Cracking the Molecular Origin of Intrinsic Tyrosine Kinase Activity through Analysis of Pathogenic Gain-of-Function Mutations

Huaibin Chen1,2,#, Zhifeng Huang1,2,#, Kaushik Dutta3,#, Steven Blais4, Thomas A. Neubert1,4, Xiaokun Li5, David Cowburn5, Nathaniel J. Traaseth6, and Moosa Mohammadi1,*

1Department of Biochemistry & Molecular Pharmacology, New York University School of Medicine, New York, NY 10016, USA
2School of Pharmacy, Wenzhou Medical College, Wenzhou, 325035, China
3New York Structural Biology Center, New York, NY 10027, USA
4Kimmel Center for Biology and Medicine at the Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, NY 10016, USA
5Department of Biochemistry, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY 10461, USA
6Department of Chemistry, New York University, New York, NY 10003, USA

SUMMARY

The basal (ligand-independent) kinase activity of receptor tyrosine kinases (RTKs) promotes trans-phosphorylation on activation loop tyrosines upon ligand-induced receptor dimerization, thus upregulating intrinsic kinase activity and triggering intracellular signaling. To understand the molecular determinants of intrinsic kinase activity, pathogenic FGF receptor mutants with gradations in gain-of-function activity were analyzed by X-ray crystallography and NMR spectroscopy. These structural analyses revealed a “two-state” dynamic equilibrium model whereby the kinase toggles between an ‘inhibited’, structurally rigid ground state, and a more dynamic and heterogeneous active state. The pathogenic mutations have different abilities to shift this equilibrium towards the active state. The increase in the fractional population of FGF receptors in the active state correlates with the degree of gain-of-function activity and clinical severity. Our data demonstrate that the fractional population of RTKs in the active state determines intrinsic kinase activity, and underscore how a slight increase in the active population of kinase can have grave consequences for human health.

INTRODUCTION

RTKs regulate a myriad of biological processes in mammalian development, tissue homeostasis and metabolism (Hunter, 2000; Lemmon and Schlessinger, 2010). At basal
state, RTKs possess low levels of intrinsic kinase activity. This basal activity plays a key role in RTK signal transduction as it primes trans-phosphorylation of tyrosines in the activation loop (A-loop) upon ligand binding and receptor dimerization resulting in full activation of the kinase. This in turn allows tyrosine phosphorylation of the kinase insert and flanking juxtamembrane and C-terminal tail regions of the RTKs. These secondary phosphorylation events then serve to recruit downstream signaling substrates for tyrosine phosphorylation by the activated RTK, culminating in activation of intracellular signaling pathways.

The basal, intrinsic activity of RTKs must be carefully balanced. Insufficient basal activity would diminish the ability of ligand-induced dimerization to promote A-loop tyrosine trans-phosphorylation, and hence signal transmission would stall. Conversely, excess basal activity would bypass the requirement for ligand-induced dimerization in kinase activation, resulting in ligand-independent constitutive RTK signaling. Indeed, kinase domain mutations that elevate or reduce the intrinsic activity of RTKs are common culprits in a diverse array of human diseases. An accumulated wealth of structural and biophysical data have revealed some insight into the autoinhibitory mechanisms that keep tyrosine kinase activity at bay (Hubbard, 2002), and have also elucidated the molecular basis of kinase activation by A-loop tyrosine phosphorylation (Chen et al., 2007; Hubbard and Till, 2000; Pellicena and Kuriyan, 2006; Rajakulendran and Sicheri, 2010). Despite these major strides in understanding the mechanisms of kinase regulation, a fundamental question has remained unanswered: what precisely determines basal, intrinsic kinase activity?

In FGFR2 and FGFR3, a conserved lysine located two residues away from the A-loop tyrosines is a major hotspot codon for pathogenic mutations. Substitutions of Lys650 in FGFR3 with Thr, Asn, Gln, Met, or Glu cause dwarfism syndromes of varying clinical severity (Vajo et al., 2000) (Supplemental Figure S1). Among them, neonatal lethal Thanatophoric Dysplasia type II (TDII) is the most severe and is caused by the Lys650Glu mutation (Chen et al., 2001; Tavormina et al., 1995; Webster et al., 1996; Wilcox et al., 1998). Substitution of Lys650 with Met causes Severe Achondroplasia with Developmental Delay and Acanthosis Nigricans (SADDAN) syndrome (Bellus et al., 1999; Tavormina et al., 1999). The Lys650Asn and Lys650Gln mutations lead to hypochondroplasia (HCH), a mild form of dwarfism (Bellus et al., 1995; Bellus et al., 2000). The Lys650Thr mutation causes an even milder form of HCH (Berk et al., 2007; Castro-Feijoo et al., 2008). In vitro autophosphorylation assays have shown that these mutations confer different degrees of ligand-independent activation on FGFR kinase, and that the degree of aberrant activation correlates with the clinical severity of the dwarfism syndromes caused by the mutations (Bellus et al., 2000). This naturally occurring set of pathogenic kinases with graded kinase activity provides a powerful tool to explore the molecular determinants of intrinsic kinase activity. In this report we studied the impact of these five pathogenic mutations on the structure and dynamics of FGFR kinase using X-ray crystallography and NMR spectroscopy. Our data uncover a “two-state” dynamic equilibrium model for regulation of FGFR kinase and likely other RTKs, and show that the fractional population of the kinase in the active state determines intrinsic kinase activity.

