Regulation of Ephexin1, a Guanine Nucleotide Exchange Factor of Rho Family GTPases, by Fibroblast Growth Factor Receptor-mediated Tyrosine Phosphorylation*

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Fibroblast growth factor (FGF) signal is implicated in not only cell proliferation, but cell migration and morphological changes. Several different Rho family GTPases downstream of the Ras/ERK pathway are postulated to mediate the latter functions. However, none have been recognized to be directly coupled to FGF receptors (FGFRs). We have previously reported that EphA4 and FGFRs hetero-oligomerize through their cytoplasmic domains, trans-activate each other, and transduce a signal for cell proliferation through a docking protein, FRS2α (Yokote, H., Fujita, K., Jing, X., Sawada, T., Liang, S., Yao, L., Yan, X., Zhang, Y., Schlessinger, J., and Sakaguchi, K. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 18866–18871). Here, we have found that ephexin1, a guanine nucleotide exchange factor for Rho family GTPases, constitutes another downstream component of the receptor complex. Ephexin1 directly binds to the kinase domain of FGFR mainly through its DH and PH domains. The binding appears to become weaker and limited to the DH domain when FGFRs become activated. FGFR-mediated phosphorylation of ephexin1 enhances the guanine nucleotide exchange activity toward RhoA without affecting the activity to Rac1 or Cdc42. The FGFR-mediated tyrosine phosphorylation includes, but is not limited to, the residue (Tyr-87) phosphorylated by Src family kinase, which is known to be activated following EphA4 activation. The Tyr-to-Asp mutations that mimic the tyrosine phosphorylation in some of the putative FGFR-mediated phosphorylation sites increase the nucleotide exchange activity for RhoA without changing the activity for Rac1 or Cdc42. From these results, we conclude that ephexin1 is located immediately downstream of the EphA4-FGFR complex and the function is altered by the FGFR-mediated tyrosine phosphorylation at multiple sites.

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2 The abbreviations used are: FGF, fibroblast growth factor; GEF, guanine nucleotide exchange factor; SFK, Src family kinase; WT, wild type; MBP, maltose-binding protein; GST, glutathione S-transferase; EGFP, enhanced green fluorescent protein; HEK293, human embryonic kidney 293; DMEM, Dulbecco’s minimal essential medium; HA, hemagglutinin; KD, kinase-dead; KA, kinase-active; ERK, extracellular signal-regulated kinase.
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lation, ephexin1 activates RhoA, Rac1, and Cdc42, thus leading to a balanced GTPase activation that promotes axonal outgrowth. In contrast, stimulation of Ephs by ephrin induces Src family kinases (SFK)-dependent phosphorylation of ephexin1 on an evolutionarily conserved tyrosine (Tyr-87). This tyrosine phosphorylation of ephexin1 enhances the ephexin1’s GDP/GTP exchange activity specifically toward RhoA relative to Rac1 and Cdc42, thereby changing the local balance of Rho GTPases activity within cells, leading to actin cytoskeletal changes that result in growth cone collapse (8). As ephexin1 activates Rho-family GTPases that are implicated in both attraction and repulsion, the guanine nucleotide exchange activity of ephexin1 might be controlled through interactions with additional proteins. Ephexin1 may function in combination with other factors to stimulate Rac1 or Cdc42, whereas in a complex with active Eph receptors it preferentially activates RhoA (9).

In this study, we report a physical interaction between ephexin1 and FGFRs. When ephexin1 was co-expressed with FGFRs in HEK293T cells, ephexin1 was co-immunoprecipitated with FGFRs and became tyrosine-phosphorylated by the activated receptors. In vitro binding studies using a series of deletion mutants of ephexin1 revealed that ephexin1 binds to the cytoplasmic domain of activated FGFR2 mainly through its DH domain. Similar binding studies using a series of FGFR2 deletion mutants showed that the second kinase domain of FGFR2 is the preferential binding site for ephexin1. We further examined the FGFR-induced tyrosine phosphorylation sites of ephexin1 through screening ephexin1 mutants that carry Tyr-to-Phe changes in several different loci. FGFR-induced tyrosine phosphorylation of ephexin1 appears to include multiple tyrosine residues. These results suggest that FGFRs are involved in the regulation of ephexin1 activity, which in turn regulates cytoskeletal reorganization at growth cones.

