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Constitutive activation of fibroblast growth factor receptor 3 by the transmembrane domain point mutation found in achondroplasia

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Achondroplasia, the most common genetic form of dwarfism, is an autosomal dominant disorder whose underlying mechanism is a defect in the maturation of the cartilage growth plate of long bones. Achondroplasia has recently been shown to result from a Gly to Arg substitution in the transmembrane domain of the fibroblast growth factor receptor 3 (FGFR3), although the molecular consequences of this mutation have not been investigated. By substituting the transmembrane domain of the Neu receptor tyrosine kinase with the transmembrane domains of wild-type and mutant FGFR3, the Arg380 mutation in FGFR3 is shown to activate both the kinase and transforming activities of this chimeric receptor. Residues with side chains capable of participating in hydrogen bond formation, including Glu, Asp, and to a lesser extent, Gln, His and Lys, were able to substitute for the activating Arg380 mutation. The Arg380 point mutation also causes ligand-independent stimulation of the tyrosine kinase activity of FGFR3 itself, and greatly increased constitutive levels of phosphotyrosine on the receptor. These results suggest that the molecular basis of achondroplasia is unregulated signal transduction through FGFR3, which may result in inappropriate cartilage growth plate differentiation and thus abnormal long bone development. Achondroplasia may be one of a number of congenital disorders where constitutive activation of a member of the FGFR family leads to developmental abnormalities.

Keywords: achondroplasia/FGFR3/Neu/receptor activation/transmembrane domain

Introduction

Achondroplasia is the most common form of genetic dwarfism, with an estimated frequency of 1/15 000 to 1/77 000 births. This disorder is transmitted in an autosomal dominant fashion with complete penetrance, although 80–90% of cases arise from spontaneous mutations (Oberklaid *et al.*, 1979; Andersen and Hauge, 1989; Stoll *et al.*, 1989; Iannotti *et al.*, 1994). The clinical features of heterozygous achondroplasia are very consistent among patients, and include proximal shortening of the extremities and relative macrocephaly. The underlying mechanism of the disorder is believed to be a defect in the maturation of the cartilaginous growth plate of long bones (Ponseti, 1970; Maynard *et al.*, 1981; Iannotti *et al.*, 1994).

The gene altered in achondroplasia has been mapped to chromosome 4p16.3 by linkage analysis (Francomano *et al.*, 1994) and was recently identified as the fibroblast growth factor receptor 3 (FGFR3) gene. In all sporadic and familial achondroplasia cases examined, a missense mutation at a CpG dinucleotide has converted Gly380 to an Arg within the transmembrane domain of FGFR3 (Rousseau *et al.*, 1994; Shiang *et al.*, 1994). This guanosine 1138 nucleotide has been described as the most mutable nucleotide to date in the human genome (Bellus *et al.*, 1995).

FGFRs are high affinity signalling receptors for fibroblast growth factors (FGFs), a family of polypeptides with pleiotropic actions, including an ability to promote proliferation or differentiation depending on the cell type and developmental stage (Johnson and Williams, 1993). High-level expression of FGFR3 has been observed in the developing mouse central nervous system, in prebone cartilage rudiments, as well as in resting cartilage during endochondral ossification (Peters *et al.*, 1993), although the FGF ligand(s) expressed in these tissues have not been well characterized.

The molecular consequences of introducing a hydrophilic Arg residue into the hydrophobic transmembrane domain of FGFR3 have not been investigated. It was demonstrated for rat Neu/erbB-2, a member of the epidermal growth factor receptor (EGFR) family, that a missense mutation within its transmembrane domain, which changes Val664 to Glu, leads to constitutive receptor activation (Bargmann *et al.*, 1986). The analogous substitutions in human Neu/erbB-2 (Segatto *et al.*, 1988), *Drosophila* EGFR (Wides *et al.*, 1990) and human EGFR (Miloso *et al.*, 1995), have also been shown to be activating. Structural modelling of the normal Neu transmembrane domain suggests that it exists in an α-helical conformation, with dimeric packing interactions stabilized by weak van der Waals forces (Sternberg and Gullick, 1989; Gullick *et al.*, 1992). The oncogenic activation of Neu has been proposed to involve stabilization of the receptor in a dimeric conformation due to interaction of the protonated Glu664 side chain of one transmembrane domain with a carbonyl oxygen of the other subunit to form a hydrogen bond (Sternberg and Gullick, 1989). The increased formation of ligand-independent dimers results in elevated receptor tyrosine kinase activity and cellular transformation (Stern *et al.*, 1986, 1988; Weiner *et al.*, 1989). In support of this model, residues that are capable of participating in hydrogen bond formation, including Gln and to a lesser extent Asp and Tyr, but not aliphatic residues, could functionally substitute for Glu at position 664 (Bargmann and Weinberg, 1988b). The failure of Lys or His substitutions to activate Neu is an inconsistency

that could be explained by specific steric constraints. The transmembrane domain of activated Neu, expressed in the absence of the extracellular and intracellular domains, is able to bind specifically to full-length activated Neu and compete for homodimerization, thus blocking tyrosine kinase activation and oncogenic transformation (Lofts *et al.*, 1993). Also consistent with a model of transmembrane-mediated dimerization is the observation that substitution of the insulin receptor transmembrane domain with the activated Neu (Glu664) transmembrane domain results in insulin-independent receptor dimerization and activation (Yamada *et al.*, 1992; Cheatham *et al.*, 1993). These results suggest that transmembrane domains contain critical structural information that can modulate receptor-mediated signal transduction.