**RESULTS AND DISCUSSION**

**The Lys650/659 pathogenic mutations elevate intrinsic FGFR kinase activity independent of A-loop tyrosine phosphorylation**

Bellus and colleagues (Bellus et al., 2000) were the first to study the impact of the dwarfism syndrome mutations at Lys650 on the kinase activity of FGFR3 and noticed a functional-clinical correlation between the degree of gain-of-function and severity of skeletal phenotypes (Supplemental Figure S1). In their study, wild-type full-length FGFR3 and...
mutants of it harboring the pathogenic substitutions at Lys650 were overexpressed in mammalian cells, and the activity of immuno-purified receptors was compared using an in vitro autophosphorylation assay. This approach has two limitations, however. Firstly, due to the activating nature of these mutations the overexpressed receptor mutants may contain residual A-loop tyrosine phosphorylation, which would confound conclusions about the kinase assay data. Secondly, measuring intrinsic kinase activity using autophosphorylation can be potentially misleading because the mutations could affect the latter in the absence of an effect on kinase activity. To unambiguously determine the impact of the pathogenic mutations on the intrinsic kinase activity of FGFR3 kinase, we purified phosphorylation-free wild-type and mutated FGFR3 kinase domains, and compared their intrinsic activities using a substrate phosphorylation assay. Our kinase assay data show that the mutations differentially enhance the intrinsic activity of FGFR3 kinase in the following order: Lys650Glu > Lys650Met > Lys650Asn ~ Lys650Gln > Lys650Thr (Figure 1A). This trend is generally in agreement with the data reported by Bellus and colleagues (Bellus et al., 2000) except that in this previous study, Lys650Met showed greater autophosphorylation activity than Lys650Glu, which is inconsistent with the clinical severity of the dwarfism syndromes caused by these mutations.

Having confirmed the gradation in gain-of-function imparted by the different pathogenic mutations, we next examined the structural basis of graded kinase activation by the pathogenic mutations. Since comprehensive structural studies of this scale require an abundant source of protein, we decided to use the highly homologous FGFR2 kinase as the “workhorse” for this study, as it can be expressed at higher levels in E. coli than the FGFR3 kinase. FGFR2 and FGFR3 kinases share 82% overall sequence identity, with 86% identity within the A-loop, and both are regulated by the FGFR-invariant molecular brake at the kinase hinge region (Chen et al., 2007) (Figure 1C). Indeed, Lys659 (corresponding to Lys650 in FGFR3) and molecular brake mutations in FGFR2 kinase lead to gain-of-function in craniosynostosis syndromes (Kan et al., 2002), endometrial cancer (Pollock et al., 2007) and cervical cancer (Dutt et al., 2008). As expected, our substrate phosphorylation assay data show that the corresponding mutations in FGFR2 kinase, namely Lys659Glu, Lys659Met, Lys659Asn, Lys659Gln and Lys659Thr, behave similarly and impart graded kinase activation following the same order as determined for FGFR3 kinase (Figure 1A and B). Notably, the data also show that the intrinsic activities of the pathogenic FGFR2 kinases are lower than that of FGFR2 kinase activated by A-loop phosphorylation (FGFR2KPY657), indicating that these pathogenic mutations activate the kinase in a partially ligand-independent fashion. These ‘pathogenic’ FGFR2 kinases were then crystallized in the presence of a non-hydrolyzable ATP analog (AMP-PCP) and MgCl2, and their structures were determined. Data collection and refinement statistics are summarized in Table 1.

