Association of a 85-kDa Serine Kinase with Activated Fibroblast Growth Factor Receptor-4*

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Fibroblast growth factors (FGFs) transduce a variety of biological signals via four distinct tyrosine kinase receptors. We have characterized the phosphorylation of FGFR-4 and its association with a putative substrate, p85, using transfected L6 myoblasts. FGFR-4 was phosphorylated in vivo and in vitro mainly on serine and threonine residues in several peptides and to a lower degree on tyrosine residues. When analyzed further by in-gel kinase assay, immunoprecipitates of ligand-activated FGFR-4 contained a serine autophosphorylated polypeptide doublet of 85 kDa. Analysis of the major autophosphorylation site Y754F of FGFR-4 showed that binding of p85 and its serine phosphorylation were independent of receptor autophosphorylation at this site. Okadaic acid treatment increased the basal autophosphorylation activity of p85 but decreased FGFR-4 tyrosine phosphorylation. In contrast, orthovanadate treatment increased the tyrosine phosphorylation of FGFR-4. These data show that a serine kinase is associated with activated FGFR-4 and suggest a role for serine phosphorylation in FGFR-4 function.

Distinct but structurally related receptor tyrosine kinases FGFR-1 to FGFR-4 are responsible for the specificity and redundancy of FGF signaling for eg. mitogenesis, neural differentiation, and inhibition of myoblast differentiation. Alternative splicing of transcripts generates further FGFR variants, which differ in their ligand binding capacities. Stimulation of tyrosine kinase receptors by their ligands leads to activation of the receptors and consequent phosphorylation of specific tyrosyl residues of the cytoplasmic domain. However, FGFR-4 tyrosine autophosphorylation is substantially weaker than that of the other FGF receptors in stimulated cells. In general, tyrosyl phosphorylation of the receptors generates binding sites for cellular substrates containing Src homology 2 (SH2) domains, and these interactions further trigger signal transduction pathways resulting in biological responses. This far only one conserved FGF tyrosyl residue has been identified as a binding site for cellular substrates. This residue, corresponding to Tyr-766 in FGFR-1, has been shown to be a binding site for phospholipase C-γ (PLC-γ).

A number of substrates are known that bind several types of activated receptor tyrosine kinases. These include PLC-γ and docking or adaptor proteins Grb2/Sem-5 and SHC as well as Ras GTPase-activating protein, phosphatidylinositol 3-kinase, and tyrosine phosphatase SYP (for a review, see Ref. 8). It has been shown that after aFGF stimulation of FGFR-1-transfected cells, PLC-γ and SHC are prominently phosphorylated, whereas FGFR-4 stimulation leads to a weak phosphorylation of these signal transducers. Further downstream, Raf-1 and mitogen-activated protein kinases are activated after FGFR-1 stimulation. In contrast, only a weak phosphorylation of Raf-1 and mitogen-activated protein kinases is detected after FGFR-4 activation. While FGFR-1 induced a mitogenic signal in BaF3 cells, no proliferation was obtained after FGFR-4 activation. However, in L6 and U2OSDr1 cells also FGFR-4 activation sufficed for DNA synthesis and proliferation.

In contrast to PLC-γ, several signaling molecules have been shown not to interact with FGF receptors. These include SHC, Grb2, Ras GTPase-activating protein, phosphatidylinositol 3-kinase, and SYP. Recently, it was reported that a novel 89-kDa protein is tyrosine-phosphorylated in FGF-2-stimulated FGFR-1-expressing cells and binds to Grb2. It was suggested that p89 couples activated FGFR-1 to the Ras pathway.

In this report we have characterized the intracellular and in vivo phosphorylation of FGFR-4 and show that this receptor associates with a putative novel 85-kDa polypeptide having serine kinase activity.

EXPERIMENTAL PROCEDURES

Plasmids—Site-directed mutagenesis was carried out using the Altered Sites mutagenesis system (Promega). The EcoRI-HindIII cDNA insert of the expression vector pLTR-FGFR-4 (12) encoding the full-length human FGFR-4 was subcloned to the pALTER vector. The altered cDNAs were then transferred back into the LTRpoly expression vector and confirmed by sequencing.