EXPERIMENTAL PROCEDURES

Reagents—The following are the antibodies used in the current study: mouse anti-HA monoclonal antibody 12CA5 (Roche Applied Science); mouse anti-phosphotyrosine antibody, clone 4G10 (Upstate); rabbit anti-FGFR2 polyclonal antibody (Santa Cruz Biotechnology); mouse anti-XP (Xpress monoclonal antibody (Invitrogen); rabbit anti-phospho-Src family (Tyr416) polyclonal antibody (Cell Signaling); rabbit anti-c-Src (SRC2) polyclonal antibody (Santa Cruz Biotechnology); mouse anti-phospho-Src family antibody, clone 4G10 (Upstate); rabbit anti-FGFR2 polyclonal antibody (Invitrogen); mouse anti-phosphotyrosine antibody, clone Y413 and Y7 (Cell Signaling); rabbit anti-FGFR1 polyclonal antibody against the N-terminal region of ephexin1 (1–373 amino acids) (Santa Cruz Biotechnology); rabbit polyclonal antibody against ephexin1 was raised in our laboratory. Unless indicated otherwise, all other materials were purchased from Sigma-Aldrich.

Cell Culture—HEK293T cells were maintained in Dulbecco’s minimal essential medium (DMEM, Sigma) with 10% fetal bovine serum (Equitech-Bio) supplemented with 1% penicillin and streptomycin (Amersham Biosciences) at 37 °C and 5% CO2. Rat L6 myoblasts were maintained in the same condition. PC12 cells were maintained in RPMI1640 (Sigma) containing 10% horse serum (Amersham Biosciences) and 5% fetal bovine serum supplemented with 1% penicillin and streptomycin. For morphological examination, culture plates were treated with 2 μg/ml fibronectin at 37 °C for 1 h, and washed with phosphate-buffered saline before plating cells.

For treatment with growth factors and other chemicals, HEK293T cells, 48 h after transfection, were serum-deprived for 5 h and then incubated with SU5402 or PP2 (Calbiochem) at indicated concentrations for 2 h at 37 °C in 5% CO2 prior to FGFR2 stimulation. L6 cells stably expressing FGFR2 and/or ephexin1 were kept in DMEM containing 0.5% fetal bovine serum for more than 16 h for serum starvation and incubated with FGFR2 or kinase inhibitors. Ephrin-A1-Fc was used after oligomerization as described previously (3).

DNA Constructs and Mutagenesis—The wild type (WT) and mutants of Epha4 and FGFRs were constructed as reported (3). Full-length cDNA of mouse ephexin1 was prepared by reverse transcription-PCR using total RNA from a mouse brain as template. They were subsequently subcloned into pCR®-BluntII-ToPO® vector (Invitrogen). Ephexin1 mutants were prepared by applying the recombinant PCR method or site-directed mutagenesis using WT constructs as templates and a pair of appropriate mutation primers. cDNAs for Rho family GTPases were derived from the constructs previously reported (10). The sequences of all of the PCR-amplified DNAs were confirmed by sequencing after cloning into a pCR-BluntII-TOPO cloning vector according to the manufacturer’s instructions (Invitrogen). For eukaryotic transient expression, all of the DNA constructs were incorporated into a pcDNA3.1 plasmid vector, into which a Myc, FLAG, or hemagglutinin (HA) epitope-encoding sequence was integrated at the 3’ side in-frame with the coding sequences of the incorporated cDNAs. For bacterial expression, the full-length and mutant cDNAs were incorporated into a pMalC2 vector (New England Biolabs), which produces a protein N-terminally fused with maltose-binding protein (MBP), into a pGEX4T (Amersham Biosciences) expression vector that produces a protein N-terminally fused with glutathione S-transferase (GST), or a pBAD/His vector (Invitrogen) or a pTrcHisA (Invitrogen), which produces a protein fused with an Xpress epitope and six His residues at the N terminus.

