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Identification of tyrosines 154 and 307 in the extracellular domain and 653 and 766 in the intracellular domain as phosphorylation sites in the heparin-binding fibroblast growth factor receptor tyrosine kinase (*flg*)

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Abstract

Four tyrosine residues have been identified as phosphorylation sites in the tyrosine kinase isoform of the heparin-binding fibroblast growth factor receptor *flg* (FGF-R1). Baculoviral-insect cell-derived recombinant FGF-R1 was phosphorylated and fragmented with trypsin while immobilized on heparin-agarose beads. Phosphotyrosine peptides were purified by chromatography on immobilized anti-phosphotyrosine antibody and analyzed by Edman degradation and electrospray tandem mass spectrometry. Tyrosine residue 653, which is in a homologous spatial position to major autophosphorylation sites in the catalytic domain of the *src* and insulin receptor kinases, is the major intracellular FGF-R1 phosphorylation site. Residue 766 in the COOH-terminus outside the kinase domain is a secondary site. Tyrosine residues 154 and 307, which are in the extracellular domain of transmembrane receptor isoforms and are in an unusual sequence context for tyrosine phosphorylation, were also phosphorylated.

Keywords: growth factors; oncogenes; signal transduction

Heparin-binding fibroblast growth factors (FGF) are a family of seven genetically distinct polypeptides that control cell growth and differentiation within the local tissue environment (Burgess & Maciag, 1989). Alternate splicing in four distinct genes gives rise to structural isoforms of the FGF receptor (FGF-R) that differ in the extracellular ligand-binding domain, the intracellular juxtamembrane domain, and the intracellular tyrosine kinase domain (Hou et al., 1991). An alternate acceptor site splice results in a kinase-deficient isoform of the FGF-R1 (*flg*) gene that is truncated 24 residues into the kinase catalytic domain (Hou et al., 1991). The variant is devoid of candidate tyrosine phosphorylation sites downstream of the truncation but retains a candidate site in the interkinase sequence. Splicing at an alternate acceptor site in the FGF-R2 (*bek*) gene results in a truncated isoform devoid of a candidate tyrosine homologous to Tyr-766 in the COOH-terminus of the FGF-R1 gene (Champion-Arnaud et al., 1991). Based on data using a bacterially expressed intracellular domain fragment of the FGF-R1 gene, a recent report contended that Tyr-766[1] is the single phosphorylated tyrosine in the intracellular domain of the FGF-R1 recep-

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Abbreviations: bek, bacterially expressed kinase; CSF, colony-stimulating factor; DTT, dithiothreitol; FGF, heparin-binding fibroblast growth factors; FGF-R, FGF receptors; *flg*, *fms*-like gene; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid; HFA, hexafluoroacetone; PDGF, platelet-derived growth factor; PMSF, *p*-methylsulfonyl fluoride; RP-HPLC, reverse-phase high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Sf9, *Spodoptera frugiperda* cells; TFA, trifluoroacetic acid; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

[1] FGF-R residues are numbered from the methionine initiation site of the three immunoglobulin-like loop isoform (FGF-R1α1) of the *flg* receptor gene.

tor and facilitates interaction with the signal transducer phospholipase Cγ1 (Mohammadi et al., 1991). Two subsequent reports confirmed by mutation that Tyr-766 is required for association with and phosphorylation by the full-length FGF-R1 kinase when transfected into mammalian cells (Mohammadi et al., 1992; Peters et al., 1992). In this report, we employed baculoviral-infected insect cells and the FGF receptor complex cofactor heparin (Kan et al., 1992) to express and purify the mature FGF-R1 tyrosine kinase glycoprotein in quantities sufficient for complete analysis of tyrosine phosphorylation sites. Sequence and tandem mass spectrometric analysis of receptor phosphotyrosine polypeptides showed that Tyr-653 within the catalytic domain of the kinase is the major site, and Tyr-766 is a secondary intracellular site of phosphorylation in vitro. Surprisingly, two tyrosines in the extracellular domain of the receptor were phosphorylated.

