Physical Basis behind Achondroplasia, the Most Common Form of Human Dwarfism*†

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Fibroblast growth factor receptor 3 (FGFR3) is a receptor tyrosine kinase that plays an important role in long bone development. The G380R mutation in FGFR3 transmembrane domain is known as the genetic cause for achondroplasia, the most common form of human dwarfism. Despite many studies, there is no consensus about the exact mechanism underlying the pathology. To gain further understanding into the physical basis behind the disorder, here we measure the activation of wild-type and mutant FGFR3 in mammalian cells using Western blots, and we analyze the activation within the frame of a physical-chemical model describing dimerization, ligand binding, and phosphorylation probabilities within the dimers. The data analysis presented here suggests that the mutation does not increase FGFR3 dimerization, as proposed previously. Instead, FGFR3 activity in achondroplasia is increased due to increased probability for phosphorylation of the unliganded mutant dimers. This finding has implications for the design of targeted molecular treatments for achondroplasia.

Fibroblast growth factor receptor 3 (FGFR3) is a receptor tyrosine kinase that consists of an extracellular domain with three immunoglobulin-like motifs, a single transmembrane (TM)2 domain, and an intracellular split tyrosine kinase domain. Ligands (fibroblast growth factors (fgfs)) and heparin bind to the extracellular domains and stabilize the dimer, with the ligand inducing a conformational change in the extracellular domain (1). The contact stimulates catalytic activity and results in the cross-phosphorylation of the two receptors. This activates the catalytic domains for the phosphorylation of cytoplasmic substrates and triggers signaling cascades (2, 3).

Mutations in FGFR3 are known to affect long bone development, which proceeds via endochondral ossification along a pathway involving differentiation of mesenchymal stem cells into cartilage, followed by bone invasion (as the chondrocytes of the cartilage die, the space is invaded by bone). FGFR3 works as a negative regulator of bone development by mediating pro-differentiation signals in chondrocytes (4–6). FGFR3 overactivation because of mutations alters the terminal differentiation into hypertrophic chondrocytes, effectively shortening the proliferation phase. Furthermore, FGFR3 overactivation can cause cancers of the epithelium (7).

The G380R mutation in FGFR3 TM domain has been linked to achondroplasia, the most common form of human dwarfism (8). The incidence rate of achondroplasia is about 1/15,000 live births (5). It is an autosomal dominant disorder that interferes with the maturation of the cartilage growth plate of long bones (6, 9). The phenotype is characterized by short stature, narrowing of the lumbar spinal canal, accentuated bowing of the mid- and lower part of the back, and trident-shaped hands (5). Affected individuals often exhibit other skeletal as well as neurological complications.

The physical basis underlying achondroplasia is still under debate, and multiple mechanisms may be contributing to pathogenesis. One established mechanism that contributes to FGFR3 over-activation in achondroplasia is the compromised down-regulation of the mutant receptor. For instance, Monsonego-Ornan et al. (10, 11) have found that the rates of internalization and degradation of the wild-type and the mutant receptors are different, and as a result, the mutant accumulates at the cell surface and signals over a longer time than the wild type. Furthermore, Cho et al. (12) have reported that the achondroplasia mutation increases the activity of FGFR3 by disrupting c-Cbl-mediated ubiquitination that serves as a targeting signal for lysosomal degradation and termination of receptor signaling.

Other studies, however, point to a second mechanism that might contribute to the pathology, i.e. ligand-independent activation of the mutant receptor. For instance, Webster and Donoghue (13) have studied the effect of the G380R mutation on the kinase and transforming activity of full-length FGFR3 and of a chimeric Neu/FGFR3 receptor (consisting of the extracellular and catalytic domain of Neu and the TM domain of FGFR3). They have shown that the mutation increases ligand-independent FGFR3 activation. Li et al. (14) have further shown that the mutant does not need a ligand to become activated in L6 cells and induces transformations in NIH3T3 cells. These authors concluded that the mutation likely “produces a dominant oversignaling receptor that is no longer regulated by FGF binding.”

Recent work suggests that these two mechanisms may be coupled. Other mutations in FGFR3 that cause skeletal dyspla-
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sias also have been shown to impede the trafficking or down-regulation of the FGFR3 mutants, effectively prolonging signaling (11, 12, 15, 16). Importantly, the relative magnitudes of these trafficking and down-regulation defects have been shown to be proportional to the activation of the mutants, the higher the activation, the longer the lifetime of the active FGFR3 dimers in the cell (10, 11, 15). One interpretation of these findings may be that the trafficking/down-regulation defects are a consequence of the increased activation, although the opposite is also possible. The increased activation, on the other hand, is hypothesized to be due to increased FGFR3 dimerization (13, 15, 17). However, this hypothesis has not been validated thus far.

To test directly the hypothesis that the dimerization propensity of the FGFR3 TM domain changes in the presence of the achondroplasia mutation, we previously characterized the dimerization of the isolated wild-type and mutant TM domains of FGFR3 in lipid bilayers (18). Unexpectedly, we found that the dimerization free energies are the same for the wild type and the mutant. The results for this mutation contrasted with our results for a different pathogenic mutation in FGFR3 TM domain, A391E (19). The A391E mutation is known as the genetic cause for Crouzon syndrome with acanthosis nigricans characterized by the following three phenotypic features: 1) mild disturbances of the growth plate of the long bones; 2) premature ossification of the skull (craniolysostosis); and 3) skin hyperpigmentation and hyperkeratosis. Unlike the A391E mutation that increased the dimerization propensity by −1.3 kcal/mol (19), the G380R mutation did not affect the dimerization energetics of FGFR3 TM domain (18).