Comparison of the crystal structures of the pathogenic FGFR2 kinases with those of unphosphorylated and A-loop phosphorylated wild-type FGFR2 kinases reveals that all five mutants adopt the same active conformation, reminiscent of the phosphorylated wild-type kinase (Figure 2A and B). In each structure, the side chain of the mutated residue makes novel contacts with nearby residues, thus locally stabilizing an active A-loop conformation. The rearranged A-loop engages in favorable intramolecular contacts with the αC helix in the N-lobe leading to closer apposition of the N- and C-lobe and disengagement of the molecular brake at the kinase hinge region, which allows the kinase to adopt active conformation. In the Lys659Met mutant structure, the side chain of Met659 binds a hydrophobic depression formed by Leu665, Met670, Ala674, Tyr680 and Tyr657, the latter of which located in the A-loop (Figure 2C). In the remaining four mutant structures (Figure 2D to G), the side chain of the mutated residues engage in hydrogen bonding with Arg625 in the catalytic loop. Moreover, in each of these four structures, the oxygen atoms from the side chains of the mutated residues participate in oxygen-aromatic interactions with Tyr657.
in the A-loop. In all five mutant structures, Tyr657 occupies a position similar to the phosphorylated Tyr657 in activated wild-type kinase structure and makes a direct hydrogen bond with Arg649, which further supports an active A-loop conformation. These data unambiguously demonstrate that the pathogenic mutations simulate an activated A-loop conformation (Figure 2B). To ensure that the mechanism of activation observed in the FGFR2 kinase mutant structures truly reflects the effect of the actual pathogenic FGFR3 kinase mutations, we have solved the crystal structure of FGFR3 kinase harboring the Lys650Glu mutation. Similar to the Lys659Glu FGFR2 kinase structure, the carboxylate side chain of Glu650 engages in hydrogen bonding with Arg616 in the catalytic loop, while simultaneously participating in oxygen-aromatic interactions with Tyr648. These intramolecular interactions result in stabilization of the A-loop in the active conformation (Huang et al., 2013; under revision in Structure). To validate the mechanism of activation observed in the crystal structures of FGFR2 kinase mutants, the effects of mutating A-loop tyrosines (Tyr656 and Tyr657) to phenylalanine were studied. The data show that elimination of A-loop tyrosine phosphorylation only modestly reduces the activity of the pathogenic kinases (Figure 1B), which is consistent with the direct involvement of Tyr657 in the stabilization of an A-loop active conformation (Figure 2D to G).

A two-state dynamic equilibrium model underlies FGFR kinase regulation

The structural data showing that all five pathogenic mutations stabilize the same active conformation as A-loop tyrosine phosphorylation does in the wild type kinase is surprising given the differences in the intrinsic activity of the mutant kinases and the mono-phosphorylated FGFR2K\(^{pY657}\). Close inspection of the intramolecular contacts introduced by the different pathogenic mutations, however, unveils the molecular basis for the observed gradations in kinase activity of the mutants. The analysis shows a direct correlation between the number and length of hydrogen bonds and oxygen-aromatic interactions that each mutation introduces (summarized in Supplemental Table S1), and the degree of gain in intrinsic kinase activity imparted by that mutation. This is best illustrated by comparing the structures of the most active mutant (Lys659Glu, Figure 2D) and the least active mutant (Lys659Thr, Figure 2G). In the Lys659Glu structure, the carboxylate side chain of Glu659 introduces a total of three strong hydrogen bonds (≤3 Å) with Arg625 and two oxygen-aromatic interactions (≤3 Å) with Tyr657. In contrast, the Lys659Thr structure shows that Thr659 makes only a single weak hydrogen bond (>3 Å) with Arg625, and two oxygen-aromatic interactions (>3.5 Å) with Tyr657. Hence, these static crystallographic snapshots imply that the pathogenic mutations have different potentials to stabilize the same active conformation of the enzyme. Taken together with the observed gradations in kinase activity, these X-ray crystallographic data led us to propose that FGFR kinase exists in dynamic exchange between an inhibited ground state conformation and an active state conformation, and that the mutations act by increasing the population of kinase molecules in the active state. Accordingly, the fractional population of kinase molecules in the active state determines the intrinsic kinase activity.