Results

Recombinant FGF-R1βa1(*flg*) tyrosine kinase was expressed in baculoviral-infected Sf9 insect cells, cells were lysed with detergent, and the receptor was isolated by incubation of cell lysates with an anti-FGF-R1 antibody A40 complexed to protein A–agarose beads. The immunoprecipitate was incubated with [^{32}P]ATP and an aliquot analyzed by SDS-PAGE and autoradiography (Fig. 1A). The results indicated a major radioactive band that correlated with the 105-kDa insect cell-derived receptor, a band that correlated with the IgG heavy chain band and two minor bands. Immobilized ^{32}P-labeled receptor was digested on the antibody/protein A–agarose beads with trypsin and phosphopeptides which, when released from the beads, were analyzed by RP-HPLC (Fig. 1B). Six peaks of radioactivity were retained on the column, all of which exhibited phosphotyrosines when subjected to HCl hydrolysis and phosphoamino acid analysis on thin layer electrophoresis (Fig. 1C).

For large-scale enrichment of phosphorylated recombinant receptor for phosphopeptide analysis, baculoviral-infected insect cell lysates were mixed with immunopurified ^{32}P-labeled receptor, and the mixture was absorbed onto heparin–agarose beads (Fig. 2). The heparin–agarose beads were incubated with unlabeled ATP, and then the receptor was fragmented with trypsin while still immobilized on the beads. Released phosphotyrosine peptides, which accounted for over 90% of immobilized radioactivity, were then purified on a column of immobilized antiphosphotyrosine antibody. Analysis of the eluate from the immunoaffinity column by RP-HPLC revealed six radioactive protein peaks (Fig. 2C). A pool of each radioactive peak from multiple chromatographic runs was further purified by a second round of chromatography on C18 RP-HPLC or pH-stable C8 HPLC columns (Fig. 3). NH_2-terminal sequence analysis revealed that five of the phosphopeptides were tyrosine-containing sequences in

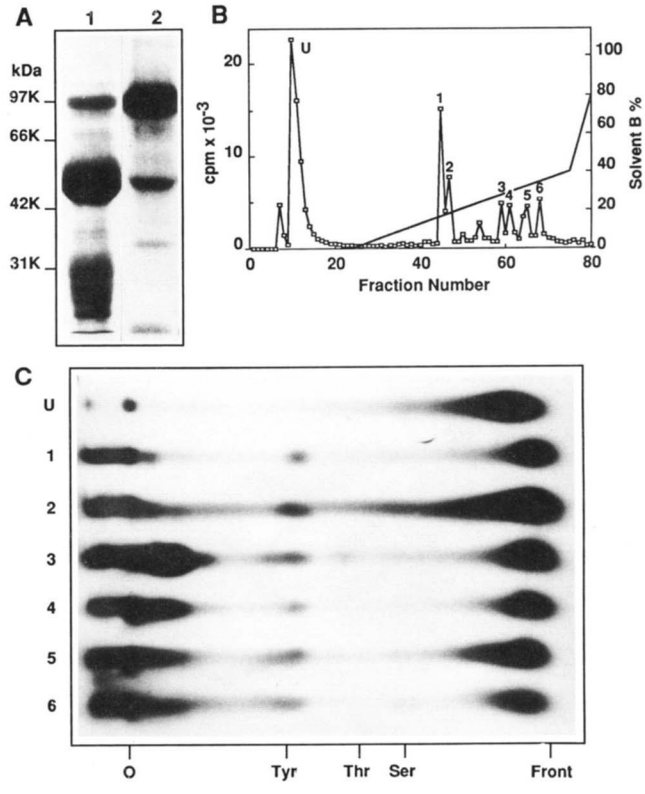

Fig. 1. Tyrosine phosphorylation of recombinant FGF-R kinase. **A:** ^{32}P-labeled-FGF-R1βa1 immobilized on anti-FGF-R1 antibody–protein A–agarose beads. Lane 1 is the extract of an aliquot of the beads stained with Coomassie blue and lane 2 is an autoradiograph of the same lane after exposure for 24 h. **B:** RP-HPLC of tryptic digest of immobilized ^{32}P-labeled FGF-R on a C18 column (4.6 × 250 mm). The indicated gradient was applied at a flow rate of 0.50 mL/min, and fractions were collected every minute and counted. Solvent A was 0.1% TFA and solvent B was 84% acetonitrile and 0.1% TFA. **C:** Phosphoamino acid analysis of tryptic peptides. Lane 0 is the unretained fraction and lanes 1–6 are corresponding peaks from B.