In our search for the physical basis behind achondroplasia, here we revisit the effect of the G380R mutation in cellular systems. We seek to determine whether the increase in FGFR3 activation occurs due to enhanced dimerization or due to a different physical mechanism. We do this using a new approach that bridges biophysics and cell biology and has the power to provide mechanistic understanding of the effect of pathogenic mutations on different steps in FGFR3 activation. In this approach, FGFR3 dimerization is considered as a two-step process, ligand-independent dimerization followed by ligand-mediated dimer stabilization, and liganded and unliganded dimers are assigned different phosphorylation probabilities.

We have already demonstrated the feasibility of such an approach in our previous work by demonstrating that RTK activity can be modeled and predicted based on the laws of mass action (20). In this previous work, we investigated the A391E mutation within a Neu_FGFR3 chimeric system, and we demonstrated that the A391E mutation increases the activation propensity of the chimeric Neu_FGFR3 receptor by −0.7 kcal/mol (20). Here, we first assess the effect of the achondroplasia mutation on the phosphorylation of the chimeric Neu_FGFR3 receptor in CHO cells. Next, we investigate the effect of the mutation on the activation of the full-length FGFR3 receptor, as a function of ligand ($fgf1$) concentration in HEK 293 cells. We observe higher phosphorylation of the mutant FGFR3 than the wild type in the absence of ligand and at low ligand concentrations. We demonstrate that the achondroplasia mutation does not increase the dimerization propensity of the receptor. Instead, the data analysis presented here suggests that the mutation affects the cross-phosphorylation within the unliganded dimer. Thus, we propose that the underlying reason for the increase in FGFR3 activation in achondroplasia is not increased dimerization but an elevated phosphorylation within the unliganded FGFR3 dimers, most probably due to a structural change.

EXPERIMENTAL PROCEDURES

Materials and Experimental Methods

Plasmids—The plasmids used in this study, Neu_FGFR3/WT, Neu_FGFR3/G380R, FGFR3/WT, and FGFR3/G380R, were a generous gift from D. J. Donoghue, University of California, San Diego. Neu_FGFR3 is a chimeric receptor composed of the extracellular and intracellular domain of Neu and the TM domain of FGFR3. The chimeric Neu_FGFR3 receptors were encoded in the pSV2 vector. The FGFR3/WT and FGFR3/G380R plasmids were in the pcDNA 3.1+ vector.

Western Blots—Chinese hamster ovary (CHO) cells were transfected with various amounts of plasmids encoding Neu_FGFR3/WT and Neu_FGFR3/G380R using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s protocol. Cells were cultured for 24 h following transfection, and then treated with lysis buffer (25 mM Tris-Cl, 0.5% Triton X-100, 20 mM NaCl, 2 mM EDTA, 2 mM NaVO₄, and protease inhibitor; Roche Applied Science). The supernatant was collected following centrifugation at 15,000 × g for 15 min at 4 °C. Lysates were loaded into 3–8% NuPAGE™ Bis-Tris gel (Invitrogen). After the proteins were transferred onto a nitrocellulose membrane, they were stained with Neu C-18 antibodies (Sc-284, Santa Cruz Biotechnology) or anti-phosphotyrosine (anti-Tyr-653/Tyr-654, Cell Signaling Technology), followed by anti-rabbit HRP-conjugated antibodies (Promega). The bands were visualized using ECL™ detection reagent (GE Healthcare) and quantified using ImageQuant TL.

Human embryonic kidney cells (HEK 293) were transfected with plasmids encoding FGFR3/WT and FGFR3/G380R using FuGENE HD (Roche Applied Science) according to the manufacturer’s protocol. Cells were cultured in normal medium for 24 h following transfection and then starved in serum-free medium for 24 h. The effect of ligand was monitored by incubating the cells in medium supplemented with $fgf1$ (Millipore, MA). The receptors in the HEK 293 cell lysates were probed with FGFR3 (H-100) antibodies (sc-9007, Santa Cruz Biotechnology) or phospho-Tyr-FGF receptor antibodies (anti-Tyr-653/Tyr-654, Cell Signaling Technology), followed by anti-rabbit HRP-conjugated antibodies (Promega). Anti-actin antibodies (Sigma) were used to stain actin and thus confirm equal loading of protein lysates from different samples.

Immunostaining—Cells were cultured in the incubator for 24 h after transfection. After fixing with 3.7% paraformaldehyde, the cells were blocked using bovine serum albumin (BSA) for 1 h. Surface localization of Neu_FGFR3 in CHO cells was detected after incubation with anti-ErbB2 (mouse mAb (7.16.4), Calbiochem), followed by secondary anti-mouse fluorescein-conjugated IgG. Surface localization of FGFR3 in HEK
293 cells was detected with anti-FGFR3 (H-100, Santa Cruz Biotechnology), followed by Alexa-488-conjugated anti-rabbit IgG (Invitrogen).

Cross-linking—In cross-linking experiments, dimeric receptors were cross-linked with either a membrane-permeable linker (EGS, Pierce) or a membrane-impermeable one (bis(sulfo succinimidyl) suberate, Pierce). Twenty four hours after transfection, cells were incubated with 2 mM cross-linker for 30 min to 1 h at room temperature and then quenched in 20 mM Tris-HCl for 15 min. After a rinse with ice-cold PBS, the cells were lysed, and the receptors were detected using Western blotting. The cross-linked fraction was calculated as \( S_D/S = S_D/(S_M + S_D) \), where \( S_D \) is the intensity of the dimeric band, and \( S_M \) is the intensity of the monomeric band.

Titration with fgf1—Human embryonic kidney cells were transfected with plasmids encoding FGFR3/WT or FGFR3/G380R. Cells were cultured in normal medium for 24 h following transfection and then starved in serum-free medium for 24 h. Different concentrations of fgf1 (Millipore, MA), ranging from 5 to 5000 ng/ml, were added to the serum-free medium. After incubating for 10 min with ligand, cells were lysed, as described above, and analyzed using Western blotting.

