The Role of Tyrosine Residues in Fibroblast Growth Factor Receptor 1 Signaling in PC12 Cells
SYSTEMATIC SITE-DIRECTED MUTAGENESIS IN THE ENDODOMAIN

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To assess the contribution of the intracellular domain tyrosine residues to the signaling capacity of fibroblast growth factor receptor 1 (FGFR1), stably transfected chimeras bearing the ectodomain of the platelet-derived growth factor receptor (PDGFR) and the endodomain of FGFR1 were systematically altered by a tyrosine to phenylalanine block and individual conversions. The 15 tyrosine residues of the endodomain of this construct (PFR1) were divided into four linear segments (labeled A, B, C, and D) that contained 4, 4, 2, and 5 tyrosine residues, respectively. When stimulated by platelet-derived growth factor, derivatives in which the A, B, or A + B blocks of tyrosines were mutated were about two-thirds as active as the unmodified chimera at 48 h but achieved full activity by 96 h in a neurite outgrowth assay in transfected PC12 cells. Elimination of only the two activation loop tyrosines (C block) also inactivated the receptor. All derivatives in which 4 or 5 of the D block tyrosines were mutated were inactive in producing differentiation but showed low levels of kinase activity in in vitro assays. Derivatives in which 1, 2, or 3 tyrosines of the D block in different combinations were systematically changed demonstrated that 2 residues (Tyr677 and Tyr701, using hFGFR1 numbering) were essential for bioactivity, but the remaining 3 residues, including Tyr766, the previously identified site for phospholipase Cγ (PLCγ) activation, were not. Differentiation activity was paralleled by the activation (phosphorylation) of FRS2, SOS, and ERK1/2. PLCγ activity was dependent on the presence of Tyr766 but also required Tyr677 and/or Tyr701. Although fully active chimeras did not require PLCγ, the responses of chimeras showing reduced activation of FRS2 were significantly enhanced by this activity. These results establish that PFR1 does not utilize any tyrosine residues, phosphorylated or not, to activate FRS2. However, it does require Tyr677 and/or Tyr701, which may function to stabilize the active conformation directly or indirectly.

Although divergent in detail, the receptor tyrosine kinase (RTK) family is characterized by a similar organization in which the protomeric unit is composed of a ligand binding (ecto) domain, a single pass transmembrane region, and an intracellular (endo) domain featuring an activatable tyrosine kinase. Activation generally requires ligand binding and a stabilized dimeric structure that allows interaction between the constituent monomers and trans protomer autophosphorylation (1, 2). The modifications (tyrosine phosphorylations) introduced usually contribute to stabilizing the activated form of the kinase and can provide docking sites for downstream transducers and effectors. However, not all RTKs require tyrosine phosphorylation for activation, e.g. the EGFR (3), and some of these modifications apparently do not contribute directly to functional responses.

The kinase domains of RTKs are homologs of the larger kinase family and show the characteristic bilobal structure of these enzymes (2). ATP and the protein (peptide) substrate are bound in a cleft formed by the two domains, and several key binding/catalytic residues are universally conserved (1). Generally, RTK activation is controlled by a loop (or “lip”) that contains one to three tyrosine residues that are sites of phosphorylation. This activation loop (A-loop) is apparently quite flexible and can move readily from a conformation in which it occludes all or part of the cleft to one in which substrate binding is not impeded. In most cases, phosphorylation of the A-loop tyrosines clearly stabilizes the “active” conformation, but it is less clear how ligand binding shifts the equilibrium from the “closed” to “open” form prior to the covalent modification.