FGF-R1 (*flg*) (Table 1). Peptides P1 and P2, the two most abundant phosphopeptides in the digest, had the same NH_2-terminal sequence and contained tandem tyrosine residues 653 and 654. The absence of Tyr-653 in the Edman sequence analysis of P1 suggested that Tyr-653 may be a phosphorylation site. To determine if only Tyr-653 was phosphorylated, phosphotyrosine peptide P1 was characterized by electrospray mass spectrometry (Smith et al., 1990; Carr et al., 1991). An $(M + 2H)^{2+}$ signal at *m/z* 706.4 (Fig. 4A) was indicative of the expected monophosphorylated peptide (M_r determined = 1,411.8; M_r calculated = 1,411.6). Absence of a signal at *m/z* 746.8, the $(M + 2H)^{2+}$ for the potential bis-phosphorylated peptide of M_r 1,491.6, indicated that there is only one phosphate moiety in the peptide. Tandem mass spectrometry (Carr et al., 1991) of the $(M + 2H)^{2+}$ parent ion provided the partial sequence -H-H-X-D-B-Y- (X = Leu or Ile; B = phosphotyrosine), which established the presence and location of the phosphotyrosine residue (Fig. 4B).

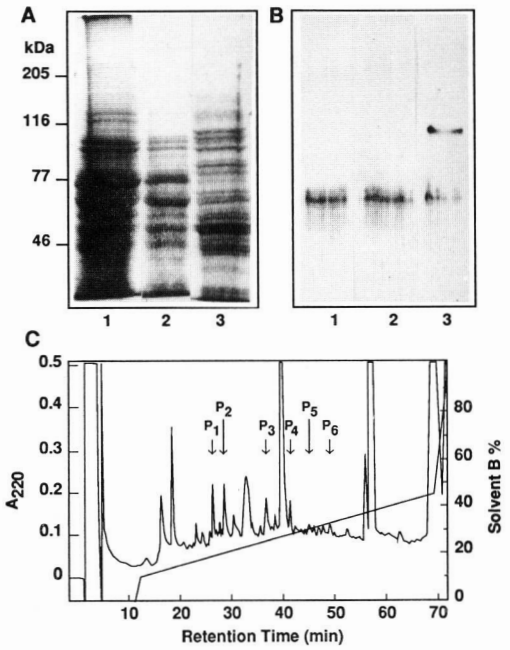

Fig. 2. Heparin–agarose chromatography and RP-HPLC purification of phosphopeptides from anti-phosphotyrosine immunoaffinity chromatography. Recombinant receptor was immobilized on heparin–agarose beads and phosphorylated and digested with trypsin in situ as described in Materials and methods. A sample of the beads was analyzed by 10% SDS-PAGE before phosphorylation and digestion. Total protein (**A**) and receptor antigen (**B**) were visualized by Coomassie blue stain and reaction with anti-receptor antibody (A40) followed by reaction with anti-rabbit IgG conjugated with alkaline phosphatase, respectively. Lane 1 is the fraction of Sf9 cell lysate that is not retained by the heparin–agarose, lane 2 is a 0.15 M NaCl eluate of the beads, and lane 3 is a 0.50 M NaCl eluate. Tryptic phosphopeptides after antiphosphotyrosine immunoaffinity chromatography were applied to an RP-HPLC C18 narrow-bore column (2.2 × 250 mm) and separated at a flow rate of 0.15 mL/min with the indicated gradient (**C**). Solvent A was 0.1% TFA and solvent B was 84% acetonitrile, 0.1% TFA. Peptide fractions were collected and the indicated radioactive peaks P1–P6 were retained for further purification and analysis (Fig. 3).