The Gly380→Arg mutation in the transmembrane domain of FGFR3 has been proposed by others to have a major effect on FGFR3 signalling, either by preventing receptor migration through the membrane, disrupting receptor dimerization with itself or with other FGFRs, or affecting the tyrosine kinase activity of the receptor (Rousseau *et al.*, 1994; Shiang *et al.*, 1994). Identifying the molecular consequences of this mutation is an important first step in understanding the role of FGFR3 signalling in normal and abnormal cartilage and bone development. We proposed that the point mutation involved in achondroplasia constitutively activates receptor signalling, in a manner analogous to the point mutation which activates Neu. To test this hypothesis, we have generated chimeric receptors with the Neu transmembrane domain substituted by the transmembrane domain of either normal FGFR3 or mutant FGFR3, allowing the Neu tyrosine kinase activity to serve as a reporter for dimerization of the transmembrane domains. Furthermore, we have examined the effect of the Arg380 point mutation on ligand-independent tyrosine kinase activity of FGFR3 itself. Results presented here suggest that the molecular basis of achondroplasia is unregulated signal transduction through constitutively activated FGFR3, which is predicted to result in abnormal cartilage growth plate proliferation and differentiation.

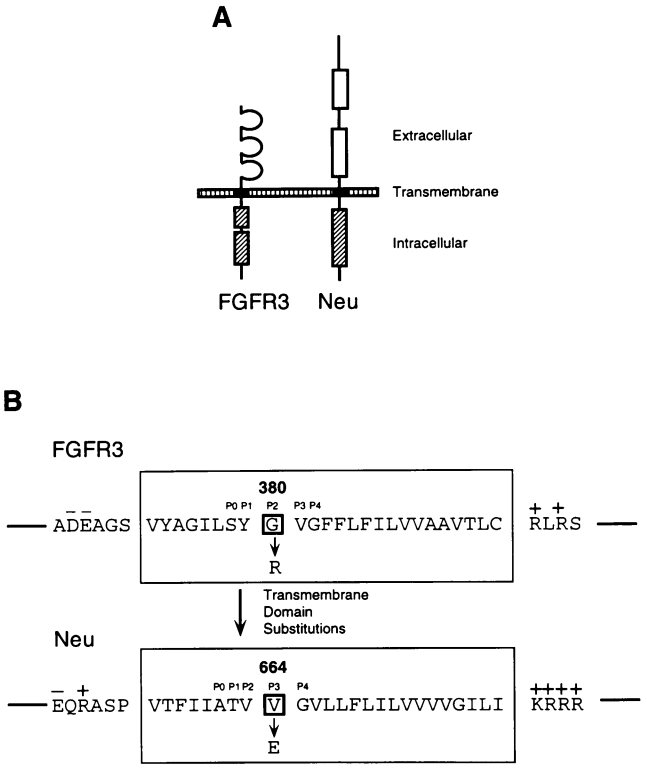

Fig. 1. (**A**) Schematic representation of FGFR3 and Neu receptor tyrosine kinases. Immunoglobulin-like domains are represented by loops, transmembrane domains by solid boxes and tyrosine kinase domains by striped boxes. Modified from Fantl *et al.* (1993). (**B**) Alignment of the transmembrane domains of FGFR3 and Neu. For the chimeric receptor constructs, the wild-type and mutant transmembrane domains of FGFR3 were substituted in place of the Neu transmembrane domain, as indicated by the boxed residues. The position of the activating mutations in both transmembrane domains is identical, relative to the positively (+) and negatively (–) charged residues flanking the transmembrane domain. The location of the five-residue sequence motif found in many receptor tyrosine kinase transmembrane domains (Sternberg and Gullick, 1990), is indicated by P0–P4. The mutation Val664→Glu provides for oncogenic activation of Neu; in FGFR3, mutation of Gly380→Arg gives rise to achondroplasia.

Results

Generation and expression of Neu/FGFR3 chimeric receptors

The EGFR family, of which rat Neu is a member, is characterized by an extracellular ligand binding domain which contains two cysteine-rich subdomains, a single membrane-spanning domain and an intracellular contiguous tyrosine kinase domain. FGF receptors possess three immunoglobulin-like motifs in their extracellular domain, a single transmembrane domain and an intracellular 'split' tyrosine kinase domain (Figure 1A) (Fantl *et al.*, 1993). Alternative splicing of the third immunoglobulin-like domain has been demonstrated for FGFR3, which affects its binding affinity for FGF ligands (Chellaiah *et al.*, 1994). Despite these structural differences, growth factor receptors of both families share an ability to dimerize in response to ligand binding, resulting in the activation of their intrinsic tyrosine kinase activities and the association of proteins which transmit biological signals (Ullrich and Schlessinger, 1990).