The sequences for various deletion mutants and point mutants are as follows (refer to the maps in Figs. 6 and 7): ephexin1 of the far N-terminal region (N-GEF, amino acids 67–186); the N-terminal DH domain (N-DH, amino acids 187–276); the C-terminal DH domain (C-DH, amino acids 277–366); the DH plus PH domains (DH-PH, amino acids 326–446); the PH domain (PH, amino acids 400–506); the PH plus SH3 domains (PH-SH3, amino acids 447–551); the SH3 domain (SH3, amino acids 525–581); and the SH3 domain plus the far C terminus region (SH3-CT, amino acids 552–620); FGFR2 (ΔK1) region (amino acids 580–821); FGFR2 (juxtamembrane-kinase1 (JM-K1) region (amino acids 400–579); FGFR2 (JM-K2) region (amino acids 400–708); FGFR2 (K2-CT (K2-tga)) region (amino acids 709–821), C-terminal (CT) region (amino acids 757–821); FGFR3 (JM1) (amino acids 392–572); FGFR3 (JM-K) (amino acids 392–693); FGFR3 (ΔK1) (amino acids 565–800); FGFR3 (K2-tga) (amino acids 694–800); and FGFR3 (CT) (amino acids 743–800).

The M series of the point mutants of ephexin1 were synthesized by changing tyrosine residues to phenylalanine. These mutants are named as ephexin1(M1) (Y87F and Y90F),
Expression and Purification of Recombinant Proteins—Fusion proteins were expressed in TOP10 bacteria and purified by amylose or glutathione affinity chromatography for the PH domains of ephexin1 and the deletion mutants of the FGFR cytoplasmic domain. The purification of recombinant proteins, the proteins expressed in TOP10 bacteria were purified using the BD-Talon cobalt-based affinity chromatography resin (BD Biosciences) according to the manufacturer’s instruction. The lysis buffer contained 20 mM Tris-HCl buffer, pH 7.5, 100 mM NaCl, 1% Triton X-100, 5% glycerol, 1 mM sodium orthovanadate, and protease inhibitors.

In Vitro Protein-Protein Interaction Assay—To determine the interaction between the ephexin1 deletion mutants and the FGFR cytoplasmic domain in vitro, 2 µg of MBP-tagged ephexin1 deletion mutants were pre-arrayed to amylose agarose at 4 °C for 60 min. The Xpress-tagged FGFR cytoplasmic domain was then added, and the mixture was incubated at 4 °C overnight. Finally, beads were washed three times with a wash buffer (50 mM HEPES and 50 mM NaCl) at 4 °C, then dissolved in 10 ml of ice-cold PBS. The protein was transferred to a polyvinylidene fluoride membrane and immunoblotted with an anti-Xpress antibody. The binding assay between the DH plus PH domains of ephexin1 and the deletion mutants of the FGFR cytoplasmic domain, ephexin1 and FGFR molecules were tagged with Xpress and MBP, respectively.

RESULTS

FGFR-mediated Ephexin1 Regulation

Ephexin1 is known to bind to EphA4, and the function of the GEF becomes modulated by EphA4 through SFK-mediated phosphorylation (7, 8, 13). We have confirmed that the ephexin1 binds to EphA4 and is phosphorylated in an EphA4 dose-dependent fashion (Fig. 1A). Because we have recently reported that EphA4 and FGFR interact with and cross-phosphorylate each other (3), we initiated studies to investigate the function of FGFR in regulating the ephexin1 function. When EphA4 was overexpressed and stimulated by its ligand, ephrin-A1, ephexin1 was phosphorylated over the basal level. Addition of SU5402, an FGFR kinase inhibitor, inhibited the phosphorylation levels of both the overexpressed EphA4 and the endoge-
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![Image](356x26 to 383x38)

**FIGURE 1.** SU5402 inhibits EphA4-mediated tyrosine phosphorylation of ephexin1. A, tyrosine phosphorylation of ephexin1 by EphA4 activation. Increasing doses (0, 1, 2, and 4 μg per 6-cm plate) of pcDNA3.1/EphA4-Myc were co-transfected with pcDNA3.1/ephexin1-HA (2 μg/plate) into 293T cells. B, inhibition of EphA4-mediated tyrosine phosphorylation of ephexin1 by an FGFR inhibitor, SU5402. Ephexin1 was co-transfected with EphA4 into HEK293T cells. Cells were pretreated with indicated concentrations of SU5402 for 1 h before 1-h treatment with ephrin-A1 (0.5 μg/ml) in the presence of SU5402. Immunoprecipitation (IP) and immunoblotting (IB) were performed using the antibodies indicated. FGFR2 and Src family kinases (SFK) are endogenous molecules.