The three-dimensional structures of several kinases, including those of the insulin receptor (IR) and fibroblast growth factor receptor 1 (FGFR1), have been particularly valuable in developing an understanding of kinase activation mechanisms (4, 5). The unphosphorylated forms of the FGFR1 and the IR kinase (IRK) domains show quite different orientations of the A-loop, although both occupy the active site cleft to some degree. In the unphosphorylated IRK, both the ATP and protein substrate binding sites are blocked, whereas in the unphosphorylated FGFR1K, an ATP analog is bound but residues of the catalytic loop hinder binding of peptide substrates. So far, there are structures available only for the IRK in both the inhibited and activated forms. There are large changes in conformation between inactive IRK and the tri-phosphorylated structure (4).
In the A-loop of the unphosphorylated IRK structure, Tyr1162 is H-bonded to the putative catalytic bases and blocks the nucleotide binding site. In this tri-phosphorylated form of IRK, pTyr1163 contributes to stabilization of the activated A-loop conformation by interaction with Arg1155, and the phosphate group of pTyr1162 H-bonds to the side chain of Arg1164. In FGFR1, mutation of either tyrosine in the A-loop sequence (Tyr653 or Tyr654), which are analogous to Tyr1162 and Tyr1163 of IR, does not inactivate the enzyme (although mutation of both does) (5). It is likely, therefore, that both tyrosines interact in the phosphorylated state, to stabilize the open form of the A-loop.

There are four major forms of the FGFR (designated FGFR1–4) that are members of the RTK family. Their expression is widely distributed in terms of tissues and developmental periods, reflecting a variety of cellular activities (6). All of the principal isoforms activate phospholipase Ca (PLC) and the RAS/MAPK (ERK) pathways, albeit with varying effectiveness (7). The former is bound to a specific phosphotyrosine (Tyr776 in FGFR1) found in the same relative position in all of the isoforms (6), and mutation of this residue (usually to phenylalanine) results in complete loss of PLC-γ-dependent responses (8, 9). The induction of ERK1/2 activation (via activated RAS) also occurs through the phosphorylation of FRS2, a signaling scaffold that binds constitutively to FGFRs regardless of their activation state (10–13). FRS2 has multiple sites for phosphorylation, which when modified allow binding of Grb2 and Shp2 leading to subsequent ERK activation as well as other responses (13).

The interaction of FRS2 with FGFR1 occurs via the binding of the phosphotyrosine binding domain of FRS2 to a 12-residue segment in the juxtamembrane region of FGFR1 (14). Because the other FGFRs also activate FRS2 and contain this sequence, the FGFRs lacking the C-terminal lobe (Tyr730) were identified. Site-directed mutagenesis established that at least one of the A-loop tyrosine residues was necessary, but the mutation of the remainder of the sites did not affect FRS2 activation nor did it affect mitogenesis and transient expression in 293 cells. Stable transfection into PC12 cells was accomplished with a retroviral vector (see below). Tyrosine to phenylalanine mutations were generated using the Clontech Site-directed mutagenesis kit. The substitutions were performed with only single base pair changes. All FGFR1 cDNA constructs used in this study were sequenced to confirm that the site-directed mutagenesis was correct.

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To further assess the specific and global contributions of the FGFR1 endodomain tyrosine residues to receptor function, these residues were systematically substituted with phenylalanine residues in a PDGFR/FGFR1 chimera and stably transfected into PC12 cells. Although none of the previously reported sites of phosphorylation outside of the A-loop were found to be required for neural differentiation, two residues adjacent to the A-loop (Tyr677 and Tyr701) were required for induction of PC12 cell differentiation by the chimeric receptors. These residues may function to stabilize the A-loop in an active conformation following ligand binding.

**Experimental Procedures**

**Cloning of FGFR1**—A full-length cDNA clone (p31) encoding a 2-lg loop form of FGFR1 was obtained from a PC12 cDNA library in λ ZAP (Stratagene). The library was screened with a cDNA probe encompassing the extracellular domain of human FGFR1 (kindly provided by Dr. Michael Jaye). The clone was sequenced by the dideoxy method using Sequenase and by automated sequencing on an ABI sequencer.

