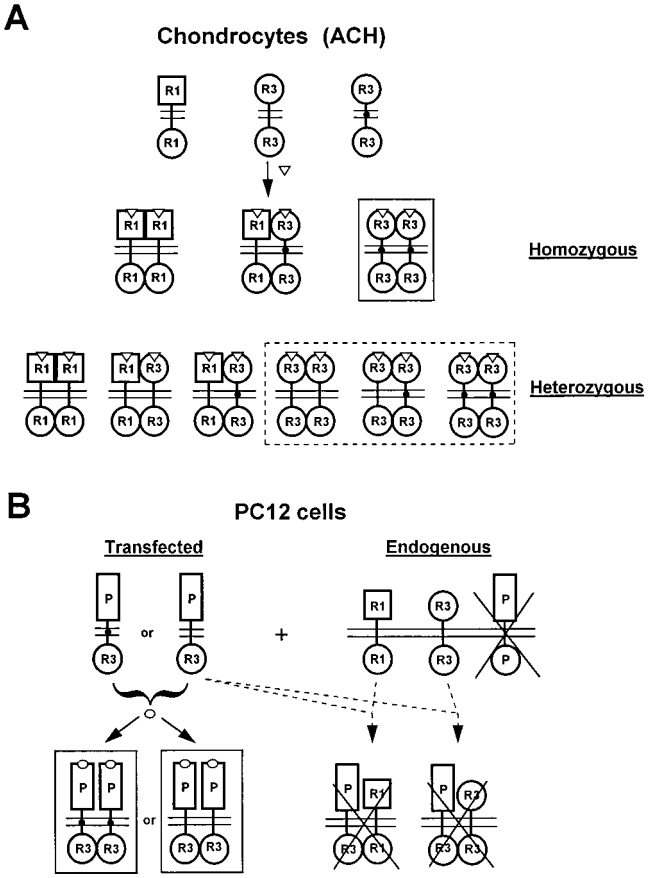

FIG. 8. Schematic profile of potential FGF (or chimeric) receptor expression and activation in chondrocytes in ACH (A) and PC12 cells in these experiments (B). Notation: R1, FGFR1; R3, FGFR3; P, PDGFR; ═, plasma membrane; ●, TM mutation (ACH); ▽, FGF; ○, PDGF; ×, species does not occur. See the text for further description.

Figure 8B illustrates the conditions of the experiments described herein. The use of chimeric receptors (and the absence of PDGFR in PC12 cells) eliminates any heterodimeric interactions, including any with FGFR1 (which was also demonstrated experimentally). Thus, in any given experiment, only a single signaling entity was present. Clearly, these data duplicate the homozygous condition (Fig. 8A). In contrast, the previously reported experiments, using native or chimeric FGFR3 receptors in various cell systems (including PC12 cells) (18, 24, 45), are all complicated by the potential for heterodimer formation with endogenous receptors (and mimic to some degree the situation shown in the bottom row of Fig. 8A).

The relatively mild effect of the G375C mutation on receptor function observed in these experiments, particularly considering that it is analogous to the more severe homozygous condition, is perhaps somewhat surprising and suggests that these chimeras (which use the extracellular domain of the PDGFR) may not be fully able to manifest the expected ligand independence. The importance of this portion of receptor tyrosine kinases in terms of ligand-induced dimer formation (14, 15) and as the site of mutations producing other skeletal dysplasias

is well appreciated (22). Thus, it may be that the full effect of the TM mutation will be observed only in certain cases. The Neu receptor is well-known to convert to a ligand-independent state by such a mutation (46), and Webster and Donoghue (45) convincingly demonstrate that at least one ACH mutation will generate essentially the same response when inserted into this molecule. This finding is also supported by the observations that other human disease mutations affecting skeletal growth are found in FGFR1 and FGFR2 (3, 21, 22). In fact, a dependence on the specific extracellular domain may also explain in part why TM mutations have not been more commonly found in other human pathologies. It should, however, be noted that some of the more severe FGFR3 mutations have been reported occur in the intracellular domain and these may not be so dependent on the nature of the extracellular structure. This is consistent with our observations that a kinase domain mutant at position 650 is strongly ligand independent (in chimeric form) as tested in the PC12 cell paradigm (43). Further experiments, now in progress, will be required to determine the importance of the extracellular domain to the manifestation of the molecular effects of these mutations.

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L.M.T. and S.R. contributed equally to this work.

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