Quantification of Western Blots—The Western blot films were scanned and processed with ImageQuant TL. At least three sets of independent experiments were performed to determine the averages and the standard deviations. The loading of the gels was adjusted such that all the band intensities were within the so-called linear range, where the staining intensities were proportional to the protein concentrations (20).

Physical-Chemical Model of FGFR3 Activation

FGFR3 lateral dimerization is a regulator of FGFR3 activity. Ligands, such as fgf1, bind to the dimer and regulate the dimerization process. We consider this dimerization process as a two-step reaction scheme describing ligand-independent dimerization followed by ligand-mediated dimer stabilization as shown in Reactions 1 and 2,

\[
K_1 = [d]/[M]^2 \quad \text{(Eq. 1)}
\]

and

\[
K_2 = [D]/[L_2][d] \quad \text{(Eq. 2)}
\]

In addition, the mass balance equations for the total receptor concentration \([TR]\) and the total ligand concentration \([TL]\) are shown in Equations 3 and 4,

\[
[TL] = 2[L_2] + 2[D] \quad \text{(Eq. 3)}
\]

\[
[TR] = [M] + 2[d] + 2[D] \quad \text{(Eq. 4)}
\]

In these equations, we define the concentrations of \([TR], [M], [D], \) and \( d \) as the average number of receptors on the cell surface per cell. Thus, \([TR]\) is the total number of receptors per cell, whereas \([M], [d], \) and \([D] \) are the number of monomers, unliganded dimers, and liganded dimers per cell, respectively. Knowing the number of cells and the three-dimensional ligand concentrations used in the experiment, we can also calculate the average number of ligands per cell, \([TL]\). The ligand exist in three different states as follows: free in solution, bound to heparin sulfate on the cell surface, and bound to the receptor dimer in the presence of heparin sulfate. \(2[L_2]\) is the average number of ligands per cell that are not bound to the receptor dimers (see equation 5). Here we do not consider explicitly the equilibrium between monomeric and dimeric ligands, as well as the equilibrium between ligands that are free in solution or bound to the cell surface.

If we know the total ligand and receptor concentrations, \([TL]\) and \([TR]\) (the two parameters that can be varied in the experiments), and if we assume that we know the two reaction constants \(K_1\) and \(K_2\), then the four equations (Equations 1–4) can be used to determine the four unknowns \([L_2]\), \([M]\), \([d]\), and \([D]\), and thus predict the monomeric and dimeric fractions of FGFR3 receptors as a function of total ligand concentration, \([TL]\) for fixed \(K_1, K_2, \) and \([TR]\). As shown in the supplemental material, the problem is reduced to a fourth-order equation for \([M]\), which yields a single real positive root. Once \([M]\) is determined from this equation, \([d]\) and \([D]\) can be determined too (see supplemental material). Fig. 1 shows the prediction for the dimeric fractions, \(2[d]\) and \(2[D]\), for different values of \(K_1\) and \(K_2\) and a fixed value of \([TR]\). The predictions show how the monomeric and dimeric fractions change when \(K_1\) and \(K_2\) are changed. They also illustrate how the ligand is reducing the monomeric fraction by depleting the unliganded dimers.

Although Reactions 1 and 2 and Equations 1–4 describe the lateral interactions between receptors as well as ligand binding, they do not account for the last step in the activation process, cross-phosphorylation within the dimer. Here, we account for this last step with the help of two additional parameters, the probabilities for phosphorylation in the unliganded and liganded dimers, \(\Phi_d\) and \(\Phi_D\). The concentration of phosphorylated receptors is then calculated according to Equation 5,

\[
[P] = 2\Phi_d[d] + 2\Phi_D[D] \quad \text{(Eq. 5)}
\]

The concentration of phosphorylated receptors \([P]\) can be measured using Western blots with antibodies that are specific.
Equation 7 shows that the phosphorylated fraction as a function of ligand concentration is the sum of the predicted liganded dimeric fraction and the unliganded dimeric fraction (predictions for both are shown in Fig. 1), the latter multiplied by \( \Phi_d/\Phi_D \).

The probabilities for phosphorylation, \( \Phi_d \) and \( \Phi_D \), are unknown, but the ratio of the two probabilities can be determined if the concentrations of phosphorylated receptors and dimeric receptors are measured. At saturating ligand concentrations, only liganded dimers are expected to be present, and the concentration of phosphorylated receptors is given by Equation 6. In the absence of ligand, the concentration of phosphorylated receptors is shown in Equation 8,

\[
[P]_0 = \Phi_d [d]_0
\]

(Eq. 8)

Taking the ratio of Equations 8 and 6, we obtain Equations 9 and 10,

\[
\frac{[P]}{[P]_0} = \frac{\Phi_d [d]_0}{\Phi_D [D]_0}
\]

(Eq. 9)

Therefore,

\[
\Phi_d = \frac{[D]_0}{[P]_0} \frac{[P]_0}{[P]_0}
\]

(Eq. 10)

The ratio \([P]/[P]_0\) is easily measured using Western blotting. As discussed above, it is the ratio of phospho-Tyr-staining intensities at saturating ligand concentration and in the absence of ligand. Dimeric fractions, however, are difficult to measure directly, and thus obtaining an estimate of \([D]_0/[d]_0\) is a challenge. Usually, dimerization is probed using cross-linking, and in this study we approximate the ratios of the dimeric fractions at saturating ligand concentrations and in the absence of ligand, \([D]_0/[d]_0\), with the ratio of cross-linked receptor fractions under the same conditions.