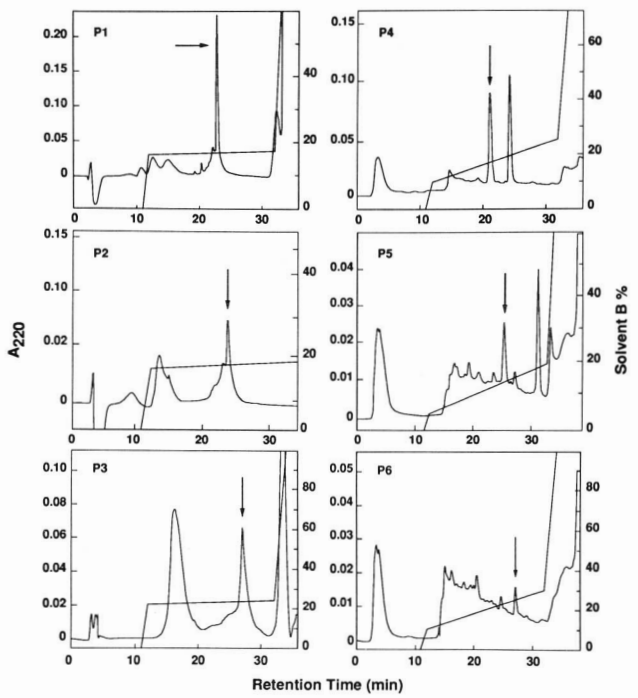

Fig. 3. Purification of phosphotyrosine peptides. Partially purified peaks P1, P2, and P3 were purified to homogeneity by narrow-bore RP-HPLC with the indicated gradients from 0.1% TFA (solvent A) to 84% acetonitrile, 0.1% TFA (solvent B). Peaks P4, P5, and P6 were purified at neutral pH on pH-stable C8 columns with the indicated gradients of acetonitrile: solvent A, 0.2% HFA/NH_3, pH 7.2; solvent B, 84% acetonitrile containing 0.03% HFA/NH_3 (Tarr & Crabb, 1983). A flow rate of 0.15 mL/min was used throughout. Arrows indicate the radioactive peaks that were selected for analysis.

Specifically, the mass increment from peak b_6 to b_7 and from y_3 to y_4 demonstrated that the phosphate group is located at position 653. Absence of a fragment ion 80 Da greater in mass than the observed y_3 further confirmed that only Tyr-653 is phosphorylated and that Tyr-654 is not.

The NH_2-terminal sequence of phosphopeptides P3 and P4 were tryptic peptides from the extracellular domain of the receptor, which contained FGF-R1 tyrosine residues 154 and 307, respectively. Gaps in the Edman sequence analysis of each peptide at Tyr-154 and Tyr-307 confirmed that these positions were probably phosphorylated. Because the COOH-terminal residue of peptide P4 was not clear from the Edman sequence analysis, the phosphopeptide was also analyzed by mass spectrometry (Fig. 5A,B). The $(M + 2H)^{2+}$ peak at 761.4 indicated the expected molecular weight of the monophosphorylated tryptic peptide (M_r determined = 1,520.8; M_r calculated = 1,520.8). The tandem mass spectrum of the $(M + 2H)^{2+}$ parent ion (Fig. 5B) exhibited y-series ions and intense internal fragment ions triggered by the presence of proline residues (Hunt et al., 1986). These internal fragments (PB, PBA, PBAQ, etc.) provided the clearest series demonstrating that FGF-R1 Tyr-307 was phosphorylated. Together with the y-ions, the partial sequence X-G-P-D-N-X-P-B-A-Q-X- was obtained.

The sequence of phosphopeptide P5 indicated that it was the tryptic peptide spanning residues 757–784 that contains Tyr-766 and Tyr-776 in the COOH-terminal sequence of FGF-R1. The absence of Tyr-766 in the Edman sequence analysis suggested that Tyr-766 was phosphorylated.

Discussion

In this report, four tyrosine residues in the full-length tyrosine kinase isoform (FGF-R1βa1) of the *flg* receptor gene family have been identified as phosphorylation sites. The recombinant baculoviral–insect cell expression system and the heparin-binding property of the FGF-R tyrosine kinase extracellular domain were exploited to-

Table 1. *Sequence of phosphopeptides*

Peptide	Sequence[a]	FGF-R1βa1(*flg*)
P1	DIHHIDXYKK	DIHHIDY[653]YKK
P2	DIHH[b]	DIHHIDY[653]YK(K)[c]
P3	XPVAPXWTXPEK	MPVAPY[154]WTSPEK
P4	IGPDNLPXAQIL	IGPDNLPY[307]AQILK
P5	XXXXXSNQEXL	IVALTSNQEY[766]LDLSMPLDQY[776]SPPSFPDTR
P6	XYILF[b]	

[a] Gas-phase Edman degradation. X = cycle too ambiguous for residue assignment.
[b] Sequence analysis was aborted at the indicated cycle.
[c] COOH-terminal lysine is presumed absent.