FGFR2 is the driving force of ephexin1 phosphorylation in the presence of FGFR2, EphA4, and ephexin1. When FGFR2 was overexpressed in L6 cells, which barely express intrinsic FGFRs (3, 14), together with ephexin1, ephexin1 was co-immunoprecipitated with FGFR2 and was phosphorylated with a peak at 60 min or later following stimulation with FGF2 (Fig. 3, A and B). Therefore, we used the time point between 30 and 60 min for evaluation of the ligand-mediated ephexin1 phosphorylation.

We further examined in HEK293T cells the effect of FGFR2 on phosphorylation of ephexin1 in the presence and absence of SU5402 following overexpression of both molecules and stimulation with FGF2. SU5402 effectively inhibited phosphorylation of FGFR2, and almost completely suppressed phosphorylation of ephexin1 at concentrations as low as 5 μM. SFK phosphorylation at Tyr-416 was not altered (Fig. 4A). Because SFK is reported to be involved in the phosphorylation of ephexin1 when stimulated by ephrin (8, 13), we used an SFK inhibitor PP2 and its non-functional control chemical PP3 in place of SU5402 to study the effect of SFK in the FGFR2-mediated phosphorylation of ephexin1. SFK phosphorylation was slightly inhibited by PP2 at 2.5 μM or higher concentrations. PP2 barely inhibited the phosphorylation of ephexin1 at concentrations up to 2.5 μM, whereas 2.5 μM PP2 clearly inhibited phosphorylation of FGFR2 and PP2 at 5 μM further inhibited phosphorylation of both FGFR2 and ephexin1 (Fig. 4B). PP2 at 10 μM inhibited phosphorylation of overexpressed FGFR2 (without FGF2 stimulation) and completely suppressed phosphorylation of ephexin1 in HEK293T cells, whereas the phosphorylation level of SFK Tyr-416 was only slightly reduced (Fig. 4C). Taken together, FGFR2 phosphorylates ephexin1 independently of SFK activation, which is reported to be required for the effect of EphA4 on ephexin1 (8, 13).

To clarify the function of PP2 on ephexin1 phosphorylation in cells other than HEK293T cells, we used L6 cells stably expressing exogenous FGFR2 and ephexin1. Under the presence of PP2 or PP3 and a phosphatase inhibitor, orthovanadate (see Fig. 9A), cells were stimulated with FGF2 (100 ng/ml) for 1 h. PP2 clearly inhibited the phosphorylation levels of both FGFR2 and ephexin1 simultaneously in a dose-dependent fashion at concentrations between 0.1 and 20 μM (only the 2.5–20 μM range is shown in the Fig. 5A). On the other hand, the phosphorylation level of SFK at Tyr-416 was suppressed to a certain level by low concentrations of PP2 and stayed at the constant low level (Fig. 5A). PP3 did not change the phosphorylation levels of ephexin1, SFK, or FGFR2. These findings suggest that PP2 is a potent kinase inhibitor of both FGFR2 and SFK and that the ligand-activated FGFR2 is directly involved in the phosphorylation of ephexin1.

Previous studies showed that the function of EphA4 kinase in the phosphorylation of ephexin1 is to bring ephexin1 close to SFK so that SFK becomes able to phosphorylate the RhoGEF (8, 13). Both SFK and ephexin1 are known to bind to EphA4. To study whether EphA4 can directly phosphorylate ephexin1 in the absence of FGFRs, we used L6 cells expressing exogenous ephexin1 (L6/ephexin1-HA). L6 cells are known to express endogenous EphA4 (3) but do not express detectable amounts of ephexin1 or FGFRs. Exposure of L6/ephexin1-HA to 0.5 μg/ml oligomerized ephrin-A1 in the presence and absence of
orthovanadate or PP2 activated EphA4 as shown in Fig. 5B, whereas any such treatments did not phosphorylate ephexin1. These findings suggest that the ligand-mediated EphA4 activation, which is reported to be followed by SFK activation in other systems, does not induce ephexin1 phosphorylation in the absence of FGFRs, supporting the notion that FGF receptors play a major role in the phosphorylation of ephexin1.