RESULTS

Expression of Neu_FGFR3 and FGFR3 Is Not Affected by the G380R Mutation

First, we investigated whether the G380R mutation affects the expression of the receptors. CHO cells were transfected with plasmids encoding Neu_FGFR3 (wild-type and mutant). The chimeric receptor Neu_FGFR3 is composed of the extracellular and intracellular domains of Neu and the TM domain of FGFR3. HEK 293 cells were transfected with plasmids encoding full-length human FGFR3 (wild type and mutant). After transfection, the expression of the receptors on the cell membrane was detected by immunostaining. The primary antibodies used were anti-ErbB2 and anti-FGFR3 (H-100), which detect the extracellular domains of Neu and FGFR3, respectively. Staining results showed that the localization of Neu_FGFR3 and FGFR3 in the membrane was not affected by the G380R mutation (Fig. 2, A–D). This result is consistent with work from other laboratories, documenting that the mutation does not affect immunostaining patterns (13).
The expression levels of Neu_FGFR3 and FGFR3 were further assessed using Western blots and antibodies that recognize the receptors (anti-Neu C-18 antibodies and anti-FGFR3 (H-100) antibodies, respectively). Cells, transfected with identical DNA concentrations, yielded Western blot bands of similar intensities, further demonstrating that the mutation does not affect the expression and trafficking of Neu_FGFR3 and FGFR3 (see data in Figs. 3 and 5–7).

**G380R Mutation Does Not Affect the Phosphorylation of Neu_FGFR3 in CHO Cells**

The Neu_FGFR3 chimera has been previously used by Webster and Donoghue (13) to study the consequences of the G380R mutation in NIH3T3 and COS7 cells. These authors observed that the G380R mutation substantially increases the phosphorylation of the chimera. Here, we re-examined the phosphorylation of the chimera in CHO cells, under the same conditions used in a recent study of a Neu_FGFR3 chimera carrying the A391E mutation linked to Crouzon syndrome with acanthosis nigricans (20). As demonstrated in Ref. 20, the A391E mutation increased Neu_FGFR3 phosphorylation, as probed by the anti-Tyr(P) 4G10® antibody.

CHO cells were transfected with different amounts of plasmids encoding Neu_FGFR3/WT or Neu_FGFR3/G380R. The expression levels and phosphorylation levels were measured 24 h after transfection. Fig. 3 and supplemental Fig. S1 show the band staining intensities of the anti-Neu C-18 and anti-Tyr(P) 4G10® antibodies, which report the total expression and phosphorylation levels of the receptors, respectively. We see that, in our hands, the G380R mutation does not affect the phosphorylation of the mutant Neu_FGFR3 chimera (see also supplemental Fig. S2). This result is different from our previous result for the A391E mutation, which increased Neu_FGFR3 phosphorylation (20).

We next investigated whether the cross-linking propensity of Neu_FGFR3 is affected by the G380R mutation, using EGS as a cross-linking agent and following the protocol described in Ref. 20. Note that here we use EGS, a membrane-permeable crosslinker. The rationale behind this approach is that on Western blots we cannot distinguish Neu intracellular and plasma membrane pools (unlike full-length FGFR3, where the two tools are separated by molecular weight, see below). Western blots were stained with anti-Neu C-18 antibodies and are shown in Fig. 4. Two bands were observed, corresponding to noncross-linked and cross-linked Neu_FGFR3. No apparent difference in cross-linking was observed due to the G380R mutation. After quantifying the bands, we calculated the cross-linked fraction according to

$$\frac{S_D - S}{S_M} = \frac{S_D}{S_M} \left( S_M + S_D \right),$$

where $S_D$ is the intensity of the dimeric band, and $S_M$ is the intensity of the monomeric band. The cross-linked fractions of Neu_FGFR3 and Neu_FGFR3/G380R are similar, 0.20±0.08 and 0.28±0.07, respectively. Therefore, the cross-linking propensities of Neu_FGFR3 and Neu_FGFR3/G380R are the same within experimental error. This result is in contrast to our previous study of the A391E mutant, which exhibited a substantial increase in cross-linking (20), as compared with wild-type.

**G380R Mutation Increases the Phosphorylation of Full-length FGFR3 in the Absence of Ligand**

Next, we investigated whether the G380R mutation affects the phosphorylation of full-length FGFR3. Plasmids encoding wild-type and mutant FGFR3 were transfected into HEK 293 cells using FuGENE HD. One day after transfection, cells were starved in serum-free medium for 24 h. The activation levels and the expression levels in the absence of ligand were mea-
FGFR3 and the G380R mutant as a function of high ligand concentrations

FGFR3 at low ligand concentrations, but has no effect at G380R mutation increases the phosphorylation of full-length mature band of FGFR3, in the absence of ligand.

had no effect on the 130-kDa band but eliminated the 120-kDa band and produced a single band at about 100 kDa. Endo-N-glycosidase, which cleaves all sugars, eliminated both the 130- and 120-kDa bands and produced a single band at about 100 kDa. Endo-N-glycosidase, which cleaves all sugars, eliminated both the 130- and 120-kDa bands and produced a single band at about 100 kDa. Endo-N-glycosidase, which cleaves all sugars, eliminated both the 130- and 120-kDa bands and produced a single band at about 100 kDa.

Fig. 5 shows the band staining intensities of anti-FGFR3 (H-100) and anti-Tyr-653/Tyr-654, which report the total expression and phosphorylation levels of FGFR3, respectively. In each case we observe two bands that are reactive to both antibodies. This is not surprising, because FGFR3 has three isoforms, with various degrees of N-glycosylation as follows: the immature unglycosylated 98-kDa receptor, the intermediate 120-kDa form that is sensitive to endo-β-N-acetylgalactosaminidase H and is found in the endoplasmic reticulum/cis-Golgi, and the fully glycosylated mature 130-kDa form located predominantly on the plasma membrane and activated in the presence of the ligand (23). In our own hands, treatment with peptide:N-glycosidase, which cleaves all sugars, eliminated both the 130- and 120-kDa bands and produced a single band at about 100 kDa. Endo-β-N-acetylgalactosaminidase H treatment had no effect on the 130-kDa band but eliminated the 120-kDa band. The results shown in Fig. 5 demonstrate that the G380R mutation significantly increases the activation level of the mature band of FGFR3, in the absence of ligand.