The Neu tyrosine kinase can be activated in a ligand-independent manner by a single Val664→Glu point mutation in its transmembrane domain, which has been proposed to stabilize receptors in a dimeric conformation due to inter-receptor hydrogen bond formation mediated by the carboxyl group of the Glu residues (Sternberg and Gullick, 1989). The relative positions of the Val664→Glu substitution in oncogenic Neu and the Gly380→Arg substitution in mutant FGFR3 are seemingly identical (Figure 1B), if defined by the first basic residue that terminates their respective hydrophobic transmembrane sequences (residue 681 for Neu, residue 397 for FGFR3). This observation suggests that these residues may reside in equivalent positions in the transmembrane α-helix. On the other hand, both Neu and FGFR3 possess a five-residue sequence motif that has been identified in the transmembrane domains of many receptor tyrosine kinases, and which has been proposed to mediate receptor dimerization (Sternberg and Gullick, 1990). This motif is characterized by the following pattern: position P0 exhibits a small side chain, such as Gly, Ala, Ser, Thr or Pro; P3 requires an

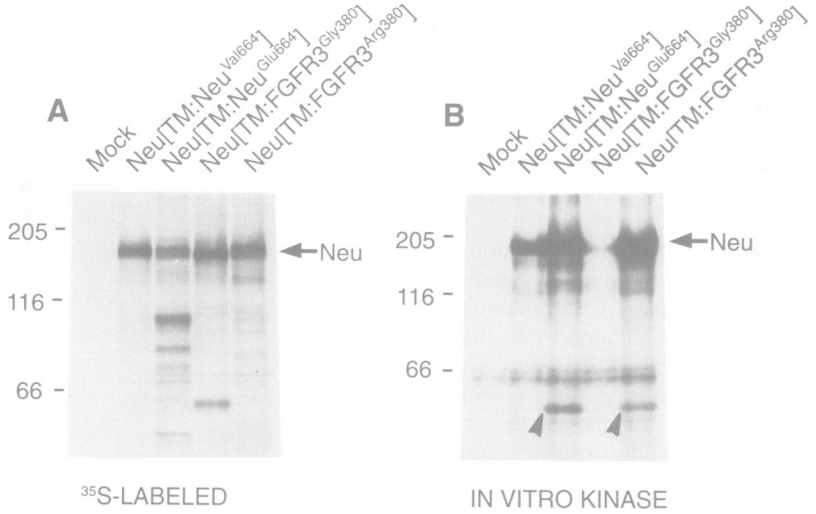

Fig. 2. (**A**) Metabolic labelling of Neu–FGFR3 chimeric receptors. Constructs were generated wherein the extracellular and intracellular domains were entirely derived from Neu and the transmembrane domains were derived from normal or mutant Neu, or normal or mutant FGFR3, as indicated within brackets. NIH 3T3-derived cell lines expressing the indicated constructs were biosynthetically labelled with [^{35}S]Cys and [^{35}S]Met and lysates were immunoprecipitated with mAb 7.16.4 directed against Neu. The arrow indicates the position of the chimeric receptor. Similar levels of each receptor protein were observed. Molecular weight markers in kDa are indicated. (**B**) Autophosphorylation of Neu–FGFR3 chimeric receptors. Parallel unlabelled cultures as in (A) were lysed, immunoprecipitated with antibody 7.16.4 and subjected to kinase reactions in the presence of [γ-^{32}P]ATP. The arrow indicates the Neu chimeric receptor and the arrowhead indicates a phosphorylated protein of 56 kDa, specifically associated with the activated receptors. Cells expressing either the Neu[TM:Neu^{Glu664}] or Neu[TM:FGFR3^{Arg380}] constructs exhibited increased autophosphorylation relative to their wild-type counterparts (4- and 25-fold, respectively).

aliphatic side chain, i.e. Ala, Val, Leu or Ile; and P4 must be a Gly or Ala residue. According to this model, the Glu664 mutation that activates Neu resides in position P3, whereas the Arg380 mutation in FGFR3 is in position P2 in this motif (Figure 1B).

Both the Arg residue of mutant FGFR3 and the Glu residue of oncogenic Neu have the structural potential to participate in hydrogen bond formation. We therefore wished to examine whether the point mutation involved in achondroplasia allows constitutive activation of intracellular signalling, in a manner analogous to the point mutation which activates Neu. To test this hypothesis we have generated chimeric receptors with the Neu transmembrane domain substituted by the transmembrane domain of either normal or mutant FGFR3, and assayed their effects on signalling through the Neu tyrosine kinase.

Silent restriction sites flanking the Neu transmembrane-encoding region were engineered into the pSV2neuN expression vector (Bargmann *et al.*, 1986). This allowed oligonucleotide pairs encoding the predicted transmembrane sequences of wild-type FGFR3^{Gly380} or the achondroplasia mutant FGFR3^{Arg380} to be ligated into the parent construct, such that the extracellular and intracellular sequences were of Neu origin. Each of the four constructs, Neu[TM:Neu^{Val664}], Neu[TM:Neu^{Glu664}], Neu[TM:FGFR3^{Gly380}] and Neu[TM:FGFR3^{Arg380}] was co-transfected with a neomycin resistance selectable marker into NIH 3T3 cells, and stable cells lines were then isolated. Cell lines expressing each chimeric receptor were biosynthetically labelled with [^{35}S]Cys and [^{35}S]Met and immunoprecipitated with Neu monoclonal antibody (mAb) 7.16.4 (Drebin *et al.*, 1985) (Figure 2A). Equivalent expression of a Neu immunoreactive band of ~185 kDa is apparent in all four cell lines.

The FGFR3^{Arg380} transmembrane domain activates a chimeric Neu/FGFR3 receptor

In parallel experiments, unlabelled lysates from each cell line were immunoprecipitated with mAb 7.16.4 and the immune complexes were tested for kinase activity in the presence of [γ-^{32}P]ATP. A significant increase in Neu receptor autophosphorylation was seen in the lines expressing the Neu[TM:Neu^{Glu664}] and Neu[TM:FGFR3^{Arg380}] constructs (4- and 25-fold, respectively) relative to their wild-type counterparts (Figure 2B). Also specific to the activated constructs was a phosphorylated band at ~56 kDa, corresponding in size to a previously identified co-immunoprecipitating substrate of activated human Neu, designated ptyr56 (Scott *et al.*, 1991).

