Trans-activation of EphA4 and FGF receptors mediated by direct interactions between their cytoplasmic domains

Hideyuki Yokote*, Koji Fujita*, Xuefeng Jing†, Takahiro Sawada†, Sitai Liang†, Li Yao*, Xiaomei Yan*, Yueqiang Zhang*, Joseph Schlessinger‡§, and Kazushige Sakaguchi*§

*Department of Molecular Cell Biology, Institute of Advanced Medicine, Wakayama Medical University, 811-1 Kimiidera, Wakayama 641-8509, Japan; and †Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06520

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A yeast two-hybrid analysis has shown that the juxtamembrane region of FGF receptor 3 (FGFR3) interacts with the cytoplasmic domain of EphA4, which is a member of the largest family of receptor tyrosine kinases. Complex formation between the two receptors was shown to be mediated by direct interactions between the juxtamembrane domain of FGFR1, FGFR2, FGFR3, or FGFR4 and the N-terminal portion of the tyrosine kinase domain of EphA4. Activation of FGFR1 in transfected cells resulted in tyrosine phosphorylation of a kinase-negative EphA4 mutant and activation of EphA4 led to tyrosine phosphorylation of a kinase-negative FGFR1 mutant. Moreover, both receptors stimulate tyrosine phosphorylation of the docking protein FRS2α and induce mitogen-activated protein kinase stimulation with a time course and intensity that depends on the ligand that is applied. We also demonstrate that FGF-receptor-mediated mitogen-activated protein kinase stimulation is potentiated in cells coexpressed with ephrin-A1. The direct interaction between EphA4 and FGFRs and the potentiation of FG response that is induced by ephrin-A1 stimulation may modulate the biochemical responses that are mediated by these receptor families in cells or tissues in which the two receptors are coexpressed.

The 22 members of the FGF family act in concert with heparan sulfate proteoglycan to mediate a great variety of cellular responses by binding to and activating four receptor tyrosine kinases (RTKs), which are designated FGFR1, FGFR2, FGFR3, and FGFR4 (1). Both FGFs and FGF receptors (FGFRs) are expressed during different stages of embryonic development, with distinct spatiotemporal expression patterns in a variety of cell types and tissues (2). It has been shown that stimulation of FGFRs in neuronal cells results in multiple responses including cell proliferation, cell survival, and cell migration, as well as initiation of directional chemoattraction of neuronal growth cones. The docking proteins, FRS2α and FRS2β, are major mediators of the signaling pathways [i.e., mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3-kinase)] downstream of FGFRs (3, 4). However, other signaling pathways, such as stimulation of phosphatidylinositol hydrolysis, are mediated by direct recruitment and tyrosine phosphorylation of phospholipase-Cγ by activated FGFRs after FG stimulation (5).

The Eph family of receptors is the largest family of RTKs. Eph receptors are stimulated by a family of membrane-linked ligands designated ephrins (6, 7). Both biochemical and genetic studies have established the central role that ephrins have in the control of cell contact repulsion, boundary formation, cell migration, and repulsive axon guidance (6). Repulsive axon guidance appears to be caused by modulation of cytoskeletal organization leading to regulation of neural growth-cone development (8). Eph-receptors also regulate cell-matrix interaction and cell proliferation by affecting signaling by integrins (9–11) and by modulation of MAPK response (12–14).

In this article, we demonstrate that EphA4 binds directly and specifically via the N-terminal portion of its protein tyrosine kinase core to the juxtamembrane (JM) region of FGFRs. In cells that express EphA4 and FGFRs, the interactions between the cytoplasmic domains of EphA4 and FGFRs can lead to transreceptor activation, resulting in tyrosine phosphorylation of FRS2α and MAPK activation. The synergistic effect of ephrin-A1 stimulation on FGF2-induced cellular responses may influence the biological outcome of the activation of these two families of RTKs.

Materials and Methods

Yeast Two-Hybrid Experiments. The yeast two-hybrid system was used as described (15). The bait used for screening was S1 aa (amino acids 398–478), derived from the JM domain of human FGFR3. A human brain cDNA library in a pJG4–5 vector consisting of 3.5 × 10⁶ primary transformants (Clontech) was used for screening for proteins that interact with the JM domain of FGFR3. Fig. L4 shows the constructs used to detect interactions between the cytoplasmic domain of EphA4 and the JM domain of FGFR3.