G380R Mutation Increases the Phosphorylation of Full-length FGFR3 at Low Ligand Concentrations, but Has No Effect at High Ligand Concentrations

We next characterized the phosphorylation of wild-type FGFR3 and the G380R mutant as a function of fgf1 concentration. HEK cells were incubated with serum-free medium supplemented by different concentrations of fgf1, ranging from 5 to 5000 ng/ml. After a 10-min incubation with fgf1, the cells were lysed. Western blotting was used to measure the phosphorylation levels and the total expression levels of wild-type FGFR3 and the G380R mutant, as a function of ligand concentration.

Fig. 6 shows one representative Western blot experiment. The Western blot shows both staining with the anti-FGFR3 antibody (monitoring expression) and with the anti-Tyr-653/Tyr-654 antibody (monitoring phosphorylation). In this experiment, as expected, only the mature FGFR3 located predominantly on the cell surface responds to the ligand, although the lower molecular weight FGFR3 located in the endoplasmic reticulum was not affected. Inspection of the high molecular weight band shows that the phosphorylation of the G380R mutant is higher than the phosphorylation of the wild type in the absence of ligand and at low ligand concentrations. The difference gradually decreases as the concentration of ligand increases, and no difference can be observed at the highest ligand concentrations. Thus, the G380R mutation increases the phosphorylation of the mature FGFR3 in the absence of ligand and at low ligand concentration. The ratios of the Tyr-653/Tyr-654 band intensities for the mutant and the wild type are shown in Fig. 6B, together with the standard errors from three independent experiments. By analysis of variance, there is a statistically significant difference in the phosphorylation of the mutant and the wild type at low ligand concentration (p = 0.001).

In the supplemental material, we describe the creation and characterization of two stable lines expressing the wild type and the mutant, HEK293-fWT and HEK293-fG380R. Experiments with these stable cell lines confirm that the phosphorylation of the mutant is higher than the phosphorylation of the wild type in the absence of ligand.

In Fig. 6, we see that for ligand concentrations above 1250 ng/ml (i.e. 4.8 × 10⁷ ligands per cell), the intensity of the Tyr-653/Tyr-654 bands does not increase when the concentration of ligand is further increased, i.e. a plateau in phosphorylation was observed. Such a behavior is expected based on the physical-chemical model proposed here. As shown in Fig. 1, at high ligand concentrations, the phosphorylation of the mutant receptor is higher than of the wild type.
In these experiments, special attention was paid to ensure that we achieve saturation in receptor phosphorylation and not simply gel saturation. Western blot protocols were developed such that the band intensities were always within the so-called linear range, i.e. the band intensities were proportional to the receptor concentrations. Detailed discussion of how to ensure that the data are in the linear range is given in the supplement to our previous paper (20). Furthermore, saturation was observed even when gel loading was decreased and when gel exposure times were deliberately very short, such that the weak phospho-Tyr-staining bands at low ligand concentration were barely observable. Thus, the observed saturation in Tyr-653/Tyr-654 staining intensity in Fig. 6 is true saturation and is not due to gel processing.

The fact that the intensities of the Tyr-653/Tyr-654 bands at high ligand concentration were the same for the wild type and the mutant suggests that the probability for phosphorylation within the liganded dimers, $\Phi_d$, is the same for the wild-type and the mutant (see Equation 6). Thus, the mutation is not affecting the phosphorylation of the liganded dimer.

**G380R Mutation Does Not Affect the Cross-linking Propensity of Full-length FGFR3**

We also investigated if the cross-linking propensity of FGFR3 was affected by the G380R mutation. Experiments were carried out in the absence of ligand and at saturating ligand concentrations. We used bis(sulfosuccinimidyl) suberate to cross-link the receptors on the cell membrane. After cell lysis, we ran Western blots while staining with the anti-FGFR3 antibody. The results are shown in Fig. 7. We saw a modest increase (~50%) in the intensity of the cross-linked band in the presence of saturating ligand concentration for both FGFR3 and FGFR3/G380R. The cross-linked band became wider and encompassed higher molecular weights. This is not surprising, because the cross-linker is nonspecific and can cross-link the ligand with the receptor dimer.

Fig. 7 further shows that the G380R mutation has no effect on FGFR3 cross-linking, with or without fgf1 stimulation. Quantification of the gels yielded the cross-linked fractions for the wild type and the mutant. In the absence of ligand, the cross-linked fractions were $0.23 \pm 0.04$ and $0.25 \pm 0.034$ for the wild-type and the mutant, respectively, and thus they were identical within experimental error. These results suggest that the increase in phosphorylation observed in Fig. 6 was not due to an increase in cross-linking propensities. Cross-linking propensities correlate with dimerization propensities, and the results suggest that the achondroplasia mutation does not affect dimerization.

**Quantitative Analysis of Western Blot Results**

**Calculation of $[P]_\text{sat}/[P]$ as a Function of Ligand Concentration**—To calculate $[P]_\text{sat}/[P]$, for the wild-type and the mutant, three independent titration experiments, such as the one in Fig. 6, were performed. For each titration, the point with the highest phospho-Tyr-staining band intensity was assigned the saturation value $[P]_\text{sat}$ for this particular titration. The values of $[P]/[P]_\text{sat}$ from the three experiments were averaged and are shown in Fig. 8.

**Calculation of $\Phi_d/\Phi_D$**—The values of $\Phi_d/\Phi_D$ for the wild-type and the mutant are needed to fit the data in Fig. 8 to the physical-chemical model described by Reactions 1 and 2 and...
The data in Fig. 8 yield both the phosphorylation at saturating ligand concentration, \([P]_{sat}\), and at zero ligand concentration, \([P]_0\), and thus, the ratio \([P]_{sat}/[P]_0\). The values from three independent experiments at identical conditions (same DNA amounts used for transfection and same ligand concentration) are given in Table 1.