To test this hypothesis, we employed NMR spectroscopy to investigate the effects of the mutations on the conformation and dynamics of FGFR2 kinase in solution. As expected, the overlay of HSQC spectra showed large chemical shift changes for many residues between unphosphorylated wild-type kinase and the mono-phosphorylated kinase, FGFR2K\(^{pY657}\) (Supplemental Figure S2). Importantly, many residues in the kinase mutants also experienced chemical shift changes. Mapping of these perturbations onto the crystal structure of unphosphorylated wild-type FGFR2 kinase shows that the pathogenic mutations affected a similar set of residues as those perturbed by Tyr657 phosphorylation (Figure 3 and Supplemental Figure S3). Interestingly, several residues of the pathogenic kinases show chemical shifts that fall between those of the unphosphorylated wild-type kinase and the
mono-phosphorylated FGFR2K\textsuperscript{pY657} along a straight, linear trajectory (Figure 4A and Supplemental Figure S4). Notably, the HSQC spectra for the unphosphorylated wild-type kinase and all five pathogenic mutant kinases contain a relatively narrow peak for each residue, indicating a fast chemical exchange with the two end points of the chemical shifts defined by the unphosphorylated low activity state and the mono-phosphorylated (pY657) active state. Furthermore, the assigned chemical shifts of the most active mutant kinase, namely Lys659Glu, are the closest to those of the mono-phosphorylated kinase, FGFR2K\textsuperscript{pY657}, whereas the assigned chemical shifts of the least active mutant kinase, namely Lys659Thr, are the closest to those of the unphosphorylated wild-type kinase (Figure 4A). This spectral behavior of these pathogenic kinases strongly suggests that these mutants differentially sample a common equilibrium between a structural state resembling the unphosphorylated inhibited state and another resembling the A-loop phosphorylated active state. Based on the positions of NMR chemical shifts for the five pathogenic kinases, and on the assumption that the unphosphorylated and A-loop phosphorylated FGFR2 kinases represent the pure ground state and the active state, respectively, in a fast “two-state” conformational exchange regime, the fractional population of kinase molecules occupying the active state was calculated for each mutant (Figure 4B). This approach has been previously used to study the impact of engineered gain- and loss-of-function mutations on the fractional populations of nitrogen regulatory protein C (NtrC) (Volkman et al., 2001) and guanine nucleotide exchange factor Vav1 (Li et al., 2008) in the active state. Using the populations derived from Figure 4B and the normalized activity values from Figure 1B, a quantitative correlation plot was constructed (Figure 4C). The strong correlation ($r^2 = 0.97$) reveals a direct relationship between an increase in population of kinase molecules in the active state and the degree of gain-of-function. These data support the two-state model and show that the pathogenic mutations act by altering the intrinsic conformational dynamics of the kinase to favor the active state (Figure 5).

**Free-energy landscape of FGFR kinase is biased towards the inactive state**

Interestingly, the HSQC spectrum of the A-loop phosphorylated kinase shows broader peaks for several residues within the catalytic loop that display linear chemical shift changes in the mutated kinases (Supplemental Figure S5). This spectral behavior implies that compared with the inactive state, the active state is more dynamic (Figure 5B). Consistent with this observation, $^{15}$N\textsuperscript{[1H]} R\textsubscript{2} NMR spin relaxation data on the unphosphorylated wild-type kinase, the pY657 mono-phosphorylated kinase, and unphosphorylated mutant kinases revealed that, akin to A-loop phosphorylation, the gain-of-function mutations lead to increases in R\textsubscript{2} consistent with greater conformational flexibility (Supplemental Figure S6). Taken together, these findings suggest the presence of conformational heterogeneity in the active state arising from an elevated energy landscape compared with the low energy state of the kinases in the ground state (Figure 5C). The high level of free energy associated with the active state is harmonious with the fact that the unphosphorylated wild-type kinase has never been crystallized in the active state, and suggests that the gain-of-function mutations cause an increase in the population of active state molecules that is sufficient to enable crystallization of the kinase in the active state. The instability of the active state also explains why the isolated phosphorylated FGFR kinase tends to dephosphorylate over time (H.C. and M.M., unpublished data).

Notably, the two-state model underlying FGFR kinase regulation differs from the conventional two-basin free-energy landscape model proposed for Src and CDK2 kinases (Banavali and Roux, 2009; Berteotti et al., 2009), in which the active state forms a well-defined low energy basin that is separated by a high energy barrier from the inactive state (high energy basin) (Supplemental Figure 7A). However, our findings are concordant with the NMR spectroscopy and MD simulation data showing that the phosphorylated apo form
of protein kinase A exists in a dynamically uncommitted state characterized by the presence of inhomogeneous line-broadening in several loops (Masterson et al., 2011). In the case of the EGFR kinase, molecular dynamics simulation experiments have led to the postulation of a distinct three-state energy landscape model in which a third “disordered” state forms a low energy basin intervening between the inactive and active states (Shan et al., 2012) (Supplemental Figure 7B). Similar to the FGFR kinase, the active state of the EGFR kinase also possesses a higher level of free energy and lacks the typical energy basins seen in Src and CDK2 kinases.

