Published in final edited form as:

Sci Signal. ; 2(58): ra6. doi:10.1126/scisignal.2000021.

The Precise Sequence of FGF Receptor Autophosphorylation Is Kinetically Driven and Is Disrupted by Oncogenic Mutations

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Abstract

Autophosphorylation of the tyrosine kinase domain of fibroblast growth factor receptor 1 (FGFR1) is mediated by a sequential and precisely ordered three-stage autophosphorylation reaction. First-stage autophosphorylation of an activation loop tyrosine leads to 50- to 100-fold stimulation of kinase activity and is followed by second-stage phosphorylation of three additional tyrosine residues, which are binding sites for signaling molecules. Finally, third-stage phosphorylation of a second activation loop tyrosine leads to an additional 10-fold stimulation of FGFR1 catalytic activity. In this report, we show that sequential autophosphorylation of five tyrosines in the FGFR1 kinase domain is under kinetic control, mediated by both the amino acid sequence surrounding the tyrosines and their locations within the kinase structure and, moreover, that phosphoryl transfer is the rate-limiting step. Furthermore, the strict order of autophosphorylation is disrupted by a glioblastoma-derived, oncogenic FGFR1 point mutation in the kinase domain. We propose that disrupted stepwise activation of tyrosine autophosphorylation caused by oncogenic and other activating FGFR mutations may lead to aberrant activation of and assembly of signaling molecules by the activated receptor.

INTRODUCTION

Autophosphorylation of receptor tyrosine kinases (RTKs) relieves cis-autoinhibitory constraints that maintain the tyrosine kinase domain in an autoinhibited, low-activity state and generates sites for the recruitment of signaling molecules that bind to tyrosine phosphorylation sites through their Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains. Autophosphorylation of tyrosine residues in the tyrosine kinase domain of fibroblast growth factor receptor 1 (FGFR1) is mediated by an intermolecular (trans) process that occurs in a precise sequence (1). Tyrosine autophosphorylation appears to occur in three stages. The first stage involves phosphorylation of Y⁶⁵³ (2) in the activation loop of the catalytic core; this leads to a 50- to 100-fold stimulation of FGFR1's intrinsic tyrosine kinase activity. Autophosphorylation of Y⁶⁵³ is followed by the ordered autophosphorylation of several key tyrosine residues within binding sites for the SH2 or PTB domains of signaling proteins that bind to and are phosphorylated by activated FGFR1. This second-stage autophosphorylation occurs on Y⁵⁸³, in the kinase insert region (a noncatalytic sequence within the kinase domain), followed by autophosphorylation of Y⁴⁶³ in the juxtamembrane region, Y⁷⁶⁶ in the C-terminal tail, and Y⁵⁸⁵ in the kinase insert region (1). The third-stage autophosphorylation takes place on the second tyrosine in the activation loop (Y⁶⁵⁴), resulting in an additional 10-fold increase in the intrinsic tyrosine kinase activity of FGFR1. We have proposed that the role of the third

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stage of autophosphorylation is to enable the efficient tyrosine phosphorylation of substrate proteins that are physically bound to the receptor molecule by a maximally activated FGFR1 (1).

Ordered autophosphorylation of tyrosine residues in the activation loops of several RTKs, including the insulin receptor, the insulin-like growth factor 1 (IGF-1) receptor, Tie2, and muscle-specific kinase (MuSK) has also been described (3-7). However, the mechanism of this ordered phosphorylation—and even whether it serves an *in vivo* function—remains unclear. Most research has focused on finding substrates and substrate recognition motifs for molecules downstream of the protein tyrosine kinases (PTKs) rather than on examining the tyrosine autophosphorylation sites themselves as substrates. Researchers have used high-throughput methods such as mass spectrometry (8-11), protein microarray chips (12,13), and degenerate peptide libraries (14-16) together with computational algorithms (17) to define optimal linear phosphorylation motifs for specific PTKs. In addition, the crystal structures of activated insulin receptor kinase (IRK) in complex with a peptide substrate derived from insulin receptor substrate-1 (IRS-1) and that of Abl kinase in complex with a bisubstrate analog have provided insight into the structural basis for the substrate specificity of RTKs (18-20). In scanning peptide libraries, RTKs generally select for acidic residues at positions immediately N-terminal to the tyrosine phosphorylation site, and in the IRK structure, an aspartic acid at the P-1 position of the peptide substrate forms a water-mediated hydrogen bond to K¹⁰⁸⁵ of the kinase (19). However, this residue is not highly conserved among RTKs, and scanning data for the optimal peptide substrate for Abl kinase has shown that these acidic residues may not be entirely necessary or sufficient for substrate recognition. In addition, large hydrophobic residues are selected by scanning peptide libraries at the P+1 and P+3 positions of the substrate. These two hydrophobic residues, methionines in the case of IRK, were accommodated by two adjacent hydrophobic pockets on the surface of the C-terminal lobe of the kinase. Thus, although peptide libraries and structural studies have yielded insight into RTK substrate recognition, they have not been able to fully explain the complex interactions or specificity between the kinase and the substrate molecules. For example, the recognition of Y⁵²⁷ on Src by the C-terminal Src kinase Csk occurs in the context of the intact molecule and is not recapitulated by peptide mimetics alone (21).