Based on the calculated values of \([P]_{sat}/[P]_0\) and \([D]_{sat}/[d]_0\), we next calculate the ratio \(\Phi_d/\Phi_p\) for the wild-type and the mutant. The ratio \((\Phi_d^{mut}/\Phi_d^{WT})(\Phi_p^{WT}/\Phi_p^{mut})\) was about 2.5. Because the values of \(\Phi_d\) were the same for the wild type and mutant \((\Phi_d^{mut}/\Phi_d^{WT}, \text{see above})\), we conclude that \(\Phi_d^{mut}/\Phi_d^{WT} = 2.5\), and thus the achondroplasia mutation increases \(\Phi_p\), the probability for Tyr-647/Tyr-648 phosphorylation within the unliganded dimer, by a factor of 2.5.

\textbf{Fit of the Phosphorylation Data to the Model and Calculation of} \(K_1\) \textbf{and} \(K_2\) — Using the values of \(\Phi_d/\Phi_p\) calculated above, the activation model given by Reactions 1 and 2 and Equations 1–7 was fitted to the experimental data in Fig. 8 using a Matlab code. There were three unknowns in the fit, \(K_1\), \(K_2\), and \([TR]\). Initial guesses for these three unknown parameters were inputted into the Matlab code, and the parameters were varied until the calculated \([P]_{sat}/[P]\) as a function of total ligand concentration, \([TL]\), provided the best description of the experimental data. The optimal parameters determined in the fit did not depend on the initial conditions, indicative of robust fits. The results are shown in Table 2. The number of receptors per cell, \([TR]\), determined in the fit, was \(5 \times 10^6\) for both the wild type and the mutant. Importantly, in the two independent fits for the wild type and the mutant, we obtain the same value of \([TR]\), consistent with the fact that the expression of the wild type and the mutant is the same. The value is similar to measurements of RTK concentrations in cells that overexpress them (20).

The results of the fit are shown in Table 2. The optimal \(K_2\) for the wild type and the mutant are the same within experimental error. Therefore, the value of \(K_2\) does not change because of the mutation, i.e. the mutation does not affect ligand-induced dimer stabilization and therefore ligand binding. Also, there is no statistically significant change in \(K_1\). Thus, the mutation does not increase the dimerization propensity. The latter result is consistent with the cross-linking results shown in Fig. 7, demonstrating no observable effects of the mutation on cross-linking. The increase in phosphorylation in the absence of ligand is thus primarily due to the different values of \(\Phi_p\), i.e. due to the different phosphorylations of the unliganded dimers of the wild type and the mutant.

\textbf{Effect of the Achondroplasia Mutation on FGFR3 TM Dimer Structure}

Insights into the observed change in phosphorylation probability in the unliganded FGFR3 dimer may come from the experiments of Bell et al. (24), who showed that in the absence of ligand the rotation of the RTK TM dimer interface leads to periodic oscillations in kinase activity. Furthermore, inserting residues in the C-terminal TM flanking region, which caused the kinase domain to rotate with respect to the TM domain,
restored the kinase activity of inactive receptors. These results suggested that the RTK TM dimer structure determines the positions of the two catalytic domains with respect to each other and controls their phosphorylation efficiency in the absence of ligand. Therefore, the increase in phosphorylation that we observe due to the mutation may be due to a structural change.

We have already reported a computer model of the mutant FGFR3 TM dimer structure (25) created with the program CHI (26–28). The model suggests that cation–π interactions occur between Arg-380 and three aromatic residues, Phe-384 on the same helix and Tyr-379 and Phe-383 on the neighboring helix. These cation–π interactions may compensate for the electrostatic repulsion between the two Arg residues, such that dimerization is not altered due to the mutation (25).

To gain insight into the effect of the G380R mutation on FGFR3 dimer structure, here we compared the structures of the mutant (Fig. 9, green) and wild-type (cyan) TM dimers, both determined with the program CHI, by aligning one of the helices in the wild-type dimer with the respective helix in the mutant dimer. The result is shown in Fig. 9 and reveals the differences in TM dimer structures. The amino acids that mediate helix-helix contacts in the wild-type dimer are Leu-377, Val-381, Phe-384, and Ile-387, whereas the mutant dimer interface is composed of Ile-376, Arg-380, Phe-383, Ile-387, Val-390, and Thr-394. In both structures, specific interactions between the helices are mediated by side chains.

To visualize the rotation in the dimer structure, in Fig. 9 we have colored Ala-391 (which is close to the C terminus of the TM domain) in both structures, yellow for the wild type and red for the mutant. We see that the two alanines in position 391 face each other directly in the wild-type structure but are rotated away from each other in the mutant structure. Such a rotation, according to the experiments of Bell et al. (24), leads to a rotation of the catalytic domains with respect to each other and to a change in their kinase activity.

**DISCUSSION**

**Novel Findings**—The main findings in this paper are as follows. 1) The achondroplasia mutation does not affect the phosphorylation of Neu_FGFR3, as probed by the 4G10 antibody. 2) The achondroplasia mutation does not affect the cross-linking of Neu_FGFR3. 3) The achondroplasia mutation does not affect the dimerization energetics of the mutant FGFR3. 4) The mutation increases the phosphorylation of FGFR3 at any ligand concentration, as probed by the Tyr-653/Tyr-654 antibody. 5) The mutation increases the phosphorylation of Neu_FGFR3 at no and at low ligand concentration, as probed by the Tyr-653/Tyr-654 antibody. Thus, the only effect that we observe due to the mutation is increased phosphorylation of FGFR3 at high ligand concentration, as probed by the Tyr-653/Tyr-654 antibody, which specifically recognizes Tyr-647 and Tyr-648 in FGFR3. 6) The mutation does not affect the cross-linking of FGFR3 at any ligand concentration. 7) The mutation increases the phosphorylation of FGFR3 at no and at low ligand concentration, as probed by the Tyr-653/Tyr-654 antibody.