**Chimera Subcloning and Site-directed Mutagenesis**—As described previously (18), the naturally occurring 5’ EcoRI and 3’ MseI cDNA fragment of human PDGFR-β encoding the N-terminal extracellular domain was ligated with a 5’ MseI site introduced by polymerase chain reaction at the extracellular/transmembrane junction of the cDNA encoding the rFGFR1 intracellular domain and flanked by a 3’ EcoRI site, also introduced by polymerase chain reaction. The construct, designated PFR1, was subcloned into the EcoRI site of pCMV (from Stratagene) for mutagenesis and transient expression in 293 cells. Sequenase and by automated sequencing on an ABI sequencer, Michael Jaye). The clone was sequenced by the dideoxy method using Sequenase and by automated sequencing on an ABI sequencer.

**Immunoprecipitation and Immunoblot Analysis**—Protein was extracted from cells in lysis buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% Triton X-100, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μM Na3VO4, 4 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 100 μM Na3VO4) and quantitated. One mg of lysate was immunoprecipitated with antibody to addiion of protein A-Sepharose (Amersham Pharmacia Biotech) and washed three times in lysis buffer. The samples were then subjected to SDS-PAGE before electrotransfer to polyvinylidene difluoride membranes, and exposed to Hyperfilm (Amersham Pharmacia Biotech). As described previously (18), the naturally occurring 5’ EcoRI and 3’ MseI cDNA fragment of human PDGFR-β encoding the N-terminal extracellular domain was ligated with a 5’ MseI site introduced by polymerase chain reaction at the extracellular/transmembrane junction of the cDNA encoding the rFGFR1 intracellular domain and flanked by a 3’ EcoRI site, also introduced by polymerase chain reaction. The construct, designated PFR1, was subcloned into the EcoRI site of pCMV (from Stratagene) for mutagenesis and transient expression in 293 cells. Sequenase and by automated sequencing on an ABI sequencer, Michael Jaye). The clone was sequenced by the dideoxy method using Sequenase and by automated sequencing on an ABI sequencer.

**In Vitro Kinase Assay**—Cells were incubated in eukaryotic lysate buffer and immunoprecipitated with anti-PDGFRβ (R & D Systems) and protein A-Sepharose (Amersham Pharmacia Biotech). The immune complexes were washed three times in lysis buffer and once in kinase buffer (10 mM HEPES, pH 7.4, 2 mM MgCl2, 25 mM glycerol-2-phosphate, 0.1 mM Na3VO4, 4 mM NaF, 1 mM dithiobis, and 20 μM ATP), resuspended in 20 μl of kinase buffer, and incubated for 30 min at 30 °C with 5 μM [γ-32P]ATP (600 Ci/mmol). The kinase reactions were terminated by the addition of SDS-PAGE sample buffer. The samples were analyzed by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and exposed to Hyperfilm (Amersham Pharmacia Biotech). The membranes were subsequently immuno-
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Comparison of endodomain sequences of rat FGFR1

| Position | Brain (D12498a) | Kidney (s54008b) | PC12 Cells (p31) |
|----------|----------------|-----------------|-----------------|
| 427      | Val            | Val             | Val             |
| 428      | Thr            | Thr             | Thr             |
| 701      | Asn Tyr        | Tyr             | Tyr             |
| 728      | Asn His        | His             | His             |
| 776      | Asp Tyr        | Tyr             | Tyr             |
| 801      | Phe Leu        | Leu             | Leu             |
| 819      | Asn Lys        | Lys             | Lys             |

a Numbering conforms to human FGFR1 sequence. 

b Protein sequence accession number.

blotted with anti-FGFR1 antibodies (Santa Cruz Biotechnology) to determine the relative amount of immunoprecipitated chimera.