The activated Neu^{Glu664} gene was originally identified in neuro/glioblastomas of carcinogen-treated rats due to its ability to transform NIH 3T3 fibroblasts oncogenically following transfection (Shih *et al.*, 1981). To examine whether the receptors that exhibited increased kinase activity were also able to transform cells, each of the four constructs was transfected into NIH 3T3 cells. After 2 weeks, plates were scored for the presence of foci, indicative of oncogenic transformation through the Neu signal transduction pathway. Expression of the mutant Neu[TM:Neu^{Glu664}] and Neu[TM:FGFR3^{Arg380}] constructs resulted in similar numbers of foci of transformed cells (Figure 3B and D), whereas expression of their normal counterparts resulted in no foci (Figure 3A and C). This result indicates that increased *in vitro* receptor autophosphorylation mediated through the achondroplasia transmembrane domain correlates well with increased signal transduction through a heterologous tyrosine kinase receptor.

The expression of each construct was examined by

indirect immunofluorescence using mAb 7.16.4 directed against the extracellular domain of Neu (Figure 4). Cell surface localization was confirmed for all four constructs, demonstrating that the Gly380→Arg substitution in the transmembrane domain of FGFR3 does not interfere with the ability of a chimeric receptor to localize appropriately to the plasma membrane.

Residues with the potential to participate in hydrogen bond formation can functionally substitute for Arg380

Using the chimeric Neu–FGFR3 assay system, we examined the consequences of various amino acid substitutions in the transmembrane domain of FGFR3 on focus formation in NIH 3T3 cells. Constructs with each of the transmembrane domains diagrammed in Figure 5 were assayed as described for Figure 3, and exhibited the following pattern of activity with respect to transformation: R = E = D>Q>H>K>>W, G. In comparison, various point mutations at Val664 of Neu led to the following relative receptor activities: E = Q>>D,Y>>H,K,V,G (Bargmann and Weinberg, 1988b). It was observed for human Neu–erbB-2, however, that both Glu and Asp mutations at the analogous position (Val659) were equally capable of activating the receptor (Segatto *et al.*, 1988). Dimerization mediated through the transmembrane domain of bovine papillomavirus E5 oncoprotein has the following tolerance for substitutions at amino acid 17: Q = E = K>H>>R,D,N,Y,V (Meyer *et al.*, 1994). These results suggest that for all of these proteins to dimerize constitutively, the essential feature of their transmembrane domains is an amino acid, in the appropriate position, with a side chain capable of hydrogen bond formation. Clearly, however, steric factors are also important in determining how efficiently a given residue will mediate dimerization in the context of a particular transmembrane domain.

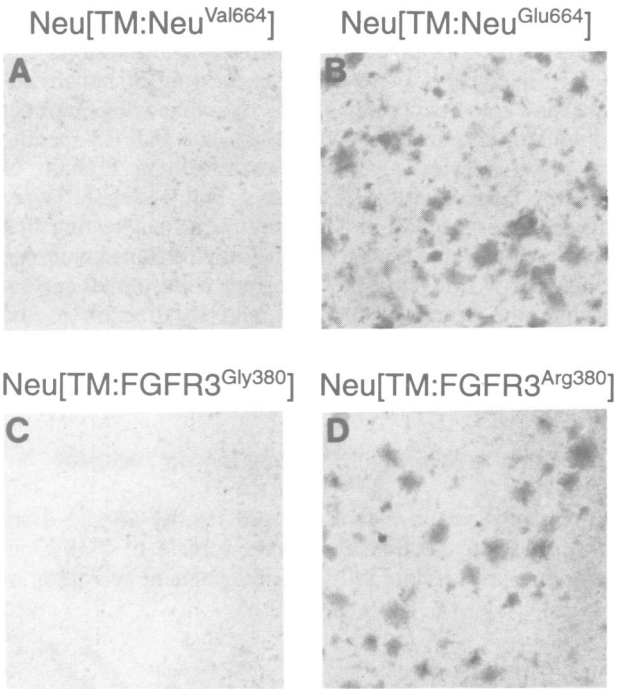

Fig. 3. Focus formation assay. NIH 3T3 cells were transfected with 10 μg plasmid DNA encoding each indicated construct. After 14 days, cells were fixed with methanol, stained with Giemsa and photographed. Expression of Neu[TM:Neu^{Glu664}] (**B**) and Neu[TM:FGFR3^{Arg380}] (**D**), but not their wild-type counterparts (**A**) and (**C**), resulted in the formation of foci of transformed cells.

Substitutions of Glu at positions P2 (Val663) or P4 (Gly665) of Neu have previously been shown to be non-activating (Bargmann and Weinberg, 1988b). Furthermore, substitutions of Val663 or Gly665 by other residues, even in the presence of Glu664, did not lead to receptor activation (Cao *et al.*, 1992), suggesting that the primary structure surrounding residue 664 plays an important role in mediating dimerization. We wished to examine whether the lateral position of the mutant residue in the FGFR3 transmembrane domain correlates with activity in the same way as it does for Neu. Substitution of Tyr379 (position P1) by Arg did not lead to transformation by the chimera (Figure 5). Replacement of Val381 (P3) by either Arg or Glu, in the presence of the wild-type Gly380 residue, did, however, activate the receptor, although less effectively than the equivalent substitutions at position 380 (P2). As a control, each construct was shown to be expressed on the cell surface by indirect immunofluorescence (data not shown).