Concluding remarks

Our X-ray crystallographic and NMR data reveal a direct correlation between gradations of intrinsic kinase activity and a shift in the equilibrium toward the active conformation of the kinase, thus demonstrating that the fractional population of enzyme in an activated state determines the intrinsic activity. We suggest that the two-state dynamic equilibrium model, uncovered here for the regulation of the class IV FGFR kinase family, is also applicable to kinases of the class III RTK subfamily. The three constituents of the molecular brake are fully conserved in class III RTKs, namely PDGFRs, C-KIT, C-FMS, and VEGFRs. In addition, molecular brake mutations in PDGFR and VEGFR also lead to receptor activation and human malignancies (Corless et al., 2005; Medeiros et al., 2004). Moreover, selective ATP-competitive inhibitors of VEGFR and PDGFR often cross-inhibit FGFR kinases (Renhowe et al., 2009) and pathogenic activating mutations in class III RTKs, when grafted into FGFR kinases, confer gain-of-function (M.M., unpublished data). In conclusion, our data demonstrate that pathogenic mutations act by corrupting the intrinsic conformational dynamics of the kinase to favor the active state, and allow us to draw an elegant correlation between the increase in population of RTK molecules in the active state and the degree of severity in clinical phenotype. In addition to underscoring the importance of tightly controlled regulation of intrinsic RTK activity in human biology, our data provide frameworks for the rational design of inhibitors to tame these hyperactive pathogenic FGFRs for treating human diseases. Lastly, our data inform us of innovative strategies for better clinical management of human skeletal disorders by suggesting that the dosage of inhibitors should be adjusted to the degree by which mutations push the kinase into the active conformation.

EXPERIMENTAL PROCEDURES

Protein Expression, Purification and Crystallization

The cDNA fragments encoding residues Pro449 to Glu759 of human FGFR3c (Accession code: P22607-1) and Pro458 to Glu768 of human FGFR2c (Accession code: P21802-1) were amplified by PCR and subcloned into a pET bacterial expression vector with an NH$_2$-terminal 6XHis-tag to aid in protein purification. All the mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene). The bacterial strain BL21(DE3) cells were transformed with the expression constructs, and kinase expression was induced with 1 mM isopropyl-L-thio-B-D-galactopyranoside overnight at 16–25°C depending on the construct. The cells were lysed, and the soluble kinase proteins were purified according to the published protocol (Chen et al., 2007). Traces of phosphorylation on wild-type and mutant kinases were removed by treating the proteins with FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific) and repurified by anion exchange chromatography (Mono Q, GE Healthcare Life Sciences). The FGFR2 kinase constructs contain five tyrosine autophosphorylation sites namely Tyr466 in the juxtamembrane region, Tyr586 and Tyr588 in the kinase insert, Tyr656 and Tyr657 in the A-loop. Of these, phosphorylation on the A-loop Tyr657 is sufficient for kinase activation. Since making large quantities of homogenously penta-phosphorylated FGFR2 kinase sample proved to be

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challenging, Tyr466, Tyr586, Tyr588, and Tyr656 were mutated to the corresponding nonphosphorylatable residues in FGFR4 kinases (Tyr586Leu and Tyr588Pro) or phenylalanine (Tyr466Phe and Tyr656Phe). This construct (FGFR2KpY657) was purified in the same fashion as the wild-type FGFR2 kinase construct, and was phosphorylated on Tyr657 by incubating it with ATP and MgCl2. The homogenously mono-phosphorylated kinase (FGFR2KpY657) was isolated by size exclusion chromatography followed by anion exchange chromatography. N-terminally His-tagged substrate peptide consisting of residues Leu761 to Thr821 of FGFR2c was expressed and purified using sequential Ni2+ chelating and size exclusion chromatographies. The substrate peptide corresponds to the C-terminal tail of FGFR2 and contains five authentic tyrosine phosphorylation sites (Tyr769, Tyr779, Tyr783, Tyr805 and Tyr812). Isotopically labeled wild-type and pathogenic kinases were expressed and purified similar to their unlabeled counterparts.

**Protein Crystallization**

The purified FGFR2 kinase mutants were concentrated to about 10–100 mg/ml using Amicon Ultra-4 10K Centrifugal Filters (Millipore). Prior to crystallization, the pathogenic kinases were mixed with ATP-analogue (AMP-PCP) and MgCl2 at a molar ratio of 1:3:15. Initial crystals of pathogenic kinases were grown by hanging drop vapor diffusion at 20°C using crystallization buffer composed of 25 mM HEPES pH 7.5, 15%–25% w/v PEG4000, 0.2–0.3 M NH4SO3 and were further optimized by appropriate additives.