To begin to understand the molecular mechanism underlying the order of FGFR1 autophosphorylation, we analyzed each tyrosine as a discrete substrate in the context of the intact FGFR1 kinase domain and asked if ordered autophosphorylation is kinetically controlled or mediated by some other regulatory mechanism (such as differential substrate affinity). Here, we show that sequential autophosphorylation of the five tyrosines in the FGFR1 catalytic core is under kinetic control and, further, that phosphoryl transfer is the rate-limiting step. In addition, we show that a mutation in the tyrosine kinase core of FGFR1 found in human glioblastoma alters the discrete order of tyrosine autophosphorylation, leading to more heterogeneous phosphorylation states. Moreover, expression of intact FGFR1 harboring this glioblastoma mutation in Rat-1 cells led to a transformed cellular phenotype. Taken together, these experiments show that ordered FGFR1 autophosphorylation is precisely controlled by structural elements within the kinase domain, suggesting not only a universal mechanism for RTK autophosphorylation, but also that disruption of ordered autophosphorylation may contribute to the pathology observed in various RTK-mediated diseases.

RESULTS

To begin to dissect the mechanism of FGFR1 ordered autophosphorylation, we compared the primary structures of the FGFR1 tyrosine phosphorylation sites to the sequence of the optimal FGFR1 peptide substrate identified by screening random substrate peptide libraries (NH₂-XEEYFFLF-COOH) (22) (Fig. 1A). Degenerate peptide library scanning has shown that

FGFR and other RTKs prefer peptide substrates with a string of acidic residues at positions N-terminal to the tyrosine phosphorylation site and large hydrophobic residues at the Y + 1 and Y + 3 positions (22). However, aside from the single acidic residue at the Y - 1 position of the first three tyrosines phosphorylated (Y⁶⁵³, Y⁵⁸³, and Y⁴⁶³), the remaining sequences surrounding the phosphorylated tyrosine sites diverged from each other and from that of the optimal substrate, suggesting that the primary structure alone could not account for the observed order of autophosphorylation.

We previously determined the order of autophosphorylation with the use of the tyrosine kinase domain of FGFR1 (1). The sequential order of phosphorylation may reflect varying degrees of accessibility of the individual tyrosine sites within the three-dimensional structure of the FGFR1 kinase domain. The location of the tyrosine autophosphorylation sites within different regions of the FGFR1 kinase domain tertiary structure would impose structural constraints for the incorporation of the tyrosine into the catalytic site. To examine each tyrosine as an individual substrate in the context of the intact kinase structure, we engineered FGFR1 kinase domain constructs to contain only a single tyrosine phosphorylation site, whereas the other four autophosphorylation sites were mutated to phenylalanine (Fig. 1B). Phosphorylation of Y⁷³⁰ occurs in low stoichiometry in vivo, presumably because it resides at the end of an α helix. In this system, phosphorylation of Y⁷³⁰ was not observed and this residue was left intact. D⁶²³ is a highly conserved amino acid in the catalytic core required for catalysis that serves as the general base catalyst to abstract the hydroxyl proton from the substrate (23-26). Because the FGFR1 kinase domain undergoes autophosphorylation, an additional point mutation, D623A, was introduced to make the FGFR1 constructs that served as substrates catalytically inactive (called Y^{xxx}KD, where Y^{xxx} is the residue that can be phosphorylated and KD means kinase dead). A subsequent kinase assay confirmed that the constructs were indeed kinase dead (Fig. 1E), and their similar migration patterns by native gel electrophoresis suggest that mutation of the tyrosines does not cause a major conformational change in the structure (Fig. 1D).

To phosphorylate the kinase-dead substrates, we generated two kinase-active FGFR1 mutants (Fig. 1C) phosphorylated at either Y⁶⁵³ only (Y⁶⁵³-1P) or bis-phosphorylated on Y⁶⁵³ and Y⁶⁵⁴ (FGFR1-3F-2P), both unable to undergo further phosphorylation as verified by an in vitro kinase assay (Fig. 1F). As previously noted, phosphorylation of Y⁶⁵³ increases FGFR1 catalytic activity 50- to 100-fold, and subsequent phosphorylation on Y⁶⁵⁴ increases its catalytic activity an additional 10-fold (1). Furthermore, under conditions in which wild-type or mutant FGFR1 are overexpressed, Y⁴⁶³, Y⁵⁸³, Y⁵⁸⁵, and Y⁷³⁰ are dispensable for tyrosine phosphorylation of Shc, the mitogen-activated protein kinase (MAPK) response, and stimulation of FGFR1-mediated cell proliferation and differentiation (27). The Y⁶⁵³-1P and FGFR1-3F-2P FGFR1 kinase domain mutants were autophosphorylated upon addition of adenosine 5'-triphosphate (ATP) and MgCl₂, and the differentially phosphorylated constructs were purified by anion-exchange chromatography. The purity and position of the phosphate group was verified by native gel electrophoresis and mass spectrometry (Fig. 1F). Subsequent experiments were carried out with the fully activated form due to the faster reaction kinetics.

We next sought to establish if the observed order of FGFR1 autophosphorylation is under kinetic control. Autophosphorylation experiments used to determine the in vitro order were carried out at saturating concentrations of substrate (35 μ M) (1), where binding affinity of the substrate to the enzyme is not rate limiting and the observed rate (k_{obs}) reflects the rate of autophosphorylation. We similarly performed a kinase assay at three saturating concentrations of kinase-dead substrate (10, 15, or 30 μ M) with 0.1 μ M of the fully activated kinase (FGFR1-3F-2P) and followed the formation of the phosphorylated species by native gel electrophoresis over time (Fig. 2A). For each substrate, similar results were obtained at each substrate concentration, confirming that all experiments were done at saturation and indicating

that the different rates observed were not due to substrate availability (Fig. 2B). Data for each substrate at the three concentrations were averaged, and the reaction rates for the formation of phosphorylated species were obtained by fitting the data to a single-exponential equation. The data were expressed as percentage of phosphorylated substrate, and the percentage for each time point was plotted as a function of time (Fig. 2, B and C). Subsequent mass spectrometry of the monophosphorylated kinase-dead substrates confirmed that the expected tyrosine site was phosphorylated (fig. S1). As illustrated in Fig. 2C, the rates of phosphorylation of Y⁶⁵³ and Y⁵⁸³ were the fastest and similar at 0.1080 and 0.1388 min⁻¹, respectively. The phosphorylation of Y⁴⁶³ and of Y⁶⁵⁴ were intermediate at 0.0209 and 0.0151 min⁻¹, respectively. Phosphorylation of Y⁵⁸⁵ was the slowest, with a rate of 0.0032 min⁻¹, and phosphorylation of Y⁷³⁰, which has been previously shown to be phosphorylated at low stoichiometry, was not observed in this system. The observed rates, in general, are in good agreement with the order of autophosphorylation observed with the exception of Y⁶⁵⁴, which is phosphorylated at a rate much faster than would be expected.