Cell Culture and Transfections—Transfected L6 rat myoblasts and NIH3T3 cells (5, 12) were grown in Dulbecco modified Eagle’s medium containing 10% calf serum. Cells were transfected using the lipofection method (DOTAP, Boehringer Mannheim), and clones were isolated for wild type (WT) and mutant FGFR-4 were selected as described (5).

Antibodies—Antiserum against FGFR-4 has been described previously (12). Monoclonal antiphosphotyrosine antibodies were obtained from Sigma.

Immunoprecipitation and Immunoblotting—Cells were starved for 2 h in serum-free Dulbecco’s modified Eagle’s medium and then stimulated for 10 min with 50 ng/ml of FGF. When indicated, the cells were treated for 30 min with 200 µM sodium orthovanadate or for 2 h with 1 µM okadaic acid, washed with 20 mE HEPES, pH 7.5, 150 mM NaCl, 1 mM NaVO₄, and lysed on ice for 15 min in immunoprecipitation buffer (20 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM NaVO₄, and 5.5 µg/ml aprotinin). The lysates were centrifuged, and the supernatants were incubated for 2 h at overnight on ice with specific antisera against

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The abbreviations used are: FGFR, fibroblast growth factor receptor; FGF, fibroblast growth factor; PLC-γ, phospholipase C-γ; aFGF, acidic fibroblast growth factor; WT, wild type; PAGE, polyacrylamide gel electrophoresis.
FGFR-4. The immune complexes were then absorbed to protein A-Sepharose CL-4B (Pharmacia Biotech Inc.), washed, and analyzed by SDS-PAGE followed by Western blotting using antibodies against phosphotyrosine or FGFR-4. Before replotting, the filters were treated for 30 min at 50 °C in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) with occasional agitation.

Metabolic Labeling—Cells were labeled for 4–5 h in phosphate-free minimum Eagle's medium, supplemented with 1–3 μCi/ml [32P]PO4. After aFGF stimulation for 10 min, immunoprecipitation was carried out as described. The proteins were resolved in 7.5% SDS-PAGE and blotted onto Hybond-C extra nitrocellulose filter.

In Vitro Kinase Reaction—The immunoprecipitates were performed as described, followed by two washes in kinase buffer. For the kinase reaction, immunoprecipitates were incubated with 1–3 μCi of [γ-32P]ATP at 30 °C for 10 min. The reactions were stopped by boiling in Laemmli buffer and analyzed in a 7.5% SDS-PAGE gel, and the gels or their blotted nitrocellulose replicates were subjected to autoradiography.

Phosphoamino Acid Analysis and Two-dimensional Phosphopeptide Mapping—After autoradiography, the regions of the phosphorylated bands were cut out and soaked in 0.5% polyvinylpyrrolidone K30 in 0.6% acetic acid at 37 °C for 30 min. The pieces of filter were then washed three times with water, 200 μl of freshly made 50 mM NH4HCO3 containing 5 μg/ml of protease K was added, and digestion was carried out at 37 °C overnight. Digestions and washings of the filters were pooled, lyophilized, dissolved in 400 μl of water, lyophilized again, and subjected to partial hydrolysis according to Boyle et al. (13).

For two-dimensional phosphopeptide mapping, the washed filters were incubated overnight in 50 mM NH4HCO3, containing 5 μg/ml sequencing grade trypsin (Promega). The supernatants were dried, oxidized in 50 μl of 50 mM performic acid for 1 h, diluted with 500 μl of water, frozen, dried, dissolved in 50 μl of 50 mM NH4HCO3, sonicated in a water bath for 10 min, and subjected to overnight digestion with 5 μg/ml trypsin. After the second trypsin digestion, 140 μl of electrophoresis buffer was added, and samples were dried and dissolved again in 7 μl of electrophoresis buffer. Two-dimensional cellulose thin layer chromatography analysis was performed according to Boyle et al. (13) using the pH 1.9 buffer for electrophoresis and isobutyric acid buffer for chromatography. The chromatography plates were analyzed in a Fuji BAS 1000 Bio-imager.