**X-ray Data Collection and Structure Determination**

Diffraction data were collected on single cryo-cooled crystals at beamlines X-4A and X-4C at the National Synchrotron Light Source, Brookhaven National Laboratory. Crystals were stabilized in mother liquor by stepwise increasing glycerol concentration to 20%, and then flash-frozen in liquid nitrogen. All diffraction data were processed using *HKL2000 Suite* (Otwonowski and Minor, 1997). Molecular replacement solutions were obtained with *AMoRe* (Navaza, 1994) using the FGFR2 kinase structure (PDB ID: 2PVY (Chen et al., 2007)) as the search model. Rigid-body refinements of the mutant kinases were performed using *CNS* (Brunger et al., 1998) by treating the N-lobe and C-lobe of the kinases as two separate entities. Model building was carried out using *O* (Jones et al., 1991) and iterative positional and B-factor refinements were completed using *CNS* (Brunger et al., 1998) or PHENIX (Adams et al., 2010). Tight non-crystallographic restraints were applied throughout the refinement cycles. Atomic superimpositions were made using *lsqkab* (Kabsch, 1976) in the *CCP4 Suite* (Collaborative, 1994) and structural representations were prepared using *PyMol* (DeLano, 2002).

**Kinase Assay**

Unphosphorylated wild-type, mono-phosphorylated FGFR2KpY657 and unphosphorylated pathogenic FGFR3 and FGFR2 kinases were mixed with kinase reaction buffer containing ATP, MgCl2 and the substrate peptide to the final concentrations of 13.5 mM (kinase), 262 mM (substrate), 10 mM (ATP) and 20 mM (MgCl2). The reactions were quenched at different time points by adding EDTA to the reaction mix with the final concentration of 33 mM. The progress of the substrate phosphorylation was monitored by native-PAGE, and the phosphate incorporation into the substrate peptide was quantified by time-resolved MALDI-TOF mass spectrometry by comparing signals from phosphorylated and the non-phosphorylated peptides as previously published (Chen et al., 2008).

**NMR Spectroscopy**

The [1H,15N] HSQC spectrum of unphosphorylated FGFR2 kinase, harboring the Ala648Thr mutation (FGFR2KpA648T), in the presence of a non-hydrolyzable ATP analog.
AMP-PCP) and MgCl₂ was assigned using a triple labeled [²H,¹³C,¹⁵N] sample at ~200 mM in a buffer consisting of 25 mM Hepes pH 7.5, and 150 mM NaCl. The rational for using this mutant rather than the wild-type kinase was that the Ala648Thr, which is a pathogenic loss-of-function mutation, increases the yield of protein expression. No significant chemical shift perturbations were observed between unphosphorylated wild-type and the Ala648Thr mutant FGFR2 kinases, so that the assignment could be transferred unambiguously. A series of TROSY based triple resonance experiments (HNCO, HN(CA)CO, HNCOCA, and HNCA) were carried out at ¹H frequencies of 800 MHz, using cryogenic probes allowing for the assignment of 81% of the non-Pro ¹H-¹⁵N amide pairs. To ascertain the accuracy of the assignments, FGFR2K¹⁶⁴⁸T were selectively labeled with ¹⁵N labeled amino acids including Tyr, Gly, Lys, Leu, Val, Phe, Ile, and Met. To compare the effects of the pathogenic mutations and A-loop phosphorylation on the conformational dynamics of the FGFR2 kinase, samples of unphosphorylated wild-type FGFR2 kinase, mono-phosphorylated FGFR2K¹⁶⁵⁷ and pathogenic FGFR2 kinases were ¹⁵N labeled and prepared at a concentration of ~300 mM. All HSQC spectra were acquired with 800 MHz spectrometer at 25°C in the presence of AMP-PCP and MgCl₂. The assignments were transferred to the unphosphorylated wild-type, mono-phosphorylated FGFR2K¹⁶⁵⁷ and each of the mutant FGFR2 kinases. The most significant perturbations were found to occur along a linear trajectory, and therefore the transfer of assignment was somewhat analogous to the peak movement in fast exchange for a ligand titration. To quantify the chemical shift perturbations between phosphorylated and unphosphorylated kinases, the following equation for the combined chemical shift (Δδ) was used:

\[ \Delta \delta = \sqrt{(0.154 \Delta \delta_N)^2 + \Delta \delta_H^2} \]  

(1)