The rate of phosphorylation can be rate-limited by ATP or adenosine 5'-diphosphate (ADP) binding and release; by the transfer of the phosphate group to the serine, threonine, or tyrosine moiety; or by product release (23). For the serine/threonine kinase adenosine 3',5'-monophosphate (cAMP)-dependent protein kinase (PKA), phosphorylation of a peptide substrate was limited by product release controlled by the dissociation of ADP, whereas phosphotransfer was rapid (28). In the case of IRK, phosphotransfer is rate-limiting in the basal state, whereas in the activated kinase, phosphotransfer and product release are each partially rate-limiting (29). For the tyrosine kinase activity of ErbB2, product release is rate-limiting (30). Having found that the ordered autophosphorylation of FGFR1 is under kinetic control, we investigated the nature of the rate-limiting step that dictates the kinetics of tyrosine phosphorylation. The kinase used to phosphorylate the different substrates was identical, and saturating concentrations of ATP were used; therefore, ATP binding and the subsequent release of ADP from the fully activated kinase must be the same and cannot account for the differences observed in the rates of catalysis. To investigate whether transfer of the phosphate group or product release is rate-limiting, we performed rapid chemical quench experiments, in which the formation of phosphorylated substrate (Y⁴⁶³KD) was monitored over time. Catalysis that is limited by product release is characterized by biphasic reaction kinetics in which a rapid burst in the initial phase is followed by a linear steady-state phase of product release. A kinase assay was performed with 3-, 5-, or 10-fold excess substrate to kinase (Fig. 3A). The formation of phosphorylated Y⁴⁶³KD was monitored by the incorporation of ³²P and plotted as a function of time (Fig. 3B). Y⁴⁶³KD was chosen as a representative substrate for pre-steady-state kinetic experiments because of its average phosphorylation kinetics relative to the other substrates. Product formation did not appear to be biphasic, and data did not converge to a steady-state burst equation, but the data fit a single-exponential equation, suggesting that phosphotransfer rather than product release is the rate-limiting step.

Mapping studies have shown that Y⁶⁵⁴ is the fifth site phosphorylated; however, the rate of Y⁶⁵⁴ phosphorylation was consistent with its being the third or fourth best substrate. Y⁶⁵⁴ is adjacent to Y⁶⁵³, the first site phosphorylated, and we postulated that phosphorylation at Y⁶⁵³ decreases the rate of Y⁶⁵⁴ phosphorylation as a consequence of charge repulsion between two adjacent negatively charged phosphate groups. To address this possibility, we constructed a kinase-dead mutant containing both Y⁶⁵³ and Y⁶⁵⁴ with the other tyrosines mutated to phenylalanine (Fig. 3C). We purified the monophosphorylated species (pY⁶⁵³/Y⁶⁵⁴ KD) by anion-exchange chromatography and used mass spectrometry to verify that the sample was indeed homogeneously phosphorylated only at Y⁶⁵³. We next performed a kinase assay at two saturating concentrations of substrate (15 and 30 μM) by incubating 0.1 μM FGFR1K-3F-2P with ATP and MgCl₂ to a final concentration of 5 and 10 mM at 25°C, respectively (Fig. 3D). We quenched the reaction samples by addition of EDTA (final concentration 50 mM), and

compared the rates of phosphorylation of Y⁶⁵⁴KD and pY⁶⁵³/Y⁶⁵⁴ KD. The reaction rates for Y⁶⁵⁴KD and pY⁶⁵³/Y⁶⁵⁴ KD phosphorylation were similar (0.0209 ± 0.001 and 0.0271 ± 0.002 min⁻¹, respectively) (Fig. 3E), suggesting that under these conditions, prior phosphorylation at Y⁶⁵³ does not slow subsequent phosphorylation at Y⁶⁵⁴ and that additional factors affect the rate of Y⁶⁵⁴ phosphorylation.

Based on the high-resolution crystal structure of FGFR1 kinase domain (31), the autophosphorylation sites are found in various regions of the kinase domain. Y⁴⁶³ is at the N terminus near the boundary of the juxtamembrane domain, Y⁵⁸³ and Y⁵⁸⁵ are in the flexible kinase insert, Y⁶⁵³ and Y⁶⁵⁴ are in the activation loop, and Y⁷³⁰ is at the end of the α H helix. Two pairs of tyrosines are adjacent to one another, Y⁵⁸³/Y⁵⁸⁵ and Y⁶⁵³/Y⁶⁵⁴; however, they are not phosphorylated sequentially, suggesting that the location of the tyrosine moieties within the tertiary structure alone does not solely dictate the order of autophosphorylation observed.