FGFR3^{Arg380} is constitutively active as a tyrosine kinase

The chimeric Neu–FGFR3 receptor constructs allowed us to address the effect of the Gly380→Arg transmembrane domain point mutation both on receptor autophosphorylation and on biological signalling, and suggested that in the context of FGFR3 itself, this point mutation would also be activating. The downstream events resulting from receptor stimulation have not been well-defined for FGFR3; however, ligand-independent FGFR3 activation should result in increased receptor tyrosine kinase activity. The achondroplasia point mutation was engineered into

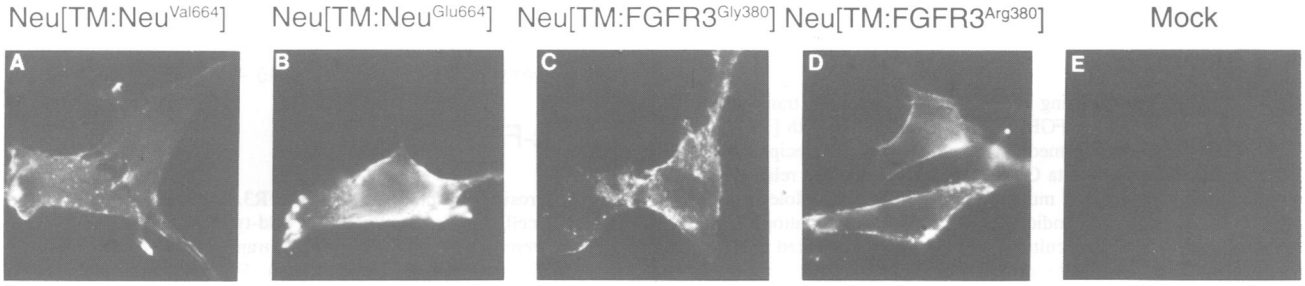

Fig. 4. Cell surface expression of Neu–FGFR3 chimeric receptors. Non-permeabilized NIH 3T3 cell lines expressing each indicated construct were examined by indirect immunofluorescence using mAb 7.16.4 (Oncogene Science) directed against the extracellular domain of Neu and a rhodamine-conjugated goat anti-mouse polyclonal secondary antibody (Boehringer-Mannheim). All constructs are localized to the cell surface.

FGFR3 and constructs expressing both wild-type and mutant proteins were transiently expressed in NIH 3T3 cells in the absence of serum or FGF. Biosynthetic ^{35}S labelling of cells expressing either construct, followed by immunoprecipitation with antiserum directed against the C-terminus of FGFR3, showed that equivalent levels of FGFR3 proteins were synthesized, represented by differentially glysosylated bands of 120–135 kDa (Figure 6A). Immune complexes from parallel, unlabelled cell lysates were subjected to *in vitro* kinase assays in the presence of [γ-^{32}P]ATP. Whereas the wild-type receptor had no detectable autophosphorylation activity, the achondroplasia mutant receptor exhibited strong ligand-independent autophosphorylation (Figure 6B).

	P1 379	P2 380	P3 381		Transformation Efficiency
VYAGILS	**Y**	**G**	**V**	GFFLFILVVAAVTLC	-
-------	-	**R**	-	----------	+++
-------	-	**K**	-	----------	+
-------	-	**E**	-	----------	+++
-------	-	**Q**	-	----------	++
-------	-	**D**	-	----------	+++
-------	-	**H**	-	----------	++
-------	-	**W**	-	----------	-
-------	**R**	-	-	----------	-
-------	-	-	**R**	----------	++
-------	-	-	**E**	----------	++

Fig. 5. Effect of amino acid substitutions in the FGFR3 transmembrane domain on the transforming activity of chimeric Neu–FGFR3 receptors. Chimeric constructs with Neu extracellular and intracellular domains and FGFR3 transmembrane domains, with the indicated amino acid substitutions, were generated as for Figure 2. NIH 3T3 cells were transfected with 10 μg of each plasmid together with 50 ng of pRSVneo. Focus formation was scored after 10 days and normalized for transfection efficiency as determined by G418-resistant colonies on parallel plates. Transformation efficiency relative to Neu[TM:FGFR3^{Arg380}] is presented, where – is 0–5%, + is 6–30%, ++ is 31–60%, and +++ is >60% of the Neu[TM:FGFR3^{Arg380}] focus number. Results are the average of three independent experiments. All constructs were shown to be expressed on the cell surface by indirect immunofluorescence with mAb 7.16.4.

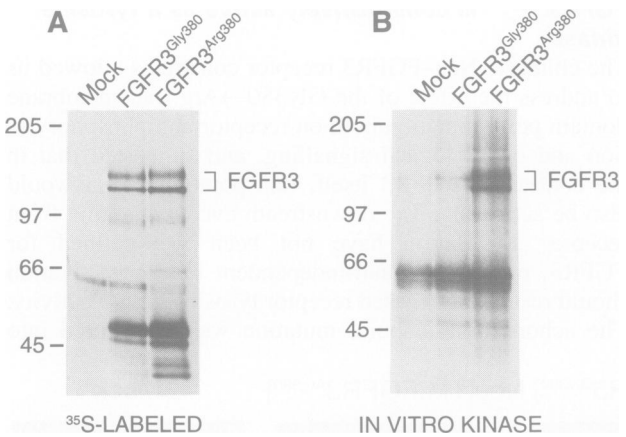

Fig. 6. (**A**) Metabolic labelling of FGFR3. NIH 3T3 cells transiently expressing FGFR3^{Gly380} or FGFR3^{Arg380} were labelled with [^{35}S]Cys and [^{35}S]Met in serum-free medium, lysed and immunoprecipitated with FGFR3 antiserum (Santa Cruz Biotechonology). The relative amounts of both normal and mutant FGFR3 are similar. Molecular weight markers in kDa are indicated. (**B**) Autophosphorylation of FGFR3. Parallel unlabelled cultures as in (A) were subjected to *in vitro* kinase reactions in the presence of [γ-^{32}P]ATP. Products were analyzed by 7.5% SDS–PAGE and autoradiography. Wild-type FGFR3^{Gly380} exhibits undetectable levels of autophosphorylation, whereas the FGFR3^{Arg380} mutant is constitutively active as a kinase.