**Determination of Binding Domains of Ephexin1 and FGFR**—We next studied the interaction between FGFR2 and ephexin1 in an *in vitro* binding assay. The binding site of ephexin1 to FGFR2 was determined to be the second kinase domain by studying the binding of Xpress-tagged ephexin1(DH plus PH) (the domain containing DH and PH) with various MBP-tagged deletion mutants of the FGFR2 cytoplasmic domain (Fig. 6A). The region of ephexin1 responsible for binding to FGFR2 was determined by examining the binding of MBP-tagged ephexin1 deletion mutants to the Xpress-tagged cytoplasmic domain of the WT and the kinase-defective (KD) mutant (K517M) of FGFR2. Ephexin1 bound to FGFR2(KD) via the N-terminal DH domain and the PH domain and to FGFR2(WT) via a confined region of the N-terminal DH domain (Fig. 6B). We also examined FGFR3 in place of FGFR2.
using the similar binding studies. FGFR3 bound to ephxin1 through its mid-cytoplasmic region encompassing the first and second kinase domains (Fig. 7A). Ephxin1 bound to the cytoplasmic domain of FGFR3(WT) and FGFR3(KD) (N540K) through a rather broad area comprising the C-terminal DH domain, PH domain, and SH3 domain, whereas it bound to a kinase-activated (KA) mutant (K650E) of FGFR3 through a rather broad area comprising the C-terminal DH domain, PH domain, and SH3 domain, whereas it bound to a kinase-defective (KD) FGFR3 mutant (FGFR3(KS17M)) or FGFR2(WT) was examined in vitro. The major binding region to FGFR2(KD) is marked by clear rectangles, and the binding region to FGFR2(WT) is marked by a dark rectangle. One-fortieth of the amount of the recombinant peptides used for the binding study was fractionated using a separate gel and stained with Coomasie Brilliant Blue to show that the peptides used in the similar amount. Cell lysates were immunoprecipitated and immunoblotted with the indicated antibodies. SFK is the endogenous molecule expressed in L6 cells. Control cells were not treated with FGFR2, PP2, or PP3. B. No EphA4-dependent ephxin1 phosphorylation was noted from methionine encoded by the initiation codon, are noted following the names of the fragments.

FIGURE 5. FGFR-dependent phosphorylation of ephxin1. A, SFK-independent ephxin1 phosphorylation following the ligand-mediated FGFR2 activation. L6 cells stably expressing FGFR2 and ephxin1 were starved in DMEM containing 0.5% serum for 20 h. Cells were pretreated with serial dilutions of PP2 or its control reagent, PP3, for 2 h, and stimulated with FGF2 (100 ng/ml) for 1 h in the presence of 0.2 mM sodium orthovanadate. PP2 or PP3 was incubated with the cells throughout the stimulation. Cell lysates were immunoprecipitated and immunoblotted with the indicated antibodies. SFK is the endogenous molecule expressed in L6 cells. L6 cells treated with 0.5 μM PP2 for 2 h and treated with 0.5 μg/ml ephrin-A1 for 1 h in the presence or absence of 0.2 mM sodium orthovanadate. Cell lysates were immunoblotted with the indicated antibodies following immunoprecipitation with the antibodies shown. EphA4 is the endogenous molecule expressed in L6 cells.

FIGURE 6. Mapping of the interaction domains between FGFR2 and ephxin1 in an in vitro binding assay. A, the ephxin1 interaction region in FGFR2. MBP-fused FGFR2 deletion mutants were synthesized as recombinant proteins, and their binding with ephxin1 (DH plus PH domains) was examined in vitro. The major ephxin1-binding region is marked by a rectangle. B, the FGFR2 binding region in ephxin1. MBP-fused ephxin1 deletion mutants were synthesized, and their binding with the kinase-defective (KD) FGFR2 mutant (FGFR2(KS17M)) or FGFR2(WT) was examined in vitro. The major binding region to FGFR2(KD) is marked by clear rectangles, and the binding region to FGFR2(WT) is marked by a dark rectangle. One-fortieth of the amount of the recombinant peptides used for the binding study was fractionated using a separate gel and stained with Coomasie Brilliant Blue to show that the peptides used in the similar amount. Stained bands of different molecular masses were put in alignment for easy comparison. Amino acid (aa) numbers of the fragments' both ends, which are counted from methionine encoded by the initiation codon, are noted following the names of the fragments.