Protein Kinase Assay Using Poly(Gly-Tyr) as a Substrate—Kinase immunoprecipitates were incubated in 5 μl of reaction mixture containing 1 mg/ml poly(Glu-Tyr), 3 μM ATP, 0.5 μCi of [γ-32P]ATP, 1 μl of 5 × kinase buffer, and 0.2 μl of H2O. The reaction was stopped after 10 min by adding 4 μl of ATP, 40 μl of EDTA, 20 μCi [32P]PO4, and 2.5 ml bovine serum albumin. Two aliquots were spotted to pieces of Whatman filter paper, which were washed in ice-cold 10% trichloroacetic acid, 8% Na2VO4 for 30 min, 3–4 times for 15 min each in 5% trichloroacetic acid, and twice in ethanol, dried, and subjected to scintillation counting.

In-gel Kinase Assay—In-gel kinase assays were performed basically as described by Hibi et al. (14) with slight modifications. The immunoprecipitates were electrophoresed in 10% SDS-PAGE, and the gel was washed twice for 30 min with 20% isopropanol alcohol, 50 mM HEPES at pH 7.5 to remove SDS. The gel was then washed twice for 30 min with buffer 1 containing 50 mM HEPES at pH 7.5 and 10 mM β-mercaptoethanol and incubated further in 6 mM urea in buffer 1, followed by successive incubations in buffer 1 containing 0.05% TWEEN 20 and 3, 1.5, or 0.75 mM urea for 45 min. After washing with buffer 1 containing 0.05% TWEEN 20 for 2 h, the gel was washed with kinase buffer containing 20 mM HEPES, pH 7.5, and 10 mM MnCl2. The gel was then incubated for 30 min in kinase buffer containing 5 μCi/ml [γ-32P]ATP and washed with 5% trichloroacetic acid and 1% sodium pyrophosphate several times, followed by fixation, drying, and autoradiography. The phosphoamino acid analysis was done as described in Ref. 15.

RESULTS

FGFR-4 is Mainly Serine Phosphorylated in Vivo—In order to analyze the phosphorylation of FGFR-4 in vivo, we carried out two-dimensional phosphopeptide mapping of FGFR-4 immunoprecipitated from transfected, [32P]PO4-labeled L6 cells. Phosphoamino acid analysis showed that the phosphoserine to phosphotyrosine ratio of the ligand-stimulated FGFR-4 was about 20:1. As can be seen from comparison of the in vivo phosphopeptide maps shown in Fig. 1, A and B, aFGF stimulation caused an increased phosphorylation of at least four peptides (a, b, c, and d). In addition, there were two constitutively phosphorylated peptides (c and d). Phosphoserine was the major phosphoamino acid residue obtained when the major peptides c, d, e, and f were analyzed (data not shown). Spot a corresponds to one of the major in vitro autophosphorylation sites of the receptor (Fig. 1D). The phosphorylation of this site was abolished when the tyrosine 754 was mutated to phenylalanine (Fig. 1E).

When cells expressing FGFR-4 were treated with the phosphotyrosyl phosphate inhibitor, 200 μM orthovanadate, for 30 min prior to ligand stimulation, FGFR-4 tyrosyl phosphorylation assessed by anti-Tyr(P) Western blotting was increased 2–3-fold (Fig. 2A, upper panel). This suggested that the weak tyrosyl phosphorylation of FGFR-4 in comparison with serine phosphorylation was due to tyrosyl phosphatase activity. However, when the cells were first treated for 2 h with 1 μM okadaic acid, an inhibitor of protein phosphatase types 1 and 2A, and then stimulated with ligand, tyrosyl phosphorylation of FGFR-4 was decreased (Fig. 2A). Neither orthovanadate nor
Okadaic acid inhibited FGFR-4 kinase activity substantially in vitro (Fig. 2B).