Thus, the only effect that we observe due to the mutation is an increase in the fraction of receptors that are phosphorylated at Tyr-653/Tyr-654 in the absence of ligand and at low ligand concentration. The two tyrosines, Tyr-647 and Tyr-648, that are recognized by the Tyr-653/Tyr-654 antibody are essential for biological function, and thus the observed increase in their phosphorylation probability is likely related to pathogenesis in achondroplasia.

Here, we do not study the effect of the G380R mutation on FGFR3 trafficking or down-regulation. We address the underlying reason for the increased FGFR3 phosphorylation because of the mutation. It has been hypothesized that this reason is increased FGFR3 dimerization. To address this hypothesis, we previously determined the dimerization propensity of the isolated FGFR3 TM domain in liposomes (18). We observed that the mutation does not alter the dimerization energetics of the

### TABLE 1

|               | \([\frac{D}{[\alpha]} | \frac{d}{[\beta]}\) | \([\frac{P}{[\alpha]} | \frac{P}{[\beta]}\) | \(\Phi_{[\alpha]} | \Phi_{[\beta]}\) |
|---------------|------------------|------------------|------------------|
| Wild type     | 1.51 ± 0.05      | 6.24 ± 0.50      | 0.24 ± 0.07      |
| G380R mutant  | 1.50 ± 0.44      | 2.50 ± 0.60      | 0.60 ± 0.09      |

### TABLE 2

Optimal values for the total receptor concentration \([T_R]\), dimerization constant \(K_1\), and ligand-binding constant \(K_2\) determined by fitting the theoretical predictions of phosphorylated fractions, given by Reactions 1 and 2 and Equations 1–7, to the experimentally measured values in Fig. 8

|               | \([T_R]\)       | \(K_1\)      | \(K_2\)      |
|---------------|----------------|-------------|-------------|
| Wild type     | 4.9 ± 0.9 \times 10^6 | 0.07 ± 0.06  | 0.2 ± 0.1    |
| G380R mutant  | 4.7 ± 0.2 \times 10^6 | 0.02 ± 0.01  | 0.3 ± 0.1    |
isolated FGFR3 TM domain. In this study, we do not see an effect of the mutation on FGFR3 and Neu_FGFR3 cross-linking. By fitting the Western blot phosphorylation data to the activation model described by Reactions 1 and 2 and Equations 1–7, we further show that the mutation does not affect the dimerization constant $K_1$. Thus, our results do not support the hypothesis that the mutation enhances FGFR3 dimerization.

The only parameter of the activation model affected by the achondroplasia mutation is $\Phi_p$, the probability for phosphorylation in the unliganded dimer. The increase in $\Phi_p$ leads to the observed increase in FGFR3 phosphorylation in the absence of ligand and at low ligand concentrations.

Discussion of the FGFR3 Activation Model—The heart of the physical-chemical model used here to fit the Western blot results are the two coupled Reactions 1 and 2 describing dimerization and ligand binding. Thus, the model accounts for what is often called “basal or constitutive” activation of FGFR3 in the absence of ligand (13). Indeed, Reaction 1 is driven to the right, toward the unliganded dimeric state, by increasing the total receptor concentration.

To account for phosphorylation within the unliganded and liganded dimers, here we introduce the probabilities for phosphorylation, $\Phi_p$ and $\Phi_p^L$. Both values are non-zero, because we observe phosphorylation both in the absence and presence of ligand. Note that even the immature FGFR3 population located in the endoplasmic reticulum can be phosphorylated (16).

In the model, $\Phi_p$ and $\Phi_p^L$ are experimentally determined. The phosphorylation level at high ligand concentrations yields information about the value of $\Phi_p^L$. Although the absolute value of $\Phi_p^L$ cannot be determined, the ratios $\Phi_p/\Phi_p^L$, for the wild type and the mutant are determined by comparing phosphorylation and cross-linking data in the absence of ligand and at saturating ligand concentrations (see Table 1). Furthermore, the fact that the Tyr-653/Tyr-654 band intensities are the same for the wild type and the mutant at saturating ligand concentrations suggests that the values of $\Phi_p$ are the same for the wild type and the mutant.

A crude assumption in the data analysis is the use of cross-linked fractions as a measure of dimeric fractions. This assumption is not always valid, because the probability for cross-linking depends on both dimerization propensity and dimer structure. The latter is an important determinant of cross-linking efficiencies, because the cross-linker reacts with amines in very close proximity. We approximate dimeric with cross-linked fractions because there are no experimental methods available that yield absolute measures of RTK dimeric fractions on cell surfaces. As quantitative FRET methods become more sophisticated, they might eventually allow us to measure $[D]_{sat}/[d]_o$ directly. When such measurements become feasible and available, the ratios $\Phi_p/\Phi_p^L$, for the wild type and the mutant can be recalculated and the Western blot data in Fig. 8 refitted, for better estimates of $K_1$ and $K_2$.

We know that the ligand induces a large structural change in the extracellular domain, and thus it is possible that the efficiency of the cross-linker is different for unliganded and liganded dimers (2, 29). Thus, our estimates of $[D]_{sat}/[d]_o$ may be very crude. At the same time, the G380R mutation is not expected to have a large effect on the structure of the extracellular domain, and thus we can expect that the ratios $[d]_o^{mut}/[d]_o^{WT}$, $[D]_{sat}^{mut}/[D]_{sat}^{WT}$, and $\Phi_d^{mut}/\Phi_d^{WT}$ are not much affected by approximating cross-linked with dimeric fractions. Thus, the conclusions of this study should not be affected by our inability to directly measure dimeric fractions.

A second crude assumption of the model is the assumption that the mature FGFR3 is located predominantly in the plasma membrane. In fact, a fraction of the mature FGFR3 is expected to be found intracellularly. For instance, FGFR3 has been shown to localize and signal in the endosomal compartments, where it can be either destroyed or recycled back to the membrane (12). The model we present here is simplified, because it does not take into account the different mature FGFR3 pools (trans-Golgi, plasma membrane, endosomes). Yet the model fits the experimental data very well, as demonstrated in Fig. 8.