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A

B

using the similar binding studies. FGFR3 bound to ephxin1 through its mid-cytoplasmic region encompassing the first and second kinase domains (Fig. 7). Ephxin1 bound to the cytoplasmic domain of FGFR3(WT) and FGFR3(KD) (N540K) through a rather broad area comprising the C-terminal DH domain, PH domain, and SH3 domain, whereas it bound to a kinase-activated (KA) mutant (K650E) of FGFR3 through a confined N-terminal DH domain area (Fig. 7B). Taken together, these findings suggest that ephxin1 binds to the cytoplasmic domain of FGFR and that the kinase-activated FGFR causes ephxin1 to make a conformation that allows it to bind orthovanadate, a phosphatase inhibitor, together with FGFR2 for 60 min. L6 cells expressing ephxin1 alone did not show any phosphorylation of ephxin1 even in the presence of FGFR2 (data not shown), and L6/FGFR2 plus ephxin1 showed barely detectable phosphorylation in the absence of FGFR2. L6/FGFR2 plus ephxin1 cells showed evidential ephxin1 phosphorylation after stimulation by FGFR2 and enhanced phosphorylation in the presence of FGFR2 and orthovanadate. The presence of orthovanadate alone moderately increased the ephxin1 phosphorylation level. SFK activity did not change with these treatments. These findings suggest that L6 cells contain a strong phosphatase system that reverses the

EphA4 is reported to induce phosphorylation of one of the tyrosines (Tyr-87) mutated in M1 through activation of SFK, which causes ephxin1 to increase its guanine nucleotide exchange activity toward RhoA without changing the activity to Rac1 or Cdc42 (8). To study which tyrosines are phosphorylated by FGFR, we constructed ephxin1 mutants (M1–M9) in a pcDNA vector that carry Tyr-to-Phe mutations along the ephxin1 molecule as described in Fig. 8A. Co-expression of these mutants with FGFR2 revealed that some mutants had a low level of phosphorylation as compared with the WT: M1, M4, M6, M7, M8, and M9 had reduced phosphorylation (Fig. 8B). These findings suggest that the tyrosine residues replaced with phenylalanine in M1, M4, M6, M7, M8, and M9 are the targets of FGFR kinase and that the function of ephxin1 phosphorylated by FGFR might be changed as in the case of phosphorylation by SFK.

Ephxin1 Phosphorylated by FGFR Enhances the Guanine Nucleotide Exchange Activity on RhoA but Not on Rac1 or Cdc42—We then examined the function of ephxin1 phosphorylated by FGFR in L6 cells that stably express exogenous FGFR2 and ephxin1 (L6/FGF2 plus ephxin1) (Fig. 9). Ephxin1 phosphorylation levels were first examined using immunoblotting of the cell lysates derived from L6 cells treated differently (Fig. 9A). Because FGFR-mediated phosphorylation of ephxin1 in L6 cells was difficult to detect, we also prepared cells incubated with sodium orthovanadate, a phosphatase inhibitor, together with FGFR2 for 60 min. L6 cells expressing ephxin1 alone did not show any phosphorylation of ephxin1 even in the presence of FGFR2 (data not shown), and L6/FGFR2 plus ephxin1 showed barely detectable phosphorylation in the absence of FGFR2. L6/FGFR2 plus ephxin1 cells showed evidential ephxin1 phosphorylation after stimulation by FGFR2 and enhanced phosphorylation in the presence of FGFR2 and orthovanadate. The presence of orthovanadate alone moderately increased the ephxin1 phosphorylation level. SFK activity did not change with these treatments. These findings suggest that L6 cells contain a strong phosphatase system that reverses the
ephexin1 phosphorylation and that SFK is not involved in the ephexin1 phosphorylation by FGFR. These results are consistent with the findings described above (Fig. 5).