Cells. HEK293 cells were maintained in DMEM supplemented with 10% calf serum. For neural differentiation, P19 cells were maintained in α-MEM supplemented with 10% FBS containing 0.5 μM retinoic acid for 3 days. Rat L6 myoblasts were maintained in DMEM supplemented with 10% FBS.

Preparation of Ephrin-A1. Ephrin-A1 fused to human IgG-Fc was purchased from Sigma-Aldrich. Before application to the cells, 5 μg of ephrin-A1-Fc was oligomerized by mixing with 12 μg of rabbit anti-human IgG-Fc (Jackson ImmunoResearch) in 1 ml of PBS at 4°C for at least 1 h. As a control, a human IgG-Fc fragment (Jackson ImmunoResearch) was also applied after oligomerization.

Expression Plasmids. Full-length cDNA of human EphA4 was prepared by RT-PCR using total RNA from a human brain extract (Clontech) as the template. The cDNA of human FGFR4 was prepared by RT-PCR using K562 cell-derived RNA as the template. The CDNA for FGFR1 and FGFR2 were provided by W. McKeehan (Texas A&M University, College Station, TX). The CDNA for FGFR3 was provided by D. E. Johnson (University of Pittsburgh, Pittsburgh). Receptor mutants were prepared by apply-

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with an Xpress epitope and six His residues at the N terminus.

TrkA (cytTrkA) (lane 1) was used as a positive control. Expression of the cytoplasmic domain of yeast. cytEphA4-1 (lane 2) and cytEphA4-2 (lane 3) in a bait vector (pEG202) AD, transcription activation domain in yeast. (FGFR3(JM) and either cytEphA4-1 or cytEphA4-2. TK, tyrosine kinase domain; EphA4 (cytEphA4-1) and cytEphA4-2, yeast (EGY48) was cotransformed with FGFR3 \[FGFR3(JM)\] as a bait yielded a truncated cytoplasmic domain of EphA4 lacking both the JM and kinase domains, and FGFR1(KD), which is a kinase-negative mutant of FGFR1 in which Lys-514 was replaced by an alanine residue (16). A pMXs-IG vector (obtained from T. Kitamura, University of Tokyo, Tokyo) linked to EGFP through the internal ribosomal entry site (IRES).

**In Vitro Protein–Protein Interaction Assay.** Recombinant proteins were produced in bacteria and purified by affinity chromatography. A nickel column was applied for purification of His-tagged proteins, and an amylose resin column was applied for purification of MBP-tagged proteins. In the in vitro interaction assay, EphA4 deletion mutants fused to MBP were bound to amylose resin and washing the pellets extensively, the pellets were analyzed by SDS/PAGE, blotted onto poly(vinylidene difluoride) membranes, incubated with primary Abs, followed by immunoblotting with secondary Abs.

**Retroviral Vector Construction.** We constructed a series of mutants of EphA4 and FGFRs, including EphA4(ΔJM,ΔK), which is a mutant EphA4 lacking both the JM and kinase domains, and FGFR1(KD), which is a kinase-negative mutant of FGFR1 in which Lys-514 was replaced by an alanine residue (16). A pMXs-IG vector (obtained from T. Kitamura, University of Tokyo, Tokyo) linked to EGFP through the internal ribosomal entry site (IRES).
sequence was used in the experiments (17). The pMXs-IG constructs were cotransfected with pCAGSVSV-G (encoding vesicular stomatitis virus surface protein under the control of chicken β-actin promoter) into HEK293/gpRES cells (obtained from T. Miyazawa, Osaka University, Osaka), HEK293 cells stably transfected with pGag-pol-ires-bsr (obtained from T. Kitamura). The resulting pseudotyped retrovirus particles were resuspended in the culture medium for the cells to be transduced. Viral transduction was usually carried out at a multiplicity of infection of 5. The transduced cells were identified easily by visualization of EGFP fluorescence.

Abs. The following Abs were used in this study: mouse anti-myc mAb MYC1-9E10.2 (American Type Culture Collection), mouse anti-HA mAb 12CA5 (Boehringer Mannheim); mouse anti-Flag mAb FLAG M2 (Sigma-Aldrich); mouse anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology); rabbit anti-EphA4 polyclonal Ab (Santa Cruz Biotechnology); rabbit anti-FGFR1 polyclonal Ab (Santa Cruz Biotechnology); rabbit anti-FGFR2 polyclonal Ab (Sigma-Aldrich); rabbit anti-FGFR3 polyclonal Ab (Sigma-Aldrich); rabbit anti-FGFR4 polyclonal Ab (Santa Cruz Biotechnology); rabbit anti-FRS2α polyclonal Ab H-91 (Santa Cruz Biotechnology); rabbit anti-phospho-p44/42 MAPK (Thr-202/Tyr-204) polyclonal Ab (Cell Signaling Technology); and rabbit anti-p44/42 MAPK polyclonal Ab (Cell Signaling Technology).