Comparison with Other Studies and New Insights—Our results with the chimeric Neu_FGFR3 receptor are different from a previous study by Webster and Donoghue (13), who observed an increase in phosphorylation due to the G380R mutation. The observed difference may be due to experimental details. For instance, we used CHO cells for these experiments, and Webster and Donoghue (13) used NIH3T3 cells and COS7 cells. They assayed phosphorylation after immunoprecipitation, while here we probe the phosphorylation in cells. It is possible that phosphorylation probabilities are different within the immunoprecipitate and the cellular membrane. Note that a similar mutation in FGFR3 TM domain, A391E, shows an increase in phosphorylation and cross-linking under the very same conditions used here to probe the effect of the G380R mutation (20).

In this study, we use a new approach to gain insight into the effect of the G380R mutation on receptor function. For the first time, we use a physical-chemical model to interpret the phosphorylation of full-length FGFR3. We fit the experimental data to the model, which describes phosphorylation in terms of propensities for dimerization and ligand binding, and in terms of phosphorylation probabilities. Although the model is relatively simple, we see that it fits the data very well. Thus, it allows us to extract apparent thermodynamic parameters and reveal the effect of the mutation on different steps in FGFR3 activation. To be able to do this work, we titrate ligand over a very wide concentration range, which allows the measurement of phosphorylated fractions. Although such high ligand concentrations are nonphysiological, they allow us to observe a plateau in receptor phosphorylation and thus measure the phosphorylated fractions at lower, physiologically relevant ligand concentrations.

By using two different receptors, the chimeric Neu_FGFR3 and the full-length FGFR3, we gain additional insight into the effect of the G380R mutation on FGFR3 activation. Although the mutation does not affect the phosphorylation within the chimeric receptor dimer, it increases the phosphorylation probability within the full-length FGFR3 unliganded dimer. Thus, the effect of the mutation appears to propagate into the kinase domain of FGFR3 but not into the kinase domain of Neu within the Neu_FGFR3 chimera. The fact that the effect is observed only in full-length FGFR3 suggests that the TM
domains and the catalytic domains of FGFR3 work synergistically to control phosphorylation.

**Physical Basis behind the Pathology in Achondroplasia**—As discussed above, the G380R mutation increases FGFR3 phosphorylation at low ligand concentrations. Here, we link this increase to an increased phosphorylation probability within the unliganded dimer. In other words, it is easier to phosphorylate Tyr-647 and Tyr-648 within the mutant unliganded dimer than the wild-type unliganded dimer. The most likely reason for this difference in phosphorylation is a difference in the structure of the unliganded dimer. Thus, we propose that the achondroplasia mutation induces a structural change in the unliganded dimer.

The computer models, presented in Fig. 9, suggest that the G380R mutation induces a rotation in the TM dimer interface. Furthermore, rotation of RTK TM dimer interfaces has been linked to changes in auto-phosphorylation propensities in the absence of ligand (24). Therefore, we propose the following: 1) positioning of the catalytic domains in the wild-type unliganded FGFR3 dimer is such that the phosphorylation efficiency is not the highest possible; and 2) rotation of the FGFR3 TM dimer interface due to the G380R mutation leads to rotation of the catalytic domains with respect to each other and to an increase in the phosphorylation probability within the unliganded mutant dimer.

Ligand binding is known to alter the structure of RTK extracellular domains (2, 30), and some argue that it alters the structure of the full-length RTK dimers (31, 32). Here, we find that the achondroplasia mutation does not alter the phosphorylation of the liganded FGFR3 dimer, consistent with the idea that ligand binding has an effect on the structure and positioning of the catalytic domain. The effect of the G380R mutation on phosphorylation is reduced in the absence of ligand, most likely due to the structural constraints imposed by the ligand.

Furthermore, it is easy to envision that although a modest change in the structure of the FGFR3 liganded dimer does not affect phosphorylation, it may affect its ubiquitination and hence its down-regulation. Thus, a Gly-380-mediated structural change may affect FGFR3 signaling in the following two ways: by increasing the phosphorylation of the unliganded dimer and by compromising the down-regulation of the liganded dimer. Both of these effects could be contributing to pathogenesis in achondroplasia.

**Implications and Future Challenges**—This work provides insights into how the achondroplasia mutation increases FGFR3 activation. The data do not support the current belief that the mutation increases FGFR3 dimerization. Rather, the mutation makes it easier for phosphorylation to occur within the unliganded dimer, as opposed to the wild type. This result has implications for future design strategies for specific FGFR3-targeted treatments for achondroplasia, suggesting that the utility of FGFR3 dimerization inhibitors may be limited. On the other hand, inhibitors that decrease the phosphorylation within the unliganded FGFR3 dimers may be useful as treatments for achondroplasia.

Although here we demonstrate a phosphorylation increase within the unliganded FGFR3 dimer in achondroplasia, we can only speculate about the underlying cause for this increase. One hypothesis shown in Fig. 9 is that the mutation changes the structure of the mutant unliganded dimer, such that it becomes easier to phosphorylate tyrosines 647 and 648. This hypothesis highlights the importance of experimentally determined structure, beyond molecular models, in understanding the molecular basis behind the pathology and for devising strategies for targeted inhibition. As discussed previously (25, 33), structural information is critical for understanding RTK signaling. Structural changes due to the achondroplasia mutation likely originate in the TM domain and propagate to the kinase domain, and thus will be observable only within the context of the full-length receptors, not the isolated kinase domains. Thus, future high resolution structures of full-length RTKs will not only elucidate the basic mechanism of RTK signaling but also shed light on how mutations impact RTK dimer structures and ultimately lead to pathologies.

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