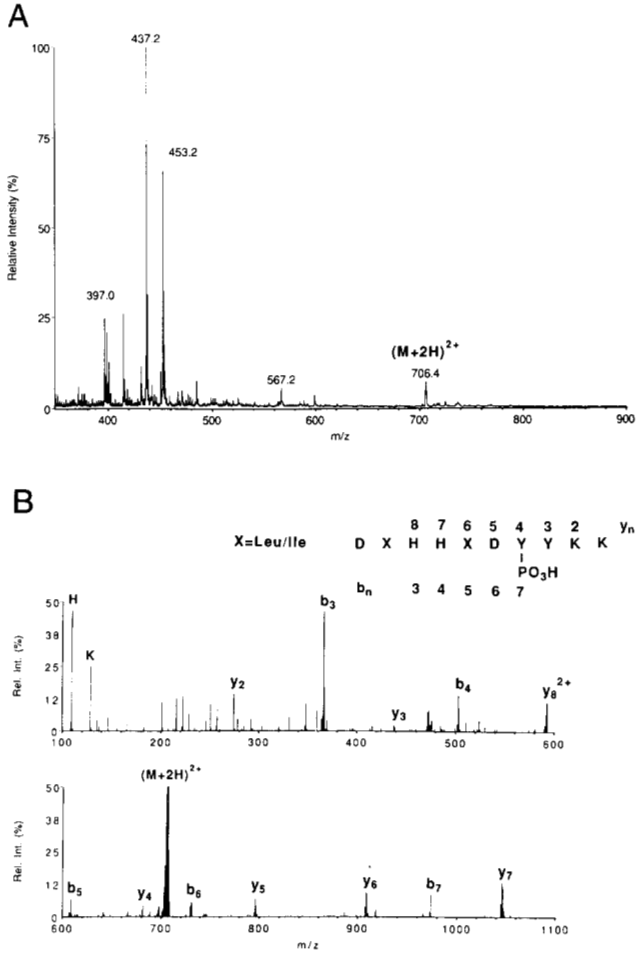

Fig. 4. Analysis of peptide P1 by electrospray mass spectrometry. **A:** Electrospray mass spectrum of 15–20 pmol of the purified peptide. **B:** Electrospray tandem mass spectrometry of the $(M + 2H)^{2+}$ (m/z 706.4) of ca. 20 pmol of peptide P1 (see A). The numbering above the printed single-letter code sequence refers to y_n-ions formed by cleavage of the peptide bond of the nth amino acid from the COOH-terminus with H-rearrangement to form a charged, COOH-terminal peptide fragment (NH_2-CHR_n-CO . . . NH-CHR_1-CO_2H + H)$^+$. Charge retention on the N-terminal acylium fragments (NH_2-CHR_1-CO . . . $NHCHR_nCO^+$) yields the b_n-ion series. B = phosphotyrosine; X = Leu or Ile, which cannot be distinguished.

gether with anti-phosphotyrosine immunoaffinity and RP-HPLC to identify and purify phosphotyrosine peptides in three steps. The glycosaminoglycan component of heparin-like extracellular matrix heparan sulfate proteoglycans has recently been identified as an obligatory component of the ternary FGF-R complex composed of the transmembrane glycoprotein tyrosine kinase, heparan sulfate, and ligand (Kan et al., 1992). Gas-phase microsequencing, electrospray mass spectrometry, and tandem mass spectrometry were used to chemically characterize the purified phosphopeptides. Digests of immunopurified and heparin–agarose-enriched phosphorylated receptor yielded six phosphotyrosine peptides. Based on combined yields of phosphopeptides P1 and P2 that were 3–10 times that of the other three phosphopeptides, the major phosphorylation site in the FGF receptor is within tryptic phosphopeptide DIHHIDYYKK containing tandem tyrosines 653 and 654. P2 probably differs from P1 by a COOH-terminal lysine residue. Both sequence and mass spectroscopic analysis clearly indicated that Tyr-653 is the single site of phosphorylation, eliminating the possibility that the more abundant phosphorylation is due to 2 moles of phosphate per mole peptide. The spatial location of Tyr-653 within the catalytic consensus domain of FGF-R1 is in the same spatial context of a major autophosphorylation site in the *src* family of tyrosine kinases (*yes*, *fgr*, *fyn*, *lyn*, *lck*, *hck*) (Hanks et al., 1988) and is conserved in all kinase isoforms of the four FGF-R genes across all species (Pasquale, 1990; Partanen et al., 1991) including *Xenopus* (Friesel & Dawid, 1991) and *Drosophila* (Glazer & Shilo, 1991). The insulin, *trk*, and *met* tyrosine kinase receptor families also exhibit tandem tyrosine residues in a homologous spatial position (Hanks et al., 1988). Although unconfirmed by mass spectrometric analysis, it has been argued that both tandem tyrosine residues are autophosphorylated in the insulin receptor kinase (White et al., 1988). In contrast, comparative cycle yields from Edman sequence analysis suggested that only the tyrosine residue homologous to Tyr-654 of FGF-R was phosphorylated in the *met* receptor kinase (Prat et al., 1991). The