Results and Discussion
To identify proteins that interact with FGFRs and may have a role in FGF signaling, we screened a human brain cDNA library by using yeast two-hybrid analysis in which the JM domain of FGFR3 was applied as a bait to identify proteins that interact with FGFR3 and potentially with other members of the FGFR family. We identified a variety of positive hits, including the cytoplasmic domain of EphA4, a member of the largest family of RTKs (Fig. 1A).

Complex Formation Between FGFR3 and EphA4 Is Mediated by Direct Interactions Between Their Cytoplasmic Domains. The region in EphA4 that is responsible for complex formation with FGFR3 was delineated by employing a pull-down assay in which the capacity of several deletion mutants of EphA4 to interact with the cytoplasmic domain of FGFR3 was compared. This experiment demonstrated that the N-terminal region of the kinase domain of EphA4 (amino acids 636–762) is responsible for complex formation with FGFR3 (Fig. 2). However, the extracellular ligand binding domain, which contains the sterile 

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95/discs large/zona occludens-1 (PDZ)-binding domain present in the C terminus of EphA4 do not seem to play a role in mediating complex formation with FGFR3 (data not shown). By using the yeast two-hybrid system to further explore the nature of the interactions between FGFR and EphA4, we have demonstrated that whereas the intact nonphosphorylated cytoplasmic domain of EphA4 (Fig. 1B) does not form a complex with the JM domain of FGFR3 and the tyrosine kinase domain of EphA4. Furthermore, a recombinant MBP fusion protein synthesized in E. coli that contains the entire cytoplasmic domain of EphA4 composed of the JM and the tyrosine kinase domains was phosphorylated at the JM tyrosines, and interacted with the JM domain of FGFR3 (Fig. 2B). Similar results were obtained when FGFR1 and FGFR2 were used in place of FGFR3 in the yeast two-hybrid and pull-down experiments (data not shown). Together, the experiments described above suggest that phosphorylation of tyrosine residues in the JM domain or deletion of the JM domain of EphA4 releases an inhibitory constraint that blocks complex formation between the tyrosine kinase domain of EphA4 and the JM domain of FGFR1, FGFR2, or FGFR3.

This conclusion was reinforced by communoprecipitation and immunoblotting experiments of lysates from HEK293 cells transiently coexpressing different pairs of a member of the FGFR family (FGFR1, FGFR2, FGFR3, or FGFR4), together with WT EphA4 or various mutants including AJM, an EphA4 deletion mutant lacking the JM domain; EphA4(KD), a kinase-negative point mutant in which Val-653 was replaced by a Met residue; EphA4(3M), a triple EphA4 mutant in which two tyrosine auto-phosphorylation sites at positions 569 and 602 in the JM were replaced by phenylalanine residues in addition to the V635M mutation; and EphA4(ΔJM, ΔK), a deletion mutant devoid of both the JM domain and a large portion of the cytoplasmic domain (Fig. 3A). This experiment shows that WT EphA4 formed a complex with FGFR3, whereas EphA4(3M) barely interacted with FGFR3, and EphA4(ΔJM, ΔK) was not found to be as competent as WT EphA4 in complex formation with FGFR3 (Fig. 3A).

Mutual Transphosphorylation Between FGFRs and EphA4 in Transfected Cells. To explore the functional consequences of complex formation between EphA4 and FGFRs, we next tested whether EphA4 can tyrosine phosphorylate FGFRs and whether FGFRs can tyrosine phosphorylate EphA4 when transiently coexpressed in
the same cells. To this end, HEK293 cells were cotransfected with expression vectors that direct the synthesis of WT EphA4 and a kinase-negative mutant of FGFR1 [FGFR1(KD)]. In a separate experiment, HEK293 cells were transiently cotransfected with an expression vector that directs the synthesis of WT FGFR1, together with an expression vector for a kinase-negative EphA4 mutant [EphA4(KD)]. Immunoprecipitation with anti-FGFR1 Abs of the kinase-negative mutant of FGFR1 in cells coexpressing WT EphA4, followed by immunoblotting with anti-phosphotyrosine Abs, have demonstrated that EphA4 activated by overexpression in HEK293 cells induces tyrosine phosphorylation of the kinase-negative FGFR1 mutant (Fig. 3C Left). Also, the experiment shown in Fig. 3C Right shows that stimulation of FGFR1 by overexpression in HEK293 cells results in tyrosine phosphorylation of the kinase-negative EphA4 mutant. Similar results were obtained in HEK293 cells overexpressing FGFR2 (as described below). Together, the cotransfection experiments using kinase-negative mutants of FGFR1 or EphA4 reveal the capacity of EphA4 to tyrosine phosphorylate FGFR1 and the capacity of FGFR1 to tyrosine phosphorylate EphA4.