Using the cell lysates taken from L6/FGFR2 plus ephexin1 treated as above, we examined the GDP/GTP exchange activity on RhoA family GTPases. Cell lysates prepared from the cells incubated with FGF2 alone or with both FGF2 and orthovanadate showed increased GDP/GTP exchange activity on RhoA, and treatment with orthovanadate alone induced similar activity (Fig. 9, B–D). The phosphorylated ephexin1 did not exhibit any change in the GDP/GTP exchange activity on either Rac1 or Cdc42 as compared with the non-phosphorylated ephexin1. To examine whether the differential activation of guanine nucleotide exchange activity is caused by ephexin1 phosphorylation, we also studied the exchange activity in L6 cells expressing FGFR2 alone (L6/FGFR2). Treatment of L6/FGFR2 with FGF2 in the presence or absence of orthovanadate did not induce any activation of Rho family GTPases as compared with the non-treated control, suggesting that the RhoA activation in L6/FGFR2 plus ephexin1 is attributed to the effect on ephexin1.

To clarify the function of ephexin1 phosphorylated at specific tyrosines, we constructed mutants simulating the phosphorylation by replacing tyrosine residues with aspartic acid as reported (15) at loci 1, 4, and 8 of Fig. 8A and designated them A1, A4, and A8. These phosphorylation-simulating mutants were expressed in L6 cells at a similar level (Fig. 10A), and the GDP/GTP exchange activities on Rho family GTPases were examined using the same protein amount of cell lysates (Fig. 10, B–D). Cell lysates from L6 cells expressing A1 and A4 had an increased GDP/GTP exchange activity on RhoA as compared with those from the cells expressing WT or A8. The same assay using these lysates on Rac1 or Cdc42 did not show any significant changes over the control. We then expressed these ephexin1 mutants in PC12 cells, which naturally extend neurites using a retrovirus vector co-expressing EGFP (Fig. 10E). Mutants A1 and A4 reduced the neurite extension. Especially the cells expressing A1 became flattened and round without any neurites. Expression of EGFP alone, WT, or A8 did not cause any change in the cell morphology. These findings strongly support the results of the nucleotide exchange assay, wherein the FGFR-mediated phosphorylation of some tyrosines augments the guanine nucleotide exchange activity of ephexin1 on RhoA without affecting the activity on Cdc42 or Rac1.

DISCUSSION

We have shown here that ephexin1 is phosphorylated by FGFR in addition to the reported SFK-mediated phosphoryla-
tion that is induced by EphA4 activation (8, 13). The current finding is consistent with our previous report that EphA4 and FGFR hetero-oligomerize and cross-phosphorylate each other through direct interactions between their cytoplasmic domains (3). We previously focused on the activation of FRS2-mediated signal transductions. In this report we have shown that a GEF for Rho family GTPases, ephexin1, is immediately downstream of the hetero-oligomer consisting of FGFR and EphA4. Non-phosphorylated ephexin1 has a balanced guanine nucleotide exchange activity toward all the members of Rho family GTPases as reported (8). The FGFR-phosphorylated ephexin1, on the other hand, has an enhanced guanine nucleotide exchange activity toward RhoA, which is similar to the reported function of the EphA4-dependent SFK-phosphorylated ephexin1. SFK is not likely to be involved in the FGFR-mediated phosphorylation. PP2, an inhibitor of SFK, surprisingly suppressed FGFR phosphorylation substantially as compared with the moderate suppression of SFK phosphorylation at Tyr-416. This might be a cell-type-specific phenomenon, but we obtained similar results in both L6 and HEK293T cells. SU5402, a specific FGFR inhibitor, showed a drastic suppression of ephexin1 phosphorylation together with a clear suppression of the ligand-induced FGFR2 phosphorylation. These findings suggest that SFK might be a predominant kinase that phosphorylates ephexin1 in cells with no FGFR expression. However, in the cells that co-express ephexin1 and FGFR, FGFR is the major player in ephexin1 phosphorylation.

FGFR has long been implicated in the extension of neurites and axons, formation of growth cones, and target recognition. Studies in neuronal cells of the developing Xenopus visual system indicate that FGFR plays a role in axon extension and target recognition of retinal ganglion cells (16, 17). In addition, posterior protrusion of the early Xenopus embryo appears to be induced by EphA4 activation through FGF signaling (18). These findings suggest the presence of signal transduction pathways leading to activation of Rho family GTPases in the downstream of FGF signal. One of the GEFs located downstream of FGFR in PC12 cells is p85 βPIX, which has a specificity for Rac1. p85 βPIX is activated after phosphorylation at Ser and Thr residues by p21-activated kinase, which is located downstream of the Ras/ERK pathway (19, 20). We have shown here for the first time that a Rho family GEF, ephexin1, directly binds to FGFR and is functionally modulated by FGFR-mediated tyrosine phosphorylation.