Δδ₅ and Δδ₄ are the chemical shift changes between the unphosphorylated and mono-phosphorylated samples for ¹⁵N and ¹H nuclei, respectively. The residues displaying resolved linear chemical shift changes among the samples were used to calculate the population of active state for each sample. We assumed the mono-phosphorylated kinase had an active state population of 1.0 and the unphosphorylated wild-type sample was a good approximation of the inactive state. Based on this, the population of active state (p_active) for each mutant was calculated from the chemical shift positions (δ) of the phosphorylated and unphosphorylated samples:

\[ p_{\text{active}} = \frac{\delta_{\text{mutant}} - \delta_{\text{phos}}}{\delta_{\text{active}} - \delta_{\text{phos}}} \]  

(2)

Residues in the aEF and aG helices showed small deviations from the linear chemical shift trend, which were attributed to their proximity to the Lys659 substitutions in the mutants and the phosphorylated Tyr657 in the mono-phosphorylated FGFR2K¹⁶⁵⁷ (Figure 3 and Supplemental Figure S3).

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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References


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HIGHLIGHTS

- RTK is in a dynamic exchange between a ground state and an active state
- Pathogenic mutations differentially shift the equilibrium towards the active state
- Fractional population of RTKs in active state determines intrinsic kinase activity
Figure 1.
Pathogenic mutations at FGFR3 Lys650 impart different degrees of constitutive (A-loop phosphorylation independent) kinase activation. (A) Fold increase in the activity of Lys650 mutants and phosphorylated wild-type FGFR3 kinase over the unphosphorylated wild-type FGFR3 kinase measured at 30s. (B) Analogous mutations targeting Lys659 in FGFR2 kinase lead to a similar pattern of graded kinase activation. The activities of the pathogenic kinases are less than that of mono-phosphorylated FGFR2K<sup>pY657</sup> (labeled as pY657), implying that the mutations activate in a partially ligand-independent fashion. Black and green bars represent Tyr656/Tyr657 and Phe656/Phe657 versions of the kinases, respectively. (C) Structure based sequence alignment of the A-loop and the autoinhibitory molecular brake among FGFR kinases. The A-loop is boxed in blue. The twin tyrosines and the lysine on the A-loop, and the three components of the molecular brake are labeled in orange, red and green, respectively.
**Figure 2.**

FGFR2 kinase Lys659 mutations have different capacities to stabilize the active state conformation of the A-loop. (A) Comparison of the A-loop conformations of unphosphorylated wild-type (in black, PDB ID: 2PSQ (Chen et al., 2007)), phosphorylated wild-type (in red, PDB ID: 2PVF (Chen et al., 2007)), Lys659Glu mutant (in blue, PDB ID: 4J97), Lys659Met mutant (in purple, PDB ID: 4J96), Lys659Gln mutant (in orange, PDB ID: 4J98), Lys659Asn mutant (in green, PDB ID: 4J95) and Lys659Thr mutant (in cyan, PDB ID: 4J99) FGFR2 kinase structures. Asp644 and Pro666 lie at the beginning and end of the A-loop and are indicated in the unphosphorylated wild-type kinase structure. The subtle difference in the C-terminal region of the A-loop between the phosphorylated wild-type...
kinase and the unphosphorylated wild-type and mutant kinases, is due to an interaction between this section of the A-loop and substrate peptide (which is not present in any of the unphosphorylated wild-type and mutant kinase structures). (B) Close-up view of intramolecular interactions introduced by A-loop tyrosine phosphorylation (Chen et al., 2008). (C to G) Close-up view of intramolecular interactions introduced by the Lys659 mutations. The mutant residues are labeled in red. Side chains of selected residues are shown as sticks. Atom colorings are as follows: oxygens (red), nitrogens (blue), phosphorus (yellow), and carbons (green). The hydrogen bonds and oxygen aromatic interactions are shown as dashed lines with distance (in Å) labeled alongside in black and red, respectively. The hydrophobic interactions are represented by semi-transparent surfaces.
Figure 3.
HSQC chemical shift perturbation plotted as a function of residue for the mutants and the mono-phosphorylated FGFR2K<sup>pY657</sup>. Δδ was calculated according to Equation 1 between the unphosphorylated wild-type kinase and the kinases indicated in the figure. This data was used to construct Supplemental Figure S3.
Figure 4.
NMR chemical shift spectra for the unphosphorylated wild-type FGFR2 kinase, unphosphorylated pathogenic FGFR2 kinases, and mono-phosphorylated FGFR2KpY657.  
(A) Selected peaks in the [\(^1\)H,\(^{15}\)N] HSQC spectra showing linear chemical shift changes. 
(B) The population of active state for each mutant, assuming that the unphosphorylated wild-type FGFR2 kinase and the mono-phosphorylated FGFR2KpY657 respectively represent the pure basal and active states in a fast two-state conformational exchange regime. 
(C) Correlation plot of the normalized kinase activity versus the active state population calculated from the NMR chemical shifts. The NMR spectra and correlation plot are color coded for each sample as indicated in panel B.