The levels of constitutive tyrosine phosphorylation on FGFR3^{Gly380} or FGFR3^{Arg380} were also examined. Wild-type or mutant FGFR3 proteins expressed in unlabelled cells were immunoprecipitated with FGFR3 antiserum and transferred to nitrocellulose filters, which were then immunoblotted either with FGFR3 antibodies (Figure 7A) or phosphotyrosine antibodies (Figure 7B). Detectable levels of phosphotyrosine were only observed on the FGFR3^{Arg380} receptor, indicating that it exists in a constitutively tyrosine-phosphorylated form. Taken together, results from *in vitro* kinase assays and phosphotyrosine immunoblots demonstrate that the Gly380→Arg point mutation is a gain-of-function mutation that results in constitutive activation of FGFR3, and imply that ligand-independent signal transduction through mutant FGFR3 is the cause of achondroplasia.

To address concerns that the Arg380 mutation could affect correct localization of FGFR3 (Rousseau *et al.*, 1994; Shiang *et al.*, 1994), the expression of both wild-type and mutant proteins was examined by indirect immunofluorescence using SB141, an FGFR3-specific antiserum directed against the extracellular domain of FGFR3. Both FGFR3Gly380 and FGFR3Arg380 were expressed on the cell surface (Figure 8), suggesting that introduction of this mutant residue into the transmembrane domain of FGFR3 does not interfere with normal sorting through the secretory pathway and localization to the plasma membrane.

Discussion

Effect of the FGFR3^{Arg380} mutation on receptor signalling

In this report, we have demonstrated that the Gly380→Arg substitution in the transmembrane domain of FGFR3 in achondroplasia results in ligand-independent activation of

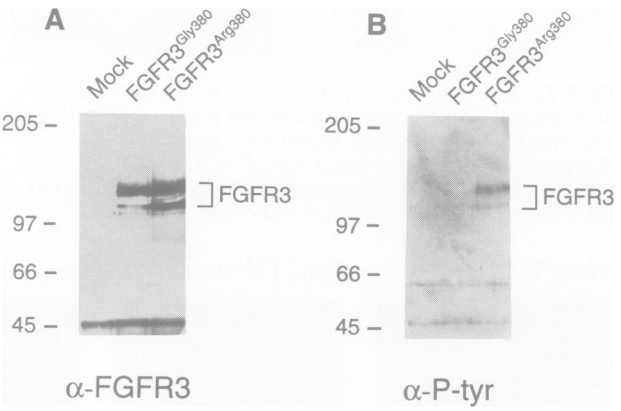

Fig. 7. Tyrosine phosphorylation of FGFR3. Immunoprecipitates from NIH 3T3 cells transiently expressing wild-type or mutant FGFR3 proteins were isolated as for Figure 5B, transferred to nitrocellulose filters, and immunoblotted either with (**A**) FGFR3 antiserum or (**B**) phosphotyrosine antiserum (mAb 4G10), followed by horseradish peroxidase-conjugated secondary antibodies and ECL development (Amersham). Constitutive levels of phosphotyrosine are only apparent on the FGFR3^{Arg380} mutant.

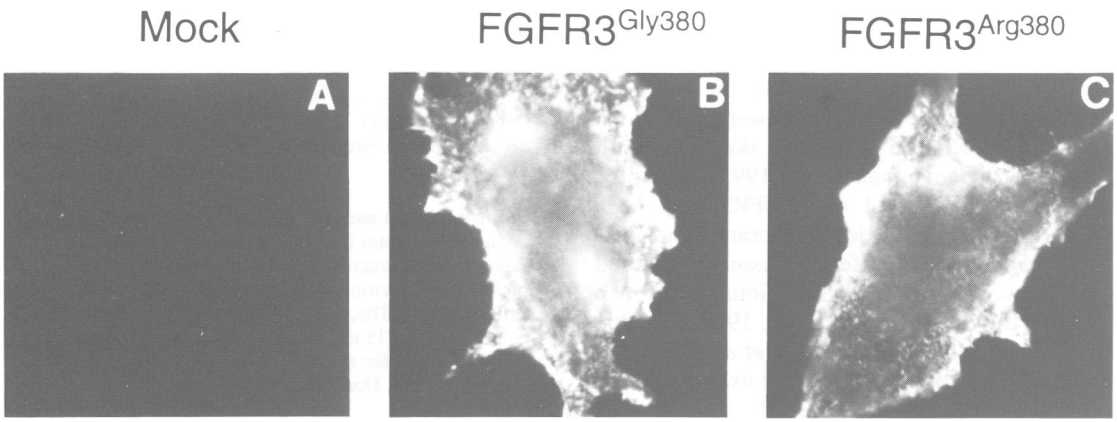

Fig. 8. Cell surface localization of wild-type and mutant FGFR3. Non-permeabilized COS-1 cells transiently transfected with the indicated constructs were examined for cell surface expression of FGFR3 by indirect immunofluorescence with antiserum SB141 directed against the extracellular domain of FGFR3 and a fluorescein-conjugated goat anti-rabbit polyclonal serum (Boehringer Mannheim). Both wild-type and mutant FGFR3 are localized to the cell surface.