RESULTS

Rat PC12 FGFR1 Clone—The rat FGFR1 clone (p31) selected to prepare the chimeras used in this study was isolated from a PC12 cDNA library in λ Zap. This clone was characterized by a 2-Ig domain ectodomain and a Thr-Val insert in the juxtamembrane region. This motif has been shown to be a protein kinase C site that is important for the regulation of receptor activity in a splice variant from Xenopus embryos. Several clones judged to contain 3-Ig domain structures from size considerations were identified but not sequenced.2 A comparison of the transmembrane and endodomains of the p31 sequence to those of two other rat FGFR1 clones revealed that they were identical to those of a rat kidney FGFR1 clone (GenBank accession no. 554008) (except for the absence of the Val-Thr insert) but differed from a rat brain sequence (GenBank accession no. D12498) at five sites (Table I). Two of these sites involve tyrosine residues (Tyr701 and Tyr776). Thus, the brain isoform contains only 13 tyrosine residues in the endodomain as compared with the 15 found in the kidney and PC12 versions.

The tyrosine residues have been numbered in this report to correspond to those of human FGFR1 (GenBank accession no. P11362) or the 3-Ig domain rat receptor. They do not correspond to the true sequential positions in the chimera (as the PDGF ectodomain is larger than that of hFGFR1) or the 2-Ig loop sequence of p31. However, using the hFGFR1 numbers allows a direct comparison to several previous studies of FGFR1 and avoids the confusion of multiple numbering systems.

PFR1 Intracellular Domain Mutagenesis: Effects on Neurite Proliferation—PFR1, constructed from the p31 clone, contains 15 tyrosines in the endodomain as illustrated at the top of Fig. 1. In studies with hFGFR1 (and a recombinant kinase domain derivative), Mohammadi et al. (15) identified six phosphorylated tyrosine residues (Tyr663, Tyr583, Tyr585, Tyr653, Tyr654, and Tyr736) in the activated receptor. Tyr766 was previously identified as a seventh site (8, 9). These sites are marked with an asterisk in Fig. 1.

To evaluate the contributions of all of the endodomain tyrosine residues to the signaling potential of PFR1, and by extrapolation of FGFR1, they were converted to phenylalanine, a substitution normally expected to maintain hydrophobic integrity but lacking the opportunity for post-translational modification or H-bonding capacity. To facilitate these changes, the tyrosine residues were subdivided into four blocs, denoted A, B, C, and D (Fig. 1). Blocs A and B contained four tyrosine sites, and C and D, five sites. The C bloc comprised only the pair of tyrosines that are situated in the A-loop and whose combined phosphorylation stabilizes the active form of the kinase (5). The Tyr to Phe mutations were introduced en bloc and the resulting derivatives stably transfected into PC12 cells. These are designated by the bloc and number of residues modified, e.g. PFR1 A4 indicates all of the tyrosine residues of the A bloc have been converted to phenylalanine. Sequence numbering is for hFGFR1.

The effect of the Tyr to Phe mutations in blocs A, B, C, and D, singly and in various combinations, on the biological response (neurite outgrowth) of the stably transfected cells is shown in Fig. 2A. All of the cells examined showed a moderate ligand-induced decrease in activity (after 48 h) as compared with unmodified PFR1, but the derivatives with modified A or B blocs (or both) eventually achieved full differentiation (data not shown). In contrast, all of the derivatives in which either the first four (D4) or all five (D5) tyrosines of the D bloc had been modified (D4, A4/D4, B4/D4, A4/B4/D4, and A4/B4/D5) were inactive. As expected, the C2 derivative, with all of the other 13 tyrosines of the endodomain intact, was also inactive. These results establish that none of the total of eight tyrosine residues in the N-terminal portion of the kinase domain (including the juxtamembrane sequence and kinase insert) are essential, although the derivatives lacking some or all of these residues are modestly less active than the native (PFR1) receptor (Fig. 2A). At the same time, the results indicate that one or more of the first four residues of the D bloc (Tyr767, Tyr770, Tyr730, and Tyr766) is required.