\[
y = (0.92 \pm 0.10)x + (0.08 \pm 0.04)
\]
\[r^2 = 0.97\]
Figure 5.
A “two-state” dynamic equilibrium model for FGFR kinase regulation deduced from the structural and biochemical data generated in this study. In this model, FGFR kinase toggles between an ‘inhibited’ and conformationally rigid ground state, and a more dynamic and heterogeneous ‘active’ state. (A) Under physiological conditions, FGFR kinases primarily populate the inhibited ground state with only a small fraction of kinases capable of adopting the active state. The pathogenic gain-of-function mutations enable the kinase to more readily attain and reside longer in the active state, thus increasing the overall population of the kinases in the active state. According to this model, the more activating the mutation is, the more the equilibrium is skewed towards the active state. This model explains the molecular basis of the correlation between the degree of gain-of-function and the severity of clinical manifestation associated with these mutations. (B) Compared to the structurally rigid kinases in the inhibited ground state, the active state kinases are conformationally more dynamic. (C) Consistent with the conformational heterogeneity within the active state, kinases in the active state are in the rugged elevated energy state, while the kinases in ground state are in the low resting energy state.
### TABLE 1

**X-ray Data Collection and Refinement Statistics**

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<th>Construct</th>
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<tr>
<td>Resolution (Å)</td>
<td>50–2.6 (2.69–2.6)</td>
<td>50–2.3 (2.34–2.3)</td>
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<td>50–2.3 (2.34–2.3)</td>
<td>50–1.85 (1.88–1.85)</td>
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<td>Space group</td>
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<td>P&lt;sub&gt;4&lt;/sub&gt;2&lt;sub&gt;2&lt;/sub&gt;</td>
<td>P&lt;sub&gt;2&lt;/sub&gt;</td>
<td>P&lt;sub&gt;4&lt;/sub&gt;2&lt;sub&gt;2&lt;/sub&gt;</td>
<td>C&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>a = 70.584</td>
<td>a = 73.852</td>
<td>a&lt;sub&gt;2&lt;/sub&gt; = 78.634</td>
<td>a = 73.785</td>
<td>a&lt;sub&gt;2&lt;/sub&gt; = 78.508</td>
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<tr>
<td>b = 70.278</td>
<td>b = 73.852</td>
<td>b = 78.122</td>
<td>b = 73.785</td>
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<td>c = 85.499</td>
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<td>c = 72.791</td>
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<td>c = 73.197</td>
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<td><strong>Unit Cell Parameters (Å, °)</strong></td>
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<td>α = 92.39</td>
<td>α = 90.00</td>
<td>α = 90.00</td>
<td>α = 90.00</td>
<td>α&lt;sub&gt;2&lt;/sub&gt; = 90.00</td>
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<td>β = 112.19</td>
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<td>β = 101.09</td>
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<td>β&lt;sub&gt;2&lt;/sub&gt; = 101.02</td>
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<td>γ = 115.98</td>
<td>γ = 90.00</td>
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<td>Measured reflections (#)</td>
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<td>1028351</td>
<td>453471</td>
<td>258430</td>
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<td>Unique Reflections (#)</td>
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<td>39689</td>
<td>60839</td>
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<td>127643</td>
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<td>Data redundancy</td>
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<td>25.9</td>
<td>7.5</td>
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<tr>
<td>Data completeness (%)</td>
<td>97.1 (84.5)</td>
<td>99.8 (100.0)</td>
<td>99.4 (99.3)</td>
<td>96.6 (99.8)</td>
<td>96.4 (89.4)</td>
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<tr>
<td>R&lt;sub&gt;sym&lt;/sub&gt; (%)</td>
<td>7.4 (33.2)</td>
<td>11.4 (49.8)</td>
<td>5.9 (41.7)</td>
<td>11.8 (35.5)</td>
<td>3.1 (31.9)</td>
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<tr>
<td>I/sig</td>
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<td>43.6</td>
<td>50.8</td>
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<td>R factor/R free</td>
<td>18.0/26.1</td>
<td>18.0/21.8</td>
<td>20.6/26.1</td>
<td>18.6/23.7</td>
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<td>Number of solvent atoms</td>
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<td>Rmsd bond angle (°)</td>
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<td>1.132</td>
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<sup>a</sup>Numbers in parenthesis refer to the highest resolution shell.

<sup>b</sup>R<sub>sym</sub> = Σ|I − <I>| / ΣI, where I is the observed intensity of a reflection, and <I> is the average intensity of all the symmetry related reflections.