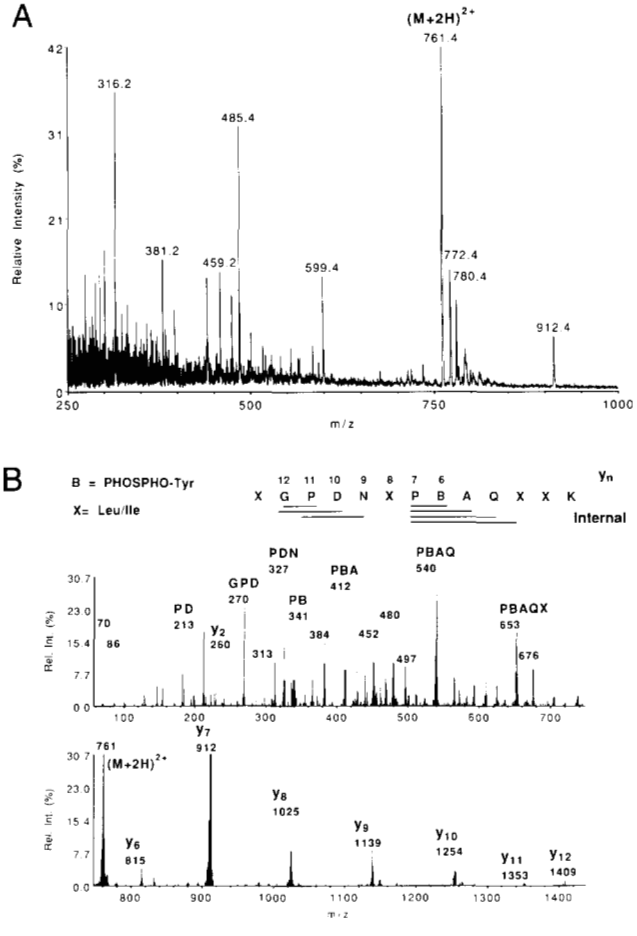

Fig. 5. Analysis of peptide P4 by electrospray mass spectrometry. **A:** Electrospray mass spectrum of 15–20 pmol of purified peptide. **B:** Electrospray tandem mass spectrometry of the $(M + 2H)^{2+}$ (m/z 761.4) of ca. 20 pmol of peptide P4 (see A). Internal fragment ions are indicated by underlining and use of the single-letter code. See legend of Figure 4B for all other nomenclature.

PDGF-R and CSF-R kinases, which have no tyrosine residue in homologous position to Tyr-653, are phosphorylated on a tyrosine in homologous spatial position to Tyr-654 in FGF-R (Kazlauskas & Cooper, 1989; Tapley et al., 1990).

Identification of Tyr-653 of FGF-R as the major phosphorylation site disagrees with a report that contends that Tyr-766 is the single phosphotyrosine site within the FGF-R1 intracellular domain (Mohammadi et al., 1991). However, our results confirm that Tyr-766 within tryptic phosphopeptide P5, which was the least abundant of the five FGF-R phosphopeptides, is an FGF-R phosphorylation site. A 28-amino acid tryptic peptide containing phosphotyrosine-766 interacts with both intact PLCγ1 and its recombinant SH2-domain sequence (Mohammadi et al., 1991). Two recent reports confirm by mutagenesis that Tyr-766 appears essential for interaction and phosphorylation of PLCγ1 with the full-length FGF-R1 kinase expressed in transfected mammalian cells (Mohammadi et al., 1992; Peters et al., 1992). Moreover, the FGF-R1 mutant with a phenylalanine substitution for Tyr-766 was autophosphorylated and exhibited two tryptic phosphopeptides with retention times on HPLC similar to phosphotyrosine-653 peptides P1 and P2 in our analysis (Mohammadi et al., 1992). In experiments to be published in detail elsewhere (E. Shi, M. Kan, J. Xu, F. Wang, J. Hou, & W.L. McKeehan, in prep.), we have shown that Tyr-653 appears to be a specific trans-intermolecular autophosphorylation site between monomers within kinase homodimers, whereas phosphorylation of Tyr-766 appears to occur by a cis-intramolecular mechanism. This is consistent with the failure to observe phosphotyrosine-653 in the truncated intracellular domain of FGF-R1 (Mohammadi et al., 1991), which unlikely forms dimers in the absence of the extracellular domain. The remarkable specificity of the phosphorylation of the Tyr-653 site relative to adjacent Tyr-654 may reflect its specificity as a trans-phosphorylation site within FGF-R kinase homodimers.