More specifically evaluate the contributions of the D bloc tyrosines, which clearly strongly affect neurite outgrowth activity, a total of 18 constructs (Fig. 3) with each site converted from Tyr to Phe in single, double, or triple combinations were prepared using the A4/B4 construct as a parent. These were stably transfected into PC12 cells and each derivative tested for neurite production by PDGF. The results of these assays are shown in Fig. 2B. The five tyrosines in the D bloc were numbered 1 to 5 (consecutively) for identification and correspond to Tyr767, Tyr770, Tyr730, Tyr766, and Tyr776 (see Fig. 1). Thus, 2 S. Raffoni, unpublished data.

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TABLE I

Comparison of endodomain sequences of rat FGFR1

| Position | Brain (D12498a) | Kidney (s54008b) | PC12 Cells (p31) |
|----------|----------------|-----------------|-----------------|
| 427      | Val            | Val             | Val             |
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| 776      | Asp Tyr        | Tyr             | Tyr             |
| 801      | Phe Leu        | Leu             | Leu             |
| 819      | Asn Lys        | Lys             | Lys             |

a Numbering conforms to human FGFR1 sequence. 

b Protein sequence accession number.

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FIG. 1. Strategy for the mutagenesis of endodomain tyrosines of PFR1. The intracellular region of FGFR1 is depicted, and the 15-tyrosine residues are labeled. The 7 tyrosine residues previously identified as sites of phosphorylation (15) in vivo are marked with asterisks. The intracellular domain has been divided into four regions: A, B, C, and D. Mutant receptors are identified by the region and the number of residues changed to phenylalanine. Sequence numbering is for hFGFR1.
The particular importance of Tyr701 is emphasized in the observations that every chimera with a double or triple substitution that includes the Y701F mutation is inactive. However, the derivative that contains only Tyr701 (D3-134) is also inactive. (Although these derivatives also contain tyrosine D1-5 (Tyr776), this residue clearly makes no contribution; cf. D2-13 with D3-135 and D2-34 with D3-345, which have effectively the same activities (Fig. 2B)). The low but quite significant activity of D3-135 as compared with D3-134 suggests that tyrosine Tyr766, which has been shown to be the site of PLCγ activation (8, 9), may contribute somewhat to differentiation (presumably via PLCγ activation) when the level of FRS2 activation is lowered significantly (see below). This would also explain the comparable activation of D2-13 and D2-5 (with D3-135) and the very low activity of D2-4.

Effects on Receptor Autophosphorylation—To correlate the cellular responses with receptor-associated molecular events, several of the stably transfected cell lines were examined with respect to receptor autophosphorylation following PDGF stimulation and by in vitro kinase assays. As shown in Fig. 4A and B, receptor autophosphorylation as measured by anti-phosphotyrosine immunoblots of anti-FGFR1-treated PC12 cell lysates closely paralleled the neurite proliferation activities (Fig. 2). Only the A4/B4/D2-23 derivative, which was inactive in the differentiation assay, showed significant autophosphorylation. Interestingly, the amount of modification observed was also generally comparable with the bioactivity even though these measurements are not strictly quantitative.

A more sensitive in vitro kinase assay was also used to examine the autophosphorylation of the A4/B4/D2-12 derivatives (Fig. 4C). As expected, the C2 chimera did not show any capacity for autophosphorylation in this test either. However, both the D4 and D2-12 derivatives were clearly weakly but significantly labeled. The similar level of modification suggests that it is the C2 tyrosines that are modified (rather than the D3 or D4 tyrosines that are absent in the A4/B4/D4 chimera). These findings also indicate that these (and probably all of the various D bloc mutants) are properly folded as they have low activity of D2-4.