Phosphorylation Pattern of FGFR-4 Wild Type and Kinase-Dead Mutant in Vitro—FGFR-4 phosphorylated by in vitro kinase assay showed a phosphoserine:phosphothreonine:phosphotyrosine ratio of about 2:2:1 (Fig. 3A). To test if the tyrosine kinase activity of FGFR-4 is needed for its serine/threonine phosphorylation in vitro, lysine 503 in FGFR-4, which is critical for ATP binding, was mutated to alanine by site-directed mutagenesis. As can be seen from Fig. 3B, this mutant (K503A), expressed in NIH3T3 or L6 cells, had no kinase activity when poly(Glu-Tyr) was used as the substrate. In vitro kinase assays, the immunoprecipitated WT receptor was prominently phosphorylated, but no phosphorylation of the K503A mutant was detected (Fig. 3A). Thus, the tyrosine kinase catalytic activity of FGFR-4 is required also for its serine/threonine phosphorylation in vitro, suggesting that a serine/threonine kinase coprecipitates with FGFR-4. Our preliminary results indicate that NIH3T3 cells transfected with the K503A mutant show decreased DNA synthesis when stimulated with aFGF, suggesting that the K503A mutant functions as a dominant negative receptor.2

Activated FGFR-4 Associates with a 85-kDa Serine Kinase—The association of FGFR-4 with a putative serine kinase was further analyzed by in-gel kinase assay. Immunoprecipitates of unstimulated and aFGF-stimulated WT and K503A FGFR-4 from L6 cells were electrophoresed in SDS-PAGE, followed by renaturation of the gel and incubation with [γ-32P]ATP. In this analysis, an autophosphorylated doublet of 85 kDa (referred hereafter as p85) was detected in immunoprecipitates of aFGF-stimulated L6 cells expressing WT receptor but not from cells expressing the K503A mutant (Fig. 4A). FGFR-4 was not detected in the in-gel kinase assay, probably because in contrast to many serine/threonine kinases, the activity of tyrosine kinases is easily lost during the assay (16). When the in-gel phosphorylated polypeptides were subjected to phosphoamino acid analysis, autophosphorylation of p85 was detected only on serine residues (Fig. 4B).

As can be seen from Fig. 4C, also FGFR-4 immunoprecipitated from transfected NIH3T3 cells associated with ligand-activated p85 kinase. Similar results were obtained when the previously described phosphorylation mutant Y754F (5) was analyzed. These results show that the association of FGFR-4 and p85 kinase occurs at least in two different cell types and this association is not mediated by the previously described autophosphorylation site, Tyr-754.

Inhibition of Serine Phosphatases Increases p85 Basal Activity—In many cases, phosphorylation is a mechanism to regulate kinase activities. Since p85 was autophosphorylated on serine residues in response to FGFR-4 stimulation, we wanted to see if serine phosphorylation has an effect on its activity. Prior to ligand stimulation, receptor-expressing NIH3T3 cells

2 S. Vainikka, V. Joukov, P. Klint, and K. Alitalo, unpublished results.
were treated with 1 μM okadaic acid and FGFR-4 immunoprecipitates were analyzed for p85 autophosphorylation. As shown in Fig. 5, okadaic acid treatment increased the activity of p85 to the level induced by ligand stimulation. However, the activity of p85 was not further increased by aFGF stimulation in the okadaic acid-treated cells.

**DISCUSSION**

In the present study we have obtained evidence that a serine kinase is associated with FGFR-4. The receptor itself is prominently serine-phosphorylated in vivo and in vitro; a 85-kDa polypeptide having serine kinase activity associates with the receptor, and in vitro autophosphorylation of this p85 protein is induced by FGFR-4 activation. These conclusions are based on in vivo labeling results as well as on in vitro kinase reactions done either directly with FGFR-4 immunocomplexes or after SDS-PAGE.