Surprisingly, phosphotyrosine peptides P3 and P4 were from the extracellular domain of the receptor. Both FGF-R extracellular tyrosines are in an unusual sequence environment for tyrosine phosphorylation, the common feature of which is an NH_2-terminal proline flanked by hydrophobic residues rather than the more common acidic residues in most tyrosine phosphorylation sites (Cantley et al., 1991). Further experiments are required to determine whether phosphorylation of the NH_2-terminal sites is functional. The results pose novel questions because there is evidence for novel activities of the extracellular domain of FGF-R in addition to ligand-binding (Shi et al., 1991a,b,c; Xu et al., 1992; Yan et al., 1992).

Tyrosine residues 583, 613, and 730 within the FGF-R kinase consensus sequence are also in a favorable sequence context for phosphorylation but were not detected in this study. Tyr-583, which is in the interkinase domain, is conserved in all FGF-R isoforms except in products of the FGF-R4 gene (Partanen et al., 1991). Autophosphorylation of a tyrosine in the interkinase domain of the PDGF-R and CSF-R kinases has been implicated in receptor–phosphoinositol-3-kinase interaction (Koch et al., 1991).

Materials and methods

Construction of recombinant baculovirus-bearing FGF-R1βa1 cDNA

The FGF-Rβa1, previously called HBGF-Rβa1, isoform of the *flg* receptor gene (Hou et al., 1991) was used throughout this study. Recombinant baculovirus was prepared, identified, and purified (Xu et al., 1992) from Sf9 insect cell hosts according to Summers and Smith (1987) using the MAXBAC kit (In Vitrogen Corp., San Diego, California). Virus stocks were standardized by resulting recombinant antigen expression levels on Western blots.

Kinase and autophosphorylation activity

Baculovirus-infected Sf9 cells ($1 \times 10^5/cm^2$) were harvested by pipetting, concentrated by centrifugation, and lysed in 20 mM Tris-HCl (pH 7.2), 150 mM NaCl, 10% glycerol, 2 mM EDTA, 1% Triton X-100, 1 mM PMSF, 0.015 units/mL aprotinin, 1 mM sodium vanadate, and 0.01 mg/mL leupeptin (20 μL cell lysis buffer/10^6 cells). The lysate from 1–5 $\times$ 10^6 cells was incubated for 90 min with 20 μL rabbit antiserum A40 (Xu et al., 1992) that had been previously immobilized on 20 μL packed protein A–agarose beads (Sigma Chemicals, St. Louis, Missouri). The receptor–antibody–protein A bead complex was washed twice with 20 mM Hepes (pH 7.0), 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol (400 μL HNTG buffer/20 μL beads) and once with 40 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 7.5 mM Mn^{2+}, 1 mM DTT, 1 mM sodium vanadate, and 0.1% Triton X-100 (phosphorylation buffer) prior to adding 20 μCi of [^{32}P]γATP. After 10 min of incubation at room temperature, the beads were collected and washed once with 20 mM Tris-HCl (pH 8.0) and 150 mM NaCl and then twice with 20 mM Tris-HCl prior to use in analyses.

Identification of phosphotyrosine peptides

^{32}P-labeled receptor immobilized on antibody–protein A beads was digested in situ with about 50 μL of 20 μg/mL of TPCK-trypsin in 20 mM Tris-HCl (pH 8.0) for 4 h at 37 °C, and the released phosphopeptides were analyzed by RP-HPLC on a 0.21 $\times$ 15-cm C18 column (Vydac Corp., Hesperia, California). For phosphoamino acid analysis, an aliquot containing 5,000 cpm of each ^{32}P-labeled peak from the column was hydrolyzed in 6 M HCl at 110 °C for 60 min, samples were dried, dissolved in 20 μL water, and applied to thin layer electrophoresis plates (Cellulose F, EM Separations, Gibbstown, New Jersey). Electrophoresis of unknowns and standard preparations of phosphotyrosine, phosphoserine, and phosphothreonine was performed in pyridine:glacial acetic acid:water (1:10:189) at pH 3.5 and run at 700 V for 60 min. Plates were dried and autoradiographed for 48 h.