To correlate the tyrosine phosphorylation of EphA4 and its binding to FGFRs, we cotransfected HEK293 cells with FGFRs and various EphA4 mutants, which are shown in Fig. 3A (Fig. 3D). Whereas weak tyrosine phosphorylation and association were detected between EphA4(3M) or EphA4(2M) and FGFRs, stronger phosphorylation and interactions were detected between EphA4(KD) or EphA4(WT) and FGFRs, suggesting that phosphorylation of the EphA4 JM tyrosines is required for complex formation between the two receptors. To identify the location of tyrosine residues that are phosphorylated in EphA4, HEK293 cells were transfected with several EphA4 mutants in the presence of increasing amounts of FGFR2, and their phosphorylation levels were examined. As shown in Fig. 3E, EphA4(KD) was modestly phosphorylated when FGFR2 expression was high, whereas the phosphorylation of EphA4(3M) was barely detected even at the very high level of FGFR2 expression. Also, in the absence of FGFR2, EphA4(JM) was weakly phosphorylated, probably because of self-phosphorylation, and the phosphorylation of EphA4(JM) was somewhat increased in cells overexpressing FGFR2. These results are consistent with the notion that the JM domain serves as a primary site of phosphorylation by FGFR2. Similar results were obtained in HEK293 cells expressing these EphA4 constructs together with FGFR1 (data not shown).

Enhanced Tyrosine Phosphorylation of FRS2α by EphA4 or FGFRs in Ectopically Expressing Cells. Biochemical and genetic studies have demonstrated that the docking protein FRS2α is a major mediator of cell signaling via all members of the FGFR family of RTKs (3, 4, 18). To examine whether FRS2α also has a role in signaling via EphA4 receptors, we first examined whether FRS2α undergoes tyrosine phosphorylation in L6 myoblasts in response to stimulation of ectopically expressed EphA4 in these cells. L6 cells express an almost undetectable amount of endogenous FGFRs (Fig. 4A) (19), and therefore, the experiment shown in Fig. 4B Left tests whether EphA4-mediated EphA4 stimulation may lead to tyrosine phosphorylation of FRS2α under very low expression of FGFRs. In this experiment, lysates from EphA4-stimulated L6 cells were subjected to immunoprecipitation with anti-FRS2α Abs, followed by immunoblotting with anti-phosphotyrosine Abs. EphA4 stimulation of L6 cells led to a weak tyrosine phosphorylation of FRS2α. FRS2α phosphorylation was not inhibited in the presence of SU5402 (25 μM), an inhibitor of FGFR kinase, suggesting that EphA4 phosphorlates FRS2α regardless of the presence of FGFR (Fig. 4B Middle). Also, unlike FGFR1 stimulation, which occurs within a few minutes (Fig. 4D), the kinetics of EphA4-induced tyrosine phosphorylation of FRS2α is slow, with a maximal stimulation after ~60 min. Fig. 4C shows that, whereas ectopic expression of FGFR1 augmented EphA4-induced tyrosine phosphorylation of FRS2α and MAPK response in L6 cells, expression of a kinase-negative FGFR1 mutant suppressed these responses. These results provide further evidence that EphA4, when highly overexpressed, is capable of mediating tyrosine phosphorylation of FRS2α and that the
EphA4-mediated response is augmented by transphosphorylation between FGFR1 and EphA4.