The low tyrosine/serine phosphorylation ratio of FGFR-4 was altered in favor of phosphotyrosine, when cells were treated with the tyrosine phosphatase inhibitor okadaic acid. Thus, serine phosphatases are at least partially responsible for the weak tyrosine phosphorylation detected. After treatment with the serine phosphatase inhibitor okadaic acid, the tyrosine phosphorylation of FGFR-4 was decreased. However, the receptor was still prominently autophosphorylated in vitro. This suggests that the activity of the tyrosine kinase was not substantially altered and that the decrease of FGFR-4 tyrosine phosphorylation is caused by an inhibition of the turnover of serine phosphorylation.

The in-gel kinase assay showed that FGFR-4 associates with a 85-kDa serine kinase. The autophosphorylation activity of p85 was clearly increased when FGFR-4 was stimulated with aFGF, but no such stimulation was seen associated with the kinase-deficient FGFR-4 mutant. Thus, stimulation of FGFR-4 causes the activation of p85 in the system used. However, when cells were treated with okadaic acid, the activity of p85 was increased to the level seen after aFGF stimulation. This shows that serine phosphorylation also apparently regulates the activity of the p85 kinase. It is thus possible that after aFGF stimulation, serine autophosphorylation or tyrosine phosphorylation by FGFR-4 increases the activity of p85. We have previously shown that a tyrosine-phosphorylated band of 85 kDa is detected in FGFR-4 immunoprecipitates from L6 cells (5). A very weakly labeled polypeptide of similar mobility is also evident in FGFR-4 immunoprecipitates from cells metabolically labeled with [32P]P04. However, without specific antisera or molecular cloning it is not possible to establish whether the tyrosine-phosphorylated 85-kDa band is the p85 serine kinase.

The C-terminal tyrosine residue Tyr-766 has been previously reported as the major in vivo and in vitro phosphorylation site of FGFR-1 and when phosphorylated, it forms the binding site for PLC-γ (7). This tyrosine residue is conserved within the FGF receptor family, and mutation of this site abolishes most of FGFR-4 autophosphorylation and all detectable PLC-γ binding (5). Furthermore, the tyrosine residue Tyr-754 of FGFR-4 is the only reported in vivo phosphorylation site thus far. Therefore, we analyzed if phosphorylation of this site is required for the association of FGFR-4 and p85. The in-gel kinase assay showed that the ligand-stimulated Y754F mutant and WT FGFR-4 induced equally well the autophosphorylation of p85, indicating that phosphorylation of Tyr-754 is not critical for the association. This suggests that the association involves some other tyrosine residue or is mediated by mechanisms where increased receptor tyrosine phosphorylation is not critical. The hypothesis that tyrosine phosphorylation of FGFR-4 is not critical for its association with p85 is further supported by the result that p85 activity was detected in immunoprecipitates of unstimulated FGFR-4 after okadaic acid treatment.

It has been reported that FGFR-1 activation leads to tyrosine phosphorylation of a 89-kDa protein and to complex formation between this p89 and the Grb2 docking protein (11). p89 and p85 reported here are distinct proteins, since no autophosphorylation activity was associated with p89. It has also been recently shown that a serine/threonine kinase, protein kinase B/Rac, is activated upon basic FGF stimulation in Rat-1 cells. However, it was concluded that phosphatidylinositol 3-kinase was essential for protein kinase B activation by platelet-derived growth factor and insulin (17, 18). The present results suggest that the differential signal transduction mechanisms of FGFR-4 involve a serine kinase differing from protein kinase B in molecular weight and properties. The characterization of this kinase will be an important task for further studies.

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**FIG. 5. OKADAIC ACID INCREASES THE BASEAL ACTIVITY OF p85.** FGFR-4 was immunoprecipitated from untreated or okadaic acid-treated NIH3T3 cells. The activity of p85 in the immunocomplexes was then measured by in-gel kinase assay. IP, immunoprecipitate.