As previously mentioned, one of the hallmarks of optimal peptide substrates for RTKs is a series of acidic residues at positions immediately N-terminal to the tyrosine phosphorylation site. As noted, the first three sites phosphorylated (and also Y⁷⁶⁶) have an acidic residue at the Y - 1 position, which may enhance the recognition of particular tyrosines by the kinase, especially in the case of adjacent tyrosine autophosphorylation sites where structural constraints should play a lesser role. To determine if these acidic residues play a role in determining the order of autophosphorylation, kinase-dead mutants of Y⁶⁵⁴, in which the Y - 1 residue was mutated to aspartic acid (Y⁶⁵⁴KD Δ), and of Y⁵⁸⁵, in which the Y - 1 residues of Y⁵⁸³ and Y⁵⁸⁵ were swapped (Y⁵⁸⁵KD Δ), were made (Fig. 3, F and G). We next performed a kinase assay at saturating concentration of substrate (30 μ M) with 0.1 μ M of fully activated kinase and compared the rate of phosphorylation of Y⁶⁵⁴KD and Y⁶⁵⁴KD Δ or Y⁵⁸⁵KD and Y⁵⁸⁵KD Δ , respectively. The rate of Y⁶⁵⁴KD Δ phosphorylation was much faster than that of Y⁶⁵⁴KD phosphorylation and occurred at a rate comparable to that observed for Y⁶⁵³KD, suggesting that the aspartic acid at the Y - 1 position plays a key role in the recognition of Y⁶⁵³ before that of Y⁶⁵⁴. On the other hand, although the rate of Y⁵⁸⁵KD Δ phosphorylation was faster than that of Y⁵⁸⁵KD phosphorylation, its rate was slower than that observed for Y⁵⁸³KD. Thus, both the amino acid sequence surrounding Y⁵⁸⁵ and the location of Y⁵⁸⁵ within the FGFR1 kinase tertiary structure appear to affect the recognition as substrate of the adjacent tyrosine residues in the kinase insert.

Having observed that the sequential order of FGFR1 autophosphorylation is dictated by both the amino acid sequence surrounding the tyrosine autophosphorylation site and the accessibility of each of the tyrosine moieties, we postulated that point mutations in the kinase domain responsible for FGFR1-mediated diseases may influence the local environment of the tyrosine and, thus, change the order of tyrosine autophosphorylation. Bidirectional dideoxy sequencing of 19 primary glioblastoma tumors has implicated two gain-of-function mutations in the FGFR1 kinase domain in human glioblastoma (32). Based on the structure of the FGFR1 kinase domain, one of these mutations, N546K, resides in the vicinity of the hinge region between the N and C lobes, and recent crystallographic evidence suggests that mutation of this residue (N549H in FGFR2) may affect the conformational dynamics of the tyrosine kinase domain by altering the flexibility of the hinge region to modulate the transition between the inactive and active states (33,34) (Fig. 4B).

The N546K point mutation was introduced into the FGFR1 kinase domain and tyrosine autophosphorylation was analyzed by a combined approach of rapid chemical quench and native gel electrophoresis with densitometric analysis (Fig. 5). As with wild-type FGFR1 kinase, the reaction proceeds via a sequential mechanism with a discrete set of phosphoproteins formed over time. The relative abundance of each phosphoprotein species at each time point was quantitated and the formation of phosphoproteins was plotted as a function of time. A

complete set of rate constants for the reaction pathway are listed in table S1. Formation of the monophosphorylated species (0P→1P) was 25 times faster with the N546K mutant (0.25 s^{-1}) than with wild-type FGFR1 kinase (0.009 s^{-1}), and the data were better fit to a biphasic reaction scheme. To determine if the order of autophosphorylation is altered by the N546K point mutation, we subjected the phosphorylated species to in-gel tryptic digest and peptide mapping by liquid chromatography electrospray ionization mass spectrometry (LC/ESI-MS) and tandem mass spectrometry (MS/MS). Representative traces for peptide mapping and MS/MS sequencing are shown in fig. S2. Peptide mapping with LC/ESI-MS and MS/MS showed that, unlike the discrete order observed for wild-type FGFR1 kinase, the order of tyrosine phosphorylation observed with FGFR1 kinase harboring the N546K mutation was heterogeneous. Specifically, Y⁶⁵³ and Y⁵⁸³ were heterogeneously the first two sites phosphorylated (1P and 2P); the third site phosphorylated (3P) was Y⁵⁸⁵ in the kinase insert, and the last sites phosphorylated (4P and 5P) were a heterogeneous mixture of Y⁴⁶³ and Y⁶⁵⁴ found in the juxtamembrane domain and activation loop, respectively (Figs. 4 and 5). Based on the intensity of the peptide peaks for the fourth and fifth phosphorylation sites, Y⁴⁶³ appeared to be the preferred phosphorylation site.

Because of the inherent difficulties in determining the in vivo autophosphorylation profile of FGFR and other RTKs, the importance of ordered autophosphorylation in the context of living cells remains largely unknown. To determine whether the enhanced tyrosine kinase activity and altered order of autophosphorylation of the FGFR1_N546K mutant affects biological outcome, we established Rat-1 cells stably expressing either wild-type FGFR1 or FGFR1 harboring the N546K point mutation. Unlike cells expressing wild-type FGFR1, the N546K-expressing cells showed morphological transformation characteristic of transformed cells characterized by decreased cell spreading and decreased adhesion to the tissue culture dish (Fig. 5C). These results suggest that mutations in the kinase domain can exert their pathogenicity in vivo.