Effects on the Activation of ERK, SOS, FRS2, and PLCγ—The ability of the various PFR1 tyrosine derivatives to activate FGFR1-sensitive signaling pathways was assessed by immunoprecipitation and Western blotting of cell lysates following PDGF stimulation. The activation of ERK1/2 was determined using antibodies specific for the phosphorylated form of their MAPK. As shown in Fig. 5A, ERK1/2 was substantially activated by PDGF in PFR1 cells but not in cells bearing either the C2 or A4/B4/D4 variants. The A4, B4, and A4/B4 derivatives also activated ERK1/2 in a ligand-dependent manner in keeping with their differentiative activity. The D2-34 derivative showed weaker activation of ERK1/2, comparable with its weaker stimulation of neurite production, whereas D2-12 showed no ERK1/2 activation (Fig. 5B).
FGFR1 as well as the other FGFRs (6) activate a scaffold protein FRS2 that interacts constitutively with FGFR1 (Fig. 6) (14). Upon phosphorylation, it binds Grb2 and Shp2, leading to the binding of SOS and activated RAS that subsequently causes ERK1/2 activation (10–13). As shown in Fig. 6, FRS2 activation of the various derivatized chimeras, as determined with anti-phosphotyrosine antibodies, is consistent with both the neurite proliferation and ERK1/2 activities of these receptors. Although clearly not quantitative, the levels of FRS2 phosphorylation closely parallel the differentiative activity. A similar profile was observed for SOS association with activated FRS2 (Fig. 7), although SOS association was not observed with the D1-1 and D1-2 derivatives (at the exposure shown).

PLCγ is specifically bound to tyrosine D4 (Tyr766) of FGFR1, and therefore derivatives lacking this residue would not be expected to show activation of this enzyme. Indeed, D4, A4/B4/D2-34, and A4/B4/D1-4 lack this capacity (Fig. 8), whereas the A4, B4, and A4/B4 derivatives all show PLCγ phosphorylation. Interestingly, the A4/B4/D2-12 chimera, which is inactive in neurite proliferation assays and ERK activation, also fails to activate PLCγ even though there is weak kinase activation (Fig. 4C), and the requisite tyrosine (Y766) is intact.

**DISCUSSION**

RTKs use a variety of strategies to recruit and activate signaling effectors but they share significant similarities in their mechanisms of auto-activation (1, 2). Generally, RTKs rely on the ligand-induced formation of phosphorylated tyrosines located in the juxtamembrane, kinase domain, or C-terminal extension to provide binding sites for PTB- or Src homology 2-containing effectors or adaptors (19). These sites can be highly selective for a particular signaling protein or be
relatively promiscuous, binding several signaling entities. Thus, mutations of these residues (usually to phenylalanine) can cause specific changes in a receptor’s signaling capacity or show little effect. At the same time, not all receptor tyrosine residues that are phosphorylated apparently act as docking sites, and it cannot be assumed that all enddomain tyrosines of a receptor that can be phosphorylated will necessarily be detected. By systematically eliminating all of the tyrosine residues in the enddomain, the role (or lack thereof) of each to the signaling capacity of a receptor can be assessed. The results reported herein indicate that there is a minimum of four tyrosines in rat FGFR1 required for function in PC12 cell differentiation: the two activation loop residues (Tyr653 and Tyr654) and two C-terminal lobe residues (Tyr677 and Tyr701), which were not previously identified as either sites of modification or necessary for activity. Because Mohammadi et al. (15) previously showed that in FGFR1 Tyr653 could also be changed to phenylalanine while still retaining activity, there may only be three tyrosines (Tyr654, Tyr677, and Tyr701) absolutely required for activity in these cells.

To some considerable degree, the contribution of the D1-1 and D1-2 tyrosines (Tyr677 and Tyr701) to both receptor activation and downstream signaling can be elucidated from the data in Figs. 2 and 4. Autophosphorylation reflects both the state of the kinase activation and the number of receptor phosphorylation sites. Although these are not likely to be equally modified, one would nevertheless expect that derivatives in which modifiable tyrosines had been converted to phenylalanine would show lower amounts of phosphorylation. There are seven sites of tyrosine phosphorylation in FGFR1 (15), and the identical sequence has a H717E substitution, although Tyr701 is conserved. The A4/B4 chimera accordingly would be expected to show lower levels of autophosphorylation for this reason alone. However, it is 25% less active in the neurite outgrowth assay at 48 h as compared with PFR1 (Fig. 2), suggesting some of the decreased autophosphorylation may reflect decreased kinase activity.