Phosphorylation and tryptic digestion of FGF-R immobilized on heparin-agarose

The lysate from 1–2 $\times$ 10^8 infected Sf9 cells, together with 1–2 μg of ^{32}P-labeled immunopurified receptor protein (2 $\times$ 10^6 cpm), was mixed with a slurry of 20 mL packed heparin-agarose beads at 4 °C for 4 h. The slurry was poured into a column and washed with 5 bed volumes of 20 mM Tris-HCl (pH 7.2), then 1.5 bed volumes of phosphorylation buffer, and then 1.5 bed volumes of phosphorylation buffer containing 2 mM ATP. The column was incubated in ATP-containing phosphorylation buffer for 10 min at room temperature and then washed with 5 bed volumes of 20 mM Tris-HCl (pH 7.2) and 150 mM NaCl and then 5 bed volumes of 20 mM Tris-HCl (pH 8.0). The heparin–agarose column was then incubated with 5 μg of TPCK-trypsin at 37 °C for 4 h on a rocker. The digested protein was then eluted from the heparin–agarose column with water, and the eluate was freeze-dried. The concentrated protein was further digested in 100 mM of Tris-HCl (pH 8.0) by addition of 25 μg TPCK-trypsin and incubated at 37 °C for 4 h.

Purification of phosphotyrosine peptides

The tryptic digest was heated in a boiling water bath for 5 min followed by addition of 1 mM PMSF and adjusted to 1 mL with 20 mM Tris-HCl (pH 7.4) and then absorbed to anti-phosphotyrosine antibody immobilized on agarose beads (1.5 mL packed beads) (UBI, Lake Placid, New York). The bead slurry was rocked at 4 °C for 60 min, poured into a column, and then washed with 10 bed volumes of 150 mM NaCl and 20 mM Tris-HCl (pH 7.4), followed by 10 bed volumes of 20 mM Tris-HCl (pH 7.4). Phosphopeptides were eluted from the column with 4 mM HCl and freeze-dried. The phosphopeptides were dissolved in 0.1% TFA and separated by RP-HPLC. Radioactive phosphopeptide peaks were then purified to homogeneity by RP-HPLC on Vydac C18 or pH-stable (C8) columns (Tarr & Crabb, 1983).

Phosphopeptide sequence analysis

Microsequence analyses were performed with an Applied Biosystems gas-phase sequencer and an on-line phenylthiohydantoin amino acid analyzer (models 470/120/900) using the Applied Biosystems O3RPTH sequencer program and the model 120A program and solvents (Crabb et al., 1986). Analyses were carried out on 70–250 pmol of peptide samples with repetitive yields of 91–93% based on standard samples of 100 pmol β-lactoglobulin. Samples were analyzed for 15 cycles unless otherwise indicated.

Electrospray and tandem mass spectrometry of phosphopeptides

Electrospray mass spectra were recorded on a Sciex API-III triple quadrupole mass spectrometer fitted with a fully articulated ion spray probe and an atmospheric pressure ionization source (Sciex, Ontario, Canada). HPLC fractions containing 50–100 pmol of phosphopeptide were concentrated to dryness on a Speed-Vac centrifuge (Savant Instruments, Farmingdale, New York) and reconstituted in 20 μL of 1:1 methanol/H_2O (v/v), 0.2% in formic acid. Approximately 5 μL of each sample was introduced into the mass spectrometer by flow injection using a Rheodyne model 8125 HPLC injector with a 20-μL sample loop connected to the ion spray probe tip by flexible fused silica tubing (100 μm inside diameter). The mobile

phase (1:1 methanol/H_2O v/v, 0.2% in formic acid) was delivered using an infusion pump (Harvard Apparatus, South Natick, Massachusetts) at a flow rate of 5 μL/min. The mass spectrometer was scanned repetitively over the range *m/z* 100–1,600 with a mass step of 0.2 Da and 2 ms dwell/step (scan duration = 15 s). Resolution was adjusted to ca. 20% valley between adjacent isotope peaks in a singly charged cluster. At this resolution, singly and doubly charged ions were directly identified by the apparent spacing between isotope peaks, and doubly charged ions were distinguished from those with higher charge states.

In tandem mass spectroscopic mode, doubly charged parent ions, $(M + 2H)^{2+}$, were selected with Q1 for collision-induced decomposition with Ar in Q2 of the triple quadrupole. Quadrupole-3 of the triple quadrupole system was scanned from *m/z* 10 to 1,450 for peptide P1, and from *m/z* 10 to 1,600 for peptide P4 with a mass step of 1.0 Da and 10 ms dwell/step. Argon was used as the collision gas with a calculated collision energy of ca. 50 eV. Parent-ion transmission was maximized by reducing the resolution of Q1 to transmit a ca. 2–3 *m/z* window about the selected parent ion. Daughter-ion spectra were obtained from an injection of 5 μL of the remaining samples (2.5–10 pmol/μL).

Acknowledgments

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