DISCUSSION

In this report, we have explored the mechanism whereby FGFR1 kinase undergoes ordered autophosphorylation on critical tyrosine residues that function to both enhance its catalytic activity and recruit downstream signaling partners. Using intact FGFR1 kinase domains, we have shown that the order of autophosphorylation (Y⁴⁶³, Y⁵⁸³, Y⁵⁸⁵, Y⁶⁵³, Y⁶⁵⁴, and Y⁷³⁰) is kinetically controlled and limited by the rate of phosphoryl transfer of the γ -phosphate group from ATP to the hydroxyl group of tyrosine. It is likely that primary structure alone does not determine the order of autophosphorylation because the amino acid sequences surrounding the tyrosine autophosphorylation sites are divergent and show minimal similarity to that of the optimal FGFR1 peptide substrate. Y⁶⁵³ and Y⁶⁵⁴, the critical tyrosines in the activation loop required for catalytic activity, are adjacent to one another; however, Y⁶⁵³ is a better substrate and is phosphorylated first. Although Y⁶⁵³ and Y⁵⁸³ have similar kinetics of phosphorylation, Y⁶⁵³ must be recognized first because its phosphorylation is required for enhanced catalytic activity as shown by in vitro and in vivo studies (1,27). Similarly, Y⁵⁸³ and Y⁵⁸⁵ are separated by one amino acid; however, Y⁵⁸³ is phosphorylated long before Y⁵⁸⁵. The primary structure of the first three sites phosphorylated contains an acidic residue at the Y - 1 position, and steady-state kinetic experiments showed enhanced phosphorylation of both Y⁶⁵⁴ and Y⁵⁸⁵ when the Y - 1 position was replaced by an acidic residue. Although Y⁶⁵⁴ with a Y - 1 acidic residue had phosphorylation kinetics comparable to those of Y⁶⁵³, Y⁵⁸⁵ with a Y - 1 acidic residue was phosphorylated faster but at a rate much slower than that observed for Y⁵⁸³. This suggests that FGFR1 kinase-ordered autophosphorylation is a function of both the amino acid sequence surrounding the tyrosine phosphorylation sites and the location of the autophosphorylation sites within the overall tertiary structure (structural constraints).

In the autophosphorylation assays, Y⁶⁵⁴ was the last site to be phosphorylated. It should be noted that Y⁶⁵⁴ is also the last site phosphorylated when a longer FGFR1 mutant containing Y⁷⁶⁶ is included (1). However, based on the data presented in this report, one would expect Y⁶⁵⁴ to be phosphorylated much earlier: The rate of Y⁶⁵⁴ phosphorylation suggests that it should be the third or fourth site phosphorylated. It does not appear that binding affinity (K_d) of Y⁶⁵⁴ to the kinase plays a role, because the sequential phosphorylation was observed at saturation (where the reaction rate does not depend on substrate concentration). Perhaps there are subtle changes in the FGFR1 kinase structure when both Y⁶⁵³ and Y⁵⁸³ are phosphorylated that are not recapitulated in the experimental design and that decrease the ability of Y⁶⁵⁴ to be a good substrate.

In the present experimental system, it is inherently difficult to precisely measure the substrate affinity (K_m) for each of the substrates (the individual tyrosine residues), although preliminary experiments suggest that for the soluble receptor substrates, the K_m is in the low nanomolar range (fig. S3). In solution, the kinase and substrate are essentially the same molecule, and the experimental system utilized does not distinguish between heterodimers of the kinase and substrate versus homodimers of either the substrate or the kinase alone, which increases the complexity of the kinetic analysis. It is therefore likely that at subsaturating concentrations of substrate utilized to determine the K_m , phosphorylation in this system cannot be explained by simple Michaelis-Menten kinetics.

Data presented here suggesting ordered autophosphorylation is intrinsically controlled by the properties of the kinase itself raise the possibility that point mutations in RTKs implicated in the pathogenesis of disease may affect the ability of a particular tyrosine to be phosphorylated by altering the microenvironment of the tyrosine, by changing the tertiary structure surrounding the tyrosine, or both. This, in turn, may change the ability of the kinase to accommodate the tyrosine and adjacent residues in the catalytic cleft and, thereby, change the order in which the kinase is phosphorylated. We investigated the order of autophosphorylation of a form of the FGFR1 kinase domain harboring a point mutation implicated in human glioblastomas and showed that an N546K mutation in the kinase core changed the order of FGFR1 kinase autophosphorylation. It is intriguing that the first and second sites phosphorylated in the N546K mutant were either Y⁵⁸³ in the kinase insert or Y⁶⁵³ in the activation loop. This suggests that the mutant had higher basal kinase activity independent of activation loop phosphorylation, a suggestion that is further supported by the greater degree of autophosphorylation observed on the native protein during expression and purification. The initial, heterogeneous phosphorylation of Y⁵⁸³ or Y⁶⁵³ interchangeably in the mutant provides additional evidence that ordered FGFR1 autophosphorylation is kinetically regulated, because both sites had similar rates of phosphorylation. Stable expression of intact FGFR1 harboring the N546K mutation in Rat-1 cells and other cell lines (fig. S4) elicited a transformed phenotype. Although it is well established that this and similar mutations in the kinase domain enhance the catalytic activity of FGFR mutants associated with pathology, it remains to be determined whether the altered order of autophosphorylation of the N546K FGFR1 mutant contributes to cell transformation by altering the program of recruitment and tyrosine phosphorylation of cellular substrates. Further biochemical characterization of the cells harboring FGFR1_N546K was not possible because of decreased adherence of the cells to the tissue culture dish under conditions of low serum concentration.

Ordered autophosphorylation of tyrosine residues in the activation loop has been observed for various tyrosine kinases (3-7,35), and data presented here suggest a general mechanism for all RTKs in which sequential autophosphorylation of tyrosine residues both in the activation loop and on other portions of the kinase domain is kinetically controlled, with the order of phosphorylation a function of the location of the tyrosine within both the primary and the tertiary structure. This report also suggests that ordered autophosphorylation may have a

physiologically relevant role in vivo for the temporal recruitment of downstream signaling partners.