The region of FGFR binding to ephexin1 was a rather broad area encompassing the kinase domains. On the other hand, the region of ephexin1 that binds to the kinase-inactive FGFR appears to include DH, PH, and SH3 domains, whereas the kinase-active cytoplasmic domain of FGFR binds weakly to a restricted N-terminal DH domain. As such, the way of ephexin1 binding to FGFR is different from that to EphA4, in which ephexin1 constitutively binds regardless of the activation state of EphA4. Furthermore, the ephexin1 phosphorylation appears to be transient, probably due to the presence of phosphatases. These findings might have important implications that...
ephexin1 is released from FGFR after phosphorylation and that ephexin1 activity is tightly modulated by kinases and phosphatases in a cell. Rho family GTPases are important switch molecules that regulate the way of actin polymerization. RhoA is shown to be involved in stress fiber formation and growth cone collapse, and Rac1 and Cdc42 stimulate neurite outgrowth through formation of lamellipodia and filopodia, respectively. It is conceivable that stress fiber formation coordinated with lamellipodia or filopodia formation is required for completing neurite outgrowth. FGFR-mediated ephexin1 phosphorylation leading to a transient activation of RhoA relative to Rac1 or Cdc42 is probably one of the important steps in the extension of neurites or axons.

From the mutation studies we have specified phosphorylation sites along the ephexin1 molecule that include not only the tyrosine (Tyr-87) targeted by SFK but other tyrosine residues. The FGFR-mediated phosphorylations of ephexin1 caused RhoA activation without affecting the activity of Rac1 or Cdc42, which is similar to the function of EphA4-dependent SFK-mediated phosphorylation. The FGF-FGFR signaling is regarded as one of the long-range diffusion-mediated systems, and controls gene expression of multiple inducers for embryonic development. In addition to the long range systems, short range contact-mediated systems are also required to complete the organ development. The ephrin-Eph system is one of the short range signals that coordinate with the long range signals (21). Thus, the duplicate regulation of the ephexin1 activity by both EphA and FGFR is a typical example that coordinates embryonic morphogenesis by integrating these two distinct signals.

In summary, we have found that ephexin1 directly binds to and is phosphorylated by FGFR and that ephexin1 activity is tightly modulated by kinases and phosphatases in a cell. Rho family GTPases are important switch molecules that regulate the way of actin polymerization. RhoA is shown to be involved in stress fiber formation and growth cone collapse, and Rac1 and Cdc42 stimulate neurite outgrowth through formation of lamellipodia and filopodia, respectively. It is conceivable that stress fiber formation coordinated with lamellipodia or filopodia formation is required for completing neurite outgrowth. FGFR-mediated ephexin1 phosphorylation leading to a transient activation of RhoA relative to Rac1 or Cdc42 is probably one of the important steps in the extension of neurites or axons.

From the mutation studies we have specified phosphorylation sites along the ephexin1 molecule that include not only the tyrosine (Tyr-87) targeted by SFK but other tyrosine residues. The FGFR-mediated phosphorylations of ephexin1 caused RhoA activation without affecting the activity of Rac1 or Cdc42, which is similar to the function of EphA4-dependent SFK-mediated phosphorylation. The FGF-FGFR signaling is regarded as one of the long-range diffusion-mediated systems, and controls gene expression of multiple inducers for embryonic development. In addition to the long range systems, short range contact-mediated systems are also required to complete the organ development. The ephrin-Eph system is one of the short range signals that coordinate with the long range signals (21). Thus, the duplicate regulation of the ephexin1 activity by both EphA and FGFR is a typical example that coordinates embryonic morphogenesis by integrating these two distinct signals.

In summary, we have found that ephexin1 directly binds to and is phosphorylated by FGFR and that the FGFR-mediated tyrosine phosphorylation sites in ephexin1 are multiple, including the Tyr-87 site that is reported to be phosphorylated by SFK following EphA activation. Some of these phosphorylations appear to increase the guanine nucleotide exchange activity on RhoA without changing the activity on Rac1 or Cdc42. We conclude that the EphA-FGFR interaction is likely to play an important role not only in cell proliferation.
as we reported earlier (3) but in migration and morphological changes.

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