the tyrosine kinase activity of FGFR3. Furthermore, this mutation also allows constitutive signal transduction through a chimeric Neu–FGFR3 receptor, suggesting that the Gly380→Arg point mutation in FGFR3 is functionally analogous to the Val664→Glu mutation that activates Neu. The Val664→Glu point mutation within the transmembrane domain of Neu causes ligand-independent receptor dimerization (Weiner *et al.*, 1989), leading to high levels of phosphorylation on tyrosine residues and increased *in vitro* kinase activity (Bargmann and Weinberg, 1988a; Stern *et al.*, 1988). Our results predict that the mechanism of activation of FGFR3^{Arg380} is also constitutive stabilization of FGFR3 in a dimeric conformation. Consistent with this idea, residues that are capable of participating in hydrogen-bond formation through either N-H or O-H groups in their side chains, such as Arg, Glu, Asp, and to a lesser degree, Gln, His and Lys, are also able to activate receptor signalling. Attempts to use chemical cross-linking reagents to detect ligand-independent dimers of mutant FGFR3 were inconclusive, possibly due to the low level of receptor expression, the transient nature of the dimeric complex and/or the low efficiency of cross-linking. Our inability directly to observe mutant FGFR3 dimers was consistent with the difficulty faced by others in detecting dimers of wild-type FGFR activated by ligand (Spivak-Kroizman *et al.*, 1994).

Either G-to-A transitions or G-to-C transversions at nucleotide 1138 of FGFR3 lead to the substitution of an Arg residue for the Gly at position 380, and individuals with achondroplasia have been identified with both mutations (Bellus *et al.*, 1995). The G1138 to T transversion, if it were to occur in nature, would result in a Gly380→Trp substitution. Our results indicate that this mutation would probably go undetected, as it is not activating (Figure 5) and such individuals would not be expected to exhibit features of achondroplasia. Our studies further predict that several other point mutations should also result in a phenotype of achondroplasia, as Glu, Asp, Gln and His at position 380, as well as Arg or Glu at position 381, strongly activate chimeric receptors. However, of these, only Gly380→Glu and Val 381→Glu substitutions could occur by single nucleotide mutations, and these events are expected to be very rare compared with the high mutation frequency at the 1137–1138 CpG dinucleotide (Bellus *et al.*, 1995).

Members of the FGF family are involved at several stages of long bone growth, a process which requires regulated chondrocyte proliferation, cell hypertrophy, matrix calcification, vascular invasion and ultimately osteoblastic bone formation (Iannotti *et al.*, 1994). FGFs act as mitogens for cultured chondrocytes, stimulating or stabilizing the deposition of cartilaginous extracellular matrix, as well as inhibiting the terminal differentiation of chondrocytes (Kato and Iwamoto, 1990; Iwamoto *et al.*, 1991), although the FGF receptor that regulates each of these responses is unknown. The FGFRs have distinct patterns of expression during mouse embryogenesis, suggesting that each receptor mediates different responses to FGFs during development. FGFR1 and FGFR2, for instance, are co-expressed with FGFR3 in cartilage rudiments of developing bone, but FGFR3 is expressed in resting chondrocytes to the exclusion of other FGFRs (Peters *et al.*, 1993). Results presented here suggest that unregulated signalling through FGFR3^{Arg380} may either inhibit the normal proliferation of chondrocytes or cause their premature differentiation, resulting in abnormal maturation at long bone growth plates. Further definition of the signal transduction pathway downstream of activated FGFR3, both at bone growth plates and in other tissues where FGFR3 is expressed, will be necessary to understand fully the developmental consequences of this activating mutation.

Relevance of FGFR3 activation to other disorders

Understanding the biological consequences of the FGFR3 mutation in achondroplasia may be of relevance to other congenital skeletal dysplasias. Recently, the skeletal disorders thanatophoric dysplasia types I and II, which have dominant neonatal lethal phenotypes resembling homozygous achondroplasia, were shown to result from point mutations in FGFR3 (Tavormina *et al.*, 1995). These mutations, which introduce an acidic residue next to a potential site of autophosphorylation in the activation loop of the tyrosine kinase domain (type II) or create an unpaired Cys residue in the extracellular domain (type I), also have the potential to constitutively activate FGFR3.

Comparison of FGFR3 signalling resulting from these independent mutations may help explain the differing severities of these dysplasias. In contrast, heterozygous loss of FGFR3 function due to a chromosomal deletion, as occurs in Wolf–Hirschhorn syndrome, does not result in skeletal dysplasia (Gandelman *et al.*, 1992). Recently, point mutations in both FGFR1 and FGFR2 have been found to cause other inherited skeletal and craniosynostosis disorders, such as Apert, Pfeiffer, Jackson–Weiss and Crouzon syndromes, although these mutations do not map to the transmembrane domain (Jabs *et al.*, 1994; Reardon *et al.*, 1994; Rutland *et al.*, 1995; Wilkie *et al.*, 1995). In many instances of Crouzon syndrome, for example, point mutations result in the gain or loss of a single Cys residue in the third immunoglobulin-like domain of FGFR2 (Reardon *et al.*, 1994). An unpaired Cys might be expected to dimerize receptors in the absence of ligand, and preliminary results indicate that the mutations found in Crouzon syndrome can indeed constitutively activate signalling through FGFR2/Neu chimeric receptors (B.D.Galvin and D.J.Donoghue, unpublished observations). Our results predict that achondroplasia is the first of many congenital developmental diseases where point mutations in FGFRs will be shown to result in constitutive receptor activation.