We also examined whether EphA4 expression will influence FGFR1-mediated tyrosine phosphorylation of FRS2α (Fig. 4D). In this experiment, L6 cells were stimulated by FGF2 and analyzed for tyrosine phosphorylation of FRS2α in cells that had been transduced with retroviral expression vector for EphA4 or vector alone. Consistent with the fact that L6 cells express very low levels of endogenous FGFRs, weak FRS2α tyrosine phosphorylation was detected in lysates from the FGF2-stimulated cells. A significant enhancement in FGFR2-induced tyrosine phosphorylation of FRS2α was observed upon ectopic expression of WT EphA4 in these cells, whereas FGFR2-induced tyrosine phosphorylation of FRS2α was suppressed by ectopic expression of a truncated dominant interfering EphA4 mutant [EphA4ΔJM, ΔK]. The experiment shown in Fig. 4B and D demonstrates a good correlation between EphA4 stimulation of tyrosine phosphorylation of FRS2α and EphA4 stimulation of MAPK. Also, the inhibitory effect elicited by coexpression of a truncated dominant interfering EphA4 mutant in cells stimulated by FGF2 as well as the same effect of the dominant interfering FGFR1 mutant in cells stimulated with ephrin-A1 reveal a good correlation between tyrosine phosphorylation of FRS2α and MAPK stimulation. In other words, both tyrosine phosphorylation of FRS2α and the MAPK response were similarly attenuated by overexpression of a dominant interfering mutant of the other RTK. However, an alternative, not mutually exclusive possibility, is that FGFR1(KD) sequesters FRS2, leading to diminished phosphorylation of FRS2 by EphA4.

Ephrin-A1-Mediated Potentiation of FGF2 Signals in Differentiated P19 Cells. We next examined whether the interactions between the two receptors occur also in cells expressing endogenous FGFRs and EphA4. For this purpose, we used P19 cells that were induced to differentiate into neural-like cells by incubating them in a medium containing retinoic acid for 3 days, followed by an additional 2-day incubation in a serum-free medium. Under these conditions, almost all of the P19 cells undergo neuronal differentiation. Northern blot analysis with specific probes revealed induction of transcripts specific for FGFR1, FGFR2, FGFR3, FRS2α, EphA4, EphA5, and EphA7 (data not shown) upon neuronal differentiation of P19 cells. Lysates of the neuronally differentiated P19 cells were subjected to immunoprecipitation with different anti-FGFR Abs, followed by immunoblotting with Abs against different FGFRs or EphA4. The experiment shown in Fig. 5A shows that a complex between EphA4 and FGFRs could not be detected in lysates of nonstimulated cells. However, in response to ephrin-A1 stimulation, complex formation between EphA4 and either FGFR1, FGFR2 or FGFR3 could be detected in lysates prepared from the stimulated cells. This experiment shows that upon ephrin-A1 stimulation endogenous EphA4 are capable of forming a complex with endogenous FGFRs in lysates from these cells.

To shed light on the biological outcome of the interactions that were detected between EphA4 and FGF in the biochemical experiments, we have analyzed tyrosine phosphorylation of FRS2α and MAPK stimulation in P19 cells that were stimulated with FGF2 or ephrin-A1 separately or in combination. The experiment presented in Fig. 5B shows that stimulation by ephrin-A1 induced a very weak and barely detectable stimulation of tyrosine phosphorylation of FRS2α. Similarly, very weak MAPK stimulation was detected when the P19 cells were stimulated with ephrin-A1 alone (Fig. 5C). However, FGF2 stimulation of both responses was robust. When FGF2 and ephrin-A1 were added together, tyrosine phosphorylation of FRS2α and MAPK responses resembled or slightly surpassed those induced by FGF2 alone. However, when ephrin-A1 was added 45 min before FGF2 stimulation, a potentiation of FGF2 stimulation of tyrosine phosphorylation of FRS2α and MAPK response was detected (Fig. 5B and C).

The experiments presented in this article show that, in vitro, FGFR1, FGFR2, FGFR3 or FGFR4 are capable of forming a direct complex with EphA4. Complex formation between the two receptors is mediated by direct interactions between the JM domain of FGFRs and the N-terminal region of catalytic core of EphA4. Phosphorylation of tyrosine residues in the EphA4 JM domain is required for complex formation with FGFRs. In cells that express these RTKs ectopically, EphA4 is capable of inducing tyrosine phosphorylation of FGFRs, tyrosine phosphorylation of FRS2α, and MAPK stimulation. Although complex formation between endogenous FGFRs and endogenous EphA4 can be detected in lysates from ephrin-A1-stimulated P19 cells, ephrin-A1 has only a minor effect on tyrosine phosphorylation of FRS2α and MAPK stimulation in differentiated P19 cells. However, ephrin-A1 stimulation of P19 cells strongly potentiates FGF2-mediated stimulation of tyrosine phosphorylation of FRS2α and MAPK stimulation, confirming that trans-activation between the two receptors takes place in the context of living cells.

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