Several other comparisons confirm that the effect of Tyr677 and Tyr701 on autophosphorylation reflects decreased kinase activity rather than just a decrease in available sites. A comparison of the A4/B4/D4 and A4/B4/D2-34 chimeras shows that the latter is well modified (comparable with the A4/B4 derivative), whereas the A4/B4/D4 modifications can be detected only with the in vitro kinase assay (Fig. 4C). Both contain only the C2 tyrosines as known modification sites (Tyr730 and Tyr766). Clearly these latter two residues are not modified when the D1 and D2 tyrosines are substituted, which is consistent with the lack of activation of PLCγ by the latter derivative (Fig. 8). It is also instructive to compare the A4/B4 derivative with the A4/B4/D1-1 and D1-2 mutants. These chimeras all contain four potential tyrosine autophosphorylation sites, but the latter are clearly modified to a lesser degree and have substantially reduced neurite proliferation activity.

Tyrosines 677 and 701 are not believed to be sites of autophosphorylation (15), which is consistent with their relatively buried positions as determined in structural analyses of the FGFR1 kinase domain (marked in blue in Fig. 9) (5). Thus, these tyrosines are likely important structurally, but when mutated to phenylalanine, they do not appear to cause misfolding or instability of the receptor. Indeed, derivatives involving these residues retain measurable catalytic activity, albeit quite low in some cases, indicating that correct folding has occurred. However, the presence of an aromatic ring is apparently insufficient to meet their roles in the fully active kinase, and a H-bonding capacity is also required either structurally or to play a more direct role in the activation process. A comparison of the FGFR1 structure with the unliganded IRK structures leads to the following conclusions (4, 5, 20). Tyr677 lies closest to the A-loop and, although not showing any interaction in the FGFR1 structure, the equivalent Phe1386 changes drastically between the two forms of the IRK. In the activated form, it lies beneath the A-loop, and a tyrosine here would have the potential to H-bond to and stabilize the loop in the activated form. In the FGFR1 structure, Tyr701 is partially buried in a group of mainly hydrophobic residues and forms a H-bond with the side chain of His717. In the IRK structures this region remains essentially unchanged between the two forms; the equivalent residue (Tyr8210) makes no contacts with side chains but is involved in water-mediated H-bonding to the carbonyl groups of 1174 and 1176 and the main chain nitrogen of 1228, thus bridging secondary structure elements. Tyr701 lies on an extended loop between α-helices F and G, which follow a similar trace in all three structures. If the A-loop adopts a similar conformation in fully activated FGFR to that in IRK, a readjustment of the αG loop would be required for Tyr701 to make a stabilizing contact. Such a feature is not, however, seen in the two known tyrosine kinase structures with activated conformation A-loop, i.e. IRK and the non-receptor Lck kinase (21).

Tyr677 and Tyr701 are well conserved in the FGFR family. Only Halocynthia noretzi, an invertebrate worm, has a Phe to Tyr substitution at residue 677. Interestingly, this same sequence has a H717E substitution, although Tyr701 is conserved. The only substitution of Tyr701 is the Asn replacement found in the rat brain form of FGFR1 (see Table I). The significance of this substitution is unclear, but this residue does retain H-bonding capacity, although it is somewhat shorter in...
FIG. 9. Representation of the kinase domain of FGFR1. α-Helices are shown in gray-green, and β-sheets are indicated by blue arrows. Tyrosine residue side chains are shown with the following colors: sites of phosphorylation are in red (Tyr<sup>653</sup> and Tyr<sup>654</sup> are not defined), A-loop tyrosines are in magenta, the three tyrosines that are unmodified are in gray, and two that are required for activity (this study) are in blue. Numbering is from hFGFR1 (GenBank<sup>™</sup> accession no. P11362).