MATERIALS AND METHODS

Chemical reagents

ATP, MgCl_2 , ammonium acetate (99.99%), ammonium bicarbonate (NH_4HCO_3), EDTA, Hepes, and ammonium acetate were purchased from Sigma. Trypsin Gold mass spectrometry grade was obtained from Promega. High-performance liquid chromatography-grade acetonitrile (ACN) and water were from J. T Baker. Trifluoroacetic acid and Slide-A-Lyzer dialysis cassettes (0.5-3 ml, 3500 molecular weight cutoff) were purchased from Pierce. The C18-packed PicoFrit column (75 μm outer diameter, 15 μm inside diameter) was obtained from New Objectives. [γ - ^{32}P]ATP was obtained from GE Healthcare (Piscataway, NJ).

FGFR1 kinase purification in unphosphorylated and phosphorylated forms

FGFR1 wild-type kinase (FGFR1K) domain was purified as previously described (1,36). A mutant form of FGFR1 kinase domain with three tyrosine to phenylalanine mutations (FGFR1K-3F) was purified as previously described in both the unphosphorylated and the phosphorylated forms (1).

Kinase-dead FGFR1 mutant

For the kinase-dead constructs, a single aspartic acid to alanine mutation, D623A, was introduced by site-directed mutagenesis (Stratagene) to make the kinase inactive. In each construct, four of the five tyrosines were mutated to phenylalanine by sequential site-directed mutagenesis steps. In the case of the $\text{Y}^{653}/\text{Y}^{654}\text{KD}$ construct, only three sites were mutated to phenylalanine, leaving the two activation loop tyrosines free to be phosphorylated. Kinase-dead substrates were purified as described for wild-type kinase. The phosphorylated form, $\text{pY}^{653}/\text{Y}^{654}\text{KD}$, was obtained by incubating 13.8 μM phosphorylated wild-type kinase (FGFR1K-4P/5P mixture), 138.8 μM $\text{Y}^{653}/\text{Y}^{654}\text{KD}$, 10 mM ATP, and 25 mM MgCl_2 at 4°C for 1 hour (final concentrations). The reaction was quenched with 50 mM EDTA to stop the reaction. The monophosphorylated form, $\text{pY}^{653}/\text{Y}^{654}\text{KD}$, was initially purified by gel-filtration chromatography on a Superdex200 HR10/30 (GE Healthcare, Piscataway, NJ) to remove nucleotides and EDTA and subsequently separated from the unphosphorylated, bis-phosphorylated, and wild-type prephosphorylated kinase by anion-exchange chromatography on a MonoQ HR 16/10 (GE Healthcare, Piscataway, NJ). The purity and location of the phosphate group was verified by native gel electrophoresis and mass spectrometry. A kinase assay was performed to verify kinase inactive status by incubating 30 μM kinase-dead substrate, 5 mM ATP, and 10 mM MgCl_2 in 20 mM Hepes buffer, pH 7.4, for various reaction times, and the reaction was quenched with 50 mM EDTA (final concentrations). Samples were separated by native gel electrophoresis to visualize the formation of differentially phosphorylated species.

Native gel electrophoresis

A 7% tris-HCl native gel was used to separate the differentially phosphorylated species present in samples generated by the substrate phosphorylation experiments. The pH of the separating gel was 8.8 and the pH of the stacking gel was 6.8. The relative intensities of the phosphorylated and unphosphorylated species were quantitated by densitometric analysis performed with a UVP Epi Chemi II Darkroom (Labworks). Data were plotted and analyzed with Graphpad Prism 4.

FGFR1 kinase substrate phosphorylation

Kinase assays were initiated by combining, at final concentrations, 0.1 μ M FGFR1-3F-2P kinase, 10, 15, or 30 μ M kinase-dead substrate, 5 mM ATP, and 10 mM MgCl_2 at 25°C for various times. Reactions were quenched with 83 mM EDTA. Kinase, substrates, nucleotides, and quench buffer were made in 10 mM Hepes (pH 7.4). Reaction samples were visualized by native gel electrophoresis.

Pre-steady-state burst kinetics

Rapid chemical quench experiments were performed with a KinTek RFQ-3 Rapid Chemical Quench Apparatus (Kintek Corporation, Austin, TX) at 25°C. The concentrations of the enzyme and substrates cited in the text are those after mixing and during the reaction. The reaction was initiated by mixing a solution containing kinase-dead substrate (Y^{463}KD) and $[\gamma\text{-}^{32}\text{P}]\text{ATP}/\text{MgCl}_2$ (15 μ l) with a solution containing fully phosphorylated kinase (FGFR1-3F-2P). For these experiments, 3 μ M phosphorylated kinase (FGFR1K-3F-2P), 9, 15, or 30 μ M Y^{463}KD substrate, 5 mM ATP (0.25 $\mu\text{Ci}/\mu\text{l}$ $[\gamma\text{-}^{32}\text{P}]\text{ATP}$), and 10 mM MgCl_2 in 10 mM Hepes (pH 7.4), final concentrations were used. After various reaction times, the reaction mixture was quenched with 83 mM EDTA. Samples were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and quantitated by PhosphorImager analysis (Image Quant, Molecular Dynamics Storm 820). Zero time points were determined by addition of EDTA to enzyme solution before mixing with substrate and were used for background correction. Data were fit by nonlinear regression in GraphPad Prism 4 to both burst equation and single-exponential equation.