Materials and methods

Neu/FGFR3 chimeric constructs

PCR-directed mutagenesis was used to create silent *Nhe*I (bases #1973–1978) and *Sac*I (bases #2114–2119) restriction sites flanking the transmembrane domain of wild-type Neu in the pSV2NeuN expression construct (Bargmann *et al.*, 1986). Pairs of long oligonucleotides encoding the FGFR3 transmembrane domains with various amino acid substitutions were designed such that, when annealed, the double-stranded DNA would contain *Nhe*I and *Sac*I cohesive overhangs. Oligonucleotides were synthesized, purified (Xu *et al.*, 1993) and ligated into the pSV2Neu(*Nhe*I/*Sac*I) vector at these novel restriction sites. The region of each construct between *Nhe*I and *Sac*I was confirmed by sequencing. The Neu[TM:Neu^{Val664}] construct is pSV2NeuN and the Neu[TM:Neu^{Glu664}] construct is pSV2NeuNT (Bargmann *et al.*, 1986).

FGFR3 constructs

PCR was used to engineer the Gly380→Arg mutation in the human FGFR3 clone 17B (Keegan *et al.*, 1991a), as well as a silent *Afl*II restriction site at residue 377, using the mutagenic PCR oligonucleotide 5′-GCAGGCATCTTAAGCTACAGGGTGGG-3′. The *Bss*HII–*Xba*I fragment of wild-type or mutant FGFR3, which removed the 5′-untranslated sequence of clone 17B, was subcloned into the pcDNAI expression vector (Invitrogen).

Transfections

Twenty-four hours prior to transfection, NIH 3T3 cells or COS-1 cells were plated at a density of 2×10^5 cells onto 60 mm plates. Cells were transfected with 10 μg plasmid DNA encoding each expression construct using a modified calcium phosphate transfection method (Chen and Okayama, 1987). Eighteen hours after transfection, cells were re-fed with DME containing 10% calf serum. For focus assays, cells were split 1:12 onto 100 mm plates 24 h later and were scored after 10 or 14 days for the presence of foci of transformed cells (Lee and Donoghue, 1992). Cell lines were established by co-transfection of NIH 3T3 cells with 10 μg plasmid DNA and 0.5 μg pRSVneo, and selection of single cell-derived subclones after 21 days of growth in 0.5 mg/ml G418-supplemented medium.

Immunoprecipitation

NIH 3T3 cells were transfected with each construct as described above. Sixteen hours after re-feeding, medium was changed to serum-free DME. Twelve hours later cells were labelled with [^{35}S]Cys and [^{35}S]Met for 4 h or not labelled, lysed in NP-40 lysis buffer (20 mM Tris, pH 7.5, 137 mM NaCl, 1% NP-40, 5 mM EDTA, 10% glycerol, 10 μg/ml aprotinin and 1 mM sodium orthovanadate) and immunoprecipitated with antiserum specific for the C-terminus of FGFR3 (Santa Cruz Biotechnology) or for the extracellular domain of Neu (mAb 7.16.4, Oncogene Science). Immune complexes were collected on protein A–Sepharose beads, which were extensively washed and analyzed by 7.5% SDS–PAGE.

In vitro kinase assays

Immunoprecipitates from unlabelled NIH 3T3 cells transfected with each expression construct were washed once in 20 mM Tris, pH 7.5, then subjected to *in vitro* kinase reactions in the presence of 40 μl kinase buffer (20 mM Tris, pH 7.5, 10 mM $MnCl_2$, 5 mM $MgCl_2$ and 5 μCi [γ-^{32}P]ATP), for 15 min at 37°C. Products were washed extensively with NP-40 lysis buffer and analyzed by 7.5% SDS–PAGE and autoradiography (Lee and Donoghue, 1992).

Immunoblotting

Immunoprecipitates from unlabelled NIH 3T3 cells transfected with each expression construct were electrophoresed through a 7.5% SDS–PAGE gel and transferred to nitrocellulose. Filters were incubated with phosphotyrosine antisera (mAb 4G10, Upstate Biotechnology Inc.) followed by horseradish peroxidase-conjugated goat anti-mouse IgG or anti-FGFR3 antisera (Santa Cruz Biotechnology) and horseradish peroxidase-conjugated goat anti-rabbit IgG, and developed by enhanced chemiluminescence (ECL, Amersham) according to the manufacturer's instructions.

Indirect immunofluorescence

To detect cell surface expression of Neu and Neu–FGFR3 chimeric receptors, stable cell lines expressing each construct were fixed with 3% paraformaldehyde and incubated without permeabilization with mAb 7.16.4 directed against the extracellular domain of Neu, followed by a rhodamine-conjugated goat anti-mouse antibody (Boehringer Mannheim). To examine expression of wild-type and mutant FGFR3 proteins, transiently transfected COS-1 cells were fixed as above and incubated with antiserum SB141 directed against the extracellular domain of FGFR3 (Keegan *et al.*, 1991b) and a fluorescein-conjugated goat anti-rabbit antibody (Boehringer Mannheim).

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