Active site cleft. The former is often accompanied by phosphorylation events that further stabilize this conformation, presumably by forming sites of interaction between phosphate groups and basic (lysine or arginine side chains) centers. The substitution of a glutamic acid for Tyr<sup>650</sup> in FGFR3 (the basis for the human disease thanatophoric dysplasia II) (22), two residues downstream from the tyrosine residues corresponding to Tyr<sup>653</sup> and Tyr<sup>654</sup>, leads to autoactivation by providing an alternative interaction of this sort that is not reversible. In a cellular milieu, the phosphorylation of the activation loop tyrosines is balanced by dephosphorylation events. The availability of these modified residues as substrates will thus depend on the degree to which the modified loop is stabilized in the open conformation. If Tyr<sup>677</sup> and Tyr<sup>701</sup> contribute directly or indirectly to this structure, their absence would likely accelerate the reverse reactions. Importantly, it cannot be stated with certainty that these derivatives do not play purely a structural role and that the kinase domains of these inactive derivatives (lacking Tyr<sup>677</sup> and/or Tyr<sup>701</sup>) are simply inactive. However, the strong autophosphorylation of the A4/B4/D2-23 derivative, which lacked measurable differentiative activity, argues against this as a general condition.

The identified sites of autophosphorylation are shown in Fig. 9 (in red). Tyr<sup>776</sup> (the PLCγ site) lies outside the domain pictured, and the kinase insert residues (Tyr<sup>583</sup> and Tyr<sup>585</sup>) were not defined because of poor resolution in this part of the structural analysis (5). Mohammadi <i>et al.</i> (15) determined that the elimination (by mutation to phenylalanine) of the modified tyrosines was without effect on L6 or PC12 cell responses (with the exception of Tyr<sup>653</sup> and Tyr<sup>654</sup> as already noted). However, Wang and Goldfarb (17), using chimeras of FGFR1 and FGFR4, found that the two tyrosines in the kinase domain insert (Tyr<sup>583</sup> and Tyr<sup>585</sup>) were required for full mitogenic activity in BaF cells. It has been shown that previously Tyr<sup>776</sup> could be altered without affecting L6 and PC12 cell responses, indicating that PLCγ was unnecessary in these cases (8, 9, 16). However, PLCγ can contribute to FGFR1 function, as has been demonstrated in neuronal growth paradigms (23) and herein. PLCγ can make a contribution, at least to PC12 differentiation, if the receptor lacks the capacity for the full activation of other pathways. This can be seen clearly in the comparison of the activity of A4/B4 and A4/B4/D1-4, with the latter lacking the PLCγ activation site. The lower but significant activity of A4/B4/D2-13 and D2-15 most likely also results from a PLCγ contribution that is absent, for instance, from the D2-14 derivative. Indeed, TrkA derivatives in PC12 cells that lack the activation site for FRS2 (or Shc) do show ligand-induced neurite outgrowth, but at greatly reduced levels, that is generated solely by PLCγ activation (23).

The elimination of the nonphosphorylated tyrosines (as well as the modified ones) also establishes that this receptor does not contain cryptic (or undetected) additional residues of phosphotyrosine, which is consistent with the view that the FGFRs signal solely through FRS2 and PLCγ activation. The former associates constitutively with FGFRs (13), as was also observed in these studies, and is independent of the state of activation. The binding site has been identified as a 12-residue sequence in the juxtamembrane domain, which is devoid of tyrosine residues (14). Whether activation of the FGFR allows for tighter binding and therefore enhanced enzyme-substrate interactions is unknown, but the elimination of the tyrosines, particularly in the A4/B4 blocks that are closest to the site of interaction, has only modest effects on the differentiation capacity (See Fig. 9).

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3 D. Tyson, L. M. Thompson, and R. A. Bradshaw, manuscript in preparation.
2). This may reflect some alteration in FRS2-FGFR1 binding and the level of activation of the signaling scaffold, or it may simply indicate that the A4/B4 derivative is not as active (because it cannot stabilize the active form as effectively).

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