Identification of tyrosine phosphorylation sites by LC/ESI-MS/MS

The electrophoretic bands from native-gel analysis corresponding to unphosphorylated and different phosphorylated states were excised and in-gel trypsin digestion was performed. The resulting tryptic digest (Trypsin Gold, Promega) for each phosphorylation state was further analyzed by nano-LC (Dionex Ultimate3000 System) coupled to a Thermo ESI LTQ mass spectrometer. A typical gradient was run for 60 min from 0 to 100% solvent B (80% ACN, 20% H_2O , and 0.1% formic acid). Solvent A consisted of 5% ACN, 95% H_2O , and 0.1% formic acid. The flow rate was set at 200 nl/min on a 75 μm by 10 cm fused silica capillary column (New Objectives) inhouse packed with Michrom Magic C18AQ (200 $\text{\AA}$, 5 μm). The ESI LTQ mass spectrometer was operated in selected ion monitoring mode for precursor ions corresponding to the peptides containing unphosphorylated and phosphorylated tyrosine residues. The peptide identification was performed automatically with the Bioworks 3.1 software. The generated peptide list was ranked by XCorr to charge state ratio and the phosphorylation sites were identified for each phosphorylation state.

Glioblastoma FGFR1 mutant purification and autophosphorylation by rapid chemical quench

The N546K glioblastoma point mutation was introduced by site-directed mutagenesis (Stratagene) into wild-type FGFR1K and purified following the protocol used for the wild-type kinase. Rapid chemical quench experiments were performed with a Kintek RFQ-3 Rapid Chemical Quench (Kintek Instruments). A final enzyme concentration of 35 μ M was utilized. The reaction was initiated by mixing the enzyme solution with ATP and MgCl_2 , 5 mM and 10 mM final concentration, respectively, and then quenching the reaction with 100 mM EDTA at different reaction times. The enzyme, ATP, MgCl_2 , and EDTA were prepared in 10 mM Hepes (pH 7.4).

Kinetic modeling of FGFR1K wild type and FGFR1K_N546K autophosphorylation reaction

Kinetic modeling of FGFR1K wild-type and glioblastoma mutant autophosphorylation was performed based on the densitometric analysis of the samples separated by native PAGE. The simulations were performed with KinTekSim and FitSim software (Kintek Instruments).

Cell culture assays

Rat-1 cells were stably infected with pBabe-puro constructs encoding either wild-type FGFR1 or FGFR1 harboring an N546K point mutation. Cell lines were maintained in Dulbecco's modified minimum essential medium supplemented with 10% fetal calf serum, 100 µg each of penicillin and streptomycin (Gibco BRL), and puromycin (2.5 µg/ml).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grants RO1-AR051448, RO1-AR051886, and P50AR054086 to J.S. and NIH grants R21 CA 125284 and R01 CA127580 to K.S.A.

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Fig. 1.

Amino acid alignment of tyrosine phosphorylation sites and overview of FGFR1 kinase constructs. **(A)** Amino acid alignment of FGFR1 tyrosine kinase autophosphorylation sites according to the observed order of phosphorylation. Phosphorylation of Y⁷³⁰ was not observed in this system. **(B)** Diagram of His-tagged, kinase-dead FGFR1 substrates, each with a single tyrosine phosphorylation site as indicated by the construct number. In the first five substrates, four other tyrosines are mutated to phenylalanine, and in the Y⁷³⁰KD construct, five tyrosines are mutated to phenylalanine as indicated by a Y→F mutation. Constructs were made kinase-dead by mutation of the catalytic base, D623A. **(C)** A schematic diagram of FGFR1 kinase-active domains used for phosphorylation of the kinase-dead substrates. The

monophosphorylated form (Y⁶⁵³-1P) is phosphorylated at Y⁶⁵³ and the bis-phosphorylated form (FGFR-3F-2P) is phosphorylated at both Y⁶⁵³ and Y⁶⁵⁴. **(D)** Native gel electrophoresis of purified FGFR1 kinase-dead substrates. **(E)** Kinase assay of FGFR1 kinase-dead substrates. The different substrates (30 μ M final concentration) were incubated at 25°C with ATP and MgCl₂ to a final concentration of 5 and 10 mM, respectively. The reaction was quenched at different reaction times with 50 mM EDTA, final. Reaction samples were separated by native gel electrophoresis to observe the formation of phosphorylated species. Autophosphorylation of the kinase-dead samples was not observed. **(F)** Native gel electrophoresis of purified FGFR kinase-active domains in either a monophosphorylated (Y⁶⁵³-1P) or a bis-phosphorylated (FGFR1-3F-2P) state as shown by the gel shift.

Fig. 2.

Quantitative analysis of phosphorylation of individual FGFR1 kinase domain tyrosine sites. (A) Phosphorylation of FGFR1 kinase-dead substrates by fully activated FGFR1 kinase. FGFR1-3F-2P (0.1 μM) was reacted with saturating concentration of FGFR1 kinase-dead substrate (10 μM shown), 5 mM ATP, and 10 mM MgCl₂ at 25°C for various amounts of time. Reactions were quenched upon addition of 50 mM EDTA and the phosphorylation states of the kinase-dead substrate at each time point separated by native gel electrophoresis. The amount of active kinase is below the limit of detection and cannot be seen on the gels. (B) Representative quantitative analysis of FGFR1-3F-2P-mediated phosphorylation of Y⁵⁸³KD substrate at three saturating concentrations of substrate: (■) 10 μM (reaction rate, 0.1523 min⁻¹), (●) 15 μM (reaction rate, 0.1139 min⁻¹), and (▼) 30 μM (reaction rate, 0.1628 min⁻¹). Data at the three concentrations were similar and averaged (inset). (C) Quantitative comparison of FGFR1-3F-2P-mediated phosphorylation of the five kinase-dead substrates. Shown is the average of the data obtained from the three saturating concentrations of each substrate. Calculated average rates of phosphorylation are as follows: (■, red) Y⁴⁶³KD, 0.0209 ± 0.004 min⁻¹; (▲, black) Y⁵⁸³KD, 0.1388 ± 0.016 min⁻¹; (▼, green) Y⁵⁸⁵KD, 0.0032 ± 0.001 min⁻¹; (◆, purple) Y⁶⁵³KD, 0.1080 ± 0.010 min⁻¹; and (●, blue) Y⁶⁵⁴KD, 0.0151 ± 0.002 min⁻¹. Data were fit to a single-exponential equation in Graphpad.

Fig. 3.

Kinetic regulation of FGFR1 autophosphorylation. **(A)** Phosphorylation of kinase-dead substrate by fully activated kinase. FGFR1-3F-2P kinase (3 μ M) was incubated with 3-fold (9 μ M), 5-fold (15 μ M), and 10-fold (30 μ M) excess Y⁴⁶³KD substrate in the presence of 5 mM [γ -³²P]ATP and 10 mM MgCl₂ in a rapid chemical quench apparatus. The formation of the monophosphorylated species over time was followed by incorporation of radiolabeled phosphate. **(B)** Quantitative analysis of Y⁴⁶³KD phosphorylation by FGFR1-3F-2P kinase. The data were fit in Graphpad to a single-exponential equation and did not converge to the burst equation. Reaction rates for 3- (■), 5- (●), and 10-fold (▼) excess substrate were 0.007 ± 0.0004 , 0.005 ± 0.0003 , and 0.004 ± 0.0003 s⁻¹, respectively. **(C)** Schematic of pY⁶⁵³/

Y⁶⁵⁴ KD substrate phosphorylated at Y⁶⁵³ with Y⁶⁵⁴ site unphosphorylated. The other three tyrosine sites are mutated to phenylalanine. **(D)** Phosphorylation of kinase-dead substrates Y⁶⁵⁴KD and pY⁶⁵³/Y⁶⁵⁴ by fully activated kinase FGFR1-3F-2P. FGFR1-3F-2P (0.1 μM) was reacted with saturating concentration of FGFR1 kinase-dead substrate (30 μM), 5 mM ATP, and 10 mM MgCl₂ for various amounts of time. Reactions were quenched with 50 mM EDTA and the phosphorylation states of the kinase-dead substrate at each time point were separated by native gel electrophoresis. The amount of the active kinase is below the limit of detection and cannot be seen on the gels. **(E)** Quantitative analysis of FGFR1-3F-2P-mediated phosphorylation of pY⁶⁵³/Y⁶⁵⁴KD and Y⁶⁵⁴KD. Shown are the average data obtained from two saturating concentrations of substrate: 15 and 30 μM. Y⁶⁵⁴KD (■) and pY⁶⁵³/Y⁶⁵⁴KD (●) were phosphorylated at a rate of 0.0209 ± 0.001 and $0.0271 \pm 0.002 \text{ min}^{-1}$, respectively. **(F)** Phosphorylation of kinase-dead substrates Y⁶⁵⁴KD and Y⁶⁵⁴KD_Δ (containing a Y653D point mutation) by fully activated kinase FGFR1-3F-2P. FGFR1-3F-2P (0.1 μM) was reacted with saturating concentration of FGFR1 kinase-dead substrate (30 μM), 5 mM ATP, and 10 mM MgCl₂ for various amounts of time. Reactions were quenched with 50 mM EDTA, and phosphorylated and unphosphorylated forms of kinase-dead substrate at each time point were separated by native gel electrophoresis. **(G)** Phosphorylation of kinase-dead substrates Y⁵⁸⁵KD and Y⁵⁸⁵KD_Δ (with Y - 1 position of Y⁵⁸³ and Y⁵⁸⁵ swapped) by fully activated kinase FGFR1-3F-2P. FGFR1-3F-2P (0.1 μM) was reacted with saturating concentration of FGFR1 kinase-dead substrate (30 μM), 5 mM ATP, and 10 mM MgCl₂ for various amounts of time. Reactions were quenched with 50 mM EDTA and phosphorylated and unphosphorylated forms of the kinase-dead substrate at each time point were separated by native gel electrophoresis. In both cases, the amount of active kinase is below the limit of detection and cannot be seen on the gels.

Fig. 4.

Molecular model of FGFR1 mutation implicated in glioblastoma, depicting order of autophosphorylation. The nucleotide binding pocket is shown in blue, the hinge region in magenta, and the catalytic cleft in yellow. The tyrosine phosphorylation sites are depicted and high-lighted in red. The observed order of autophosphorylation obtained by rapid chemical quench and subsequent LC/ESI-MS/MS is indicated. For the FGFR1 glioblastoma mutants, heterogeneous phosphorylation was observed at the fourth and fifth sites, but the intensity of the peptide peaks by LC/ESI-MS/MS suggests that the site indicated by a star was the preferred phosphorylation site. (A) FGFR1 kinase wild type (WT). (B) Glioblastoma-derived FGFR1_N546K mutation is found near the hinge region of FGFR1 kinase and is indicated on the structure.

Fig. 5.

Effect of glioblastoma-derived N546K mutation on FGFR1 autophosphorylation and cell signaling. Wild-type FGFR1 (FGFR1_WT) and FGFR1 harboring an N546K point mutation (35 μM) were reacted with 5 mM ATP and 10 mM MgCl₂ in a rapid chemical quench apparatus and quenched by addition of 83 mM EDTA. The different phosphorylation states at each time point were separated by native PAGE. The kinetic parameters are summarized in table S1.

(A) Native-PAGE and kinetic analysis of wild-type FGFR1 autophosphorylation. (B) Native PAGE and kinetic analysis of FGFR1_N546K autophosphorylation. As illustrated by the solid line fit, the data did not fit well with the monophasic mechanism used to describe the kinetics of wildtype FGFR1. Rather, a biphasic mechanism (dashed line) was required to accommodate and best describe the phosphorylation kinetics of this mutant. (C) Rat-1 cells stably expressing either wild-type FGFR1 or a glioblastoma-derived FGFR1_N546K mutant.