EphA Receptors Direct the Differentiation of Mammalian Neural Precursor Cells through a Mitogen-activated Protein Kinase-dependent Pathway*

Miwa Aoki‡, Toshihide Yamashita§, and Masaya Tohyama¶

From the Department of Neurobiology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan and the Department of Anatomy and Neuroscience, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

Ephrins are cell surface-associated ligands for Eph receptor tyrosine kinases and are implicated in repulsive axon guidance and cell migration. EphA2, 3, and 4 receptors and one of their cognate ligands, ephrin-A2, are expressed by cells in the subventricular zone and ganglionic eminence of the embryonic day 14.5 telencephalon and by neural precursor cells in vitro. Activation of EphA receptors in dissociated neural precursor cells in vitro facilitates the commitment to neuronal fates. The majority of ephrin-A1-induced neurons is immunoreactive for tyrosine hydroxylase. Blocking the signal by the extracellular domain of EphA in forebrain slices results in a decrease in neurogenesis. Extracellular signal-regulated kinase is activated by the ligand binding to EphA receptors and is involved in the neurogenesis through EphA receptors. Rap1, but not Ras, is activated in response to ephrin-A1. Our results identify EphA receptors as positive regulators of the mitogen-activated protein kinase pathway that exerts neurogenesis of neural precursor cells from the developing central nervous system.

The mammalian central nervous system is derived from a monolayer of germinal neuroepithelial cells, which are composed of self-renewing multipotent precursor cells in the ventricular zone. These most immature precursor cells generate mitotic and lineage-restricted intermediate progenitor cells. Fetal telencephalic neuroepithelial cells contain neural precursors that give rise to the neuronal lineage and the glial lineage. The fate of neural precursors in the developing brain is believed to be determined by intrinsic cellular programs and by external cues (1). Newly generated cortical neurons in the ventricular zone migrate along the surface of radial glial fibers and settle in the cortical plate to orderly layers of the cortex (2). After reaching its destination, each neuron develops a set of dendrites characteristic of its phenotype and a single long axon that extends along specific routes to reach prospective synaptic partners. The migration of either the entire neuron or its nerve growth cones is guided by the interaction between the neuron and its local environment. These navigations use similar guidance molecules.

Eph receptor tyrosine kinases/ephrins interactions are implicated in axon guidance, neural crest cell migration, establishment of segmental boundaries, and formation of angiogenic capillary plexi (3). Ephrins play important roles during axon guidance by providing a repulsive guidance signal to Eph receptors in cells. A migrating growth cone expressing a particular Eph receptors would turn away from a cell that presents the cognate ephrin ligand. Eph receptors and ephrins are divided into two subclasses, A and B, based on binding specificities. Ephrin subclasses are further distinguished by their mode of attachment to the plasma membrane; ephrin-A ligands bind EphA receptors and are anchored to the plasma membrane via a glycosylphosphatidylinositol linkage, whereas ephrin-B ligands bind EphB receptors and are anchored via a transmembrane domain. A recent study localized Eph receptors and ephrin ligands to the subventricular zone in the adult rat where neural stem cells reside (4). Activation of EphB by a 3-day infusion of the ectodomain of either EphB2 or ephrin-B2 into the lateral ventricle of the adult rat appeared to increase the number of neural stem cells, suggesting promotion of proliferation (5). On the other hand, in some non-neuronal cell lines, such as pRNS-1-1, PC-3, or MEF cells, stimulation with ephrin-A1-Fc inhibits Ras/mitogen-activated protein kinase (MAPK)1 pathway, leading to cessation of proliferation (6). These findings prompted us to hypothesize that Eph/ephrin interactions are involved in the differentiation or maintenance of the neural stem cells in the developing central nervous system. In this manuscript, we show the presence of EphA receptors and its cognate ligand ephrin-A in the neural precursor cells in vitro and in vivo and that activation of EphA receptors alters the fate of neural precursor cells to a neuronal commitment.

EXPERIMENTAL PROCEDURES

Immunohistochemistry—Mouse embryos at embryonic day 14.5 were killed by anesthetic overdose and dipped in 4% paraformaldehyde in PBS (pH 7.2). Next, they were cryoprotected overnight in SPB composed of 0.1 M phosphate-buffered saline and 3% sucrose. The bodies were embedded in Tissue Tek O.C.T. compound (Sankura Finetek, Torrance, CA), followed by cryosection at 14 μm. The following antibodies were

* This work was partly supported by the 21st Century Center of Excellence Program, 32 Grant-in-aid 14657343 from the Ministry of Education, Culture, Sports and Technology of Japan, and Research Grant 15A-2 for Nervous and Mental Disorders from the Ministry of Health, Labour and Welfare. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Neurobiology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan. Tel.: 81-43-2262024; Fax: 81-43-2262025; E-mail: t-yamashita@faculty.chiba-u.jp.

¶ This abbreviation is defined in the list of abbreviations.
Neurogenesis by EphA

used as primary antibodies: anti-EphA3 antibody (1:500; Santa Cruz), anti-EphA4 antibody (1:200; Santa Cruz), anti-ephrin-A1 antibody (1:500; Santa Cruz), and anti-nestin antibody (Rat-401, 1:300; Developmental Studies Hybridoma Bank). Ephrin-A1-Fc or EphA2-Fc preincubated with the anti-human IgG-Fc antibody (Sigma) at a fixed ratio of 0.1× indicated concentrations of Fc fusion proteins. Sections were perméabilized with 0.2% Triton X-100/PBS and then were incubated overnight with the primary antibodies, followed by incubation with corres-ponding secondary antibodies conjugated with FITC or Alexa fluor 568 for 1 h at room temperature. After rinsing with PBS, the sections were mounted with ImmunomTM (Thermo Shandon) and viewed under a SM510-V2.01 laser confocal microscope (Carl Zeiss). The specificity of the antibodies was assessed with a competition study using a ratio of 100 ng/ml of EphA3 or EphA4 (Santa Cruz Biotechnology) or by leaving out the primary antibodies. Western blot analysis of cells expressing the proteins was also performed.

Primary Cell Culture—The procedure used to generate neurospheres from the embryonic forebrain was described previously (9). Briefly, the forebrains were dissected from mouse embryos at E14 in PBS and then were transfused to defined media (MHM composed of Dulbecco’s mod-ified Eagle’s medium/Ham’s F-12 medium (1:1; Invitrogen), glucose (0.6%; Wako), NaHCO3, HEPES (0.5 mM; Sigma), -glutamine (2 mM), bovine transferrin (80 mg), insulin (20 mg), putrescine (56 mM), sodium selenite (28 mM), and progesterone (19 nM). The tissues were mechani-cally trituated into single cells in MHM with a fire-polished pipette. The cells were cultured in MHM containing 10% fetal calf serum, 500 ng/ml human bFGF (R & D Systems, Inc.) in noncoated dishes for 4 days in vitro (DIV) at a density of 2 × 10⁶ cell/ml.

Differentiation Assay—The generated neurospheres were dissociated mechanically, plated at a density of 1 × 10⁵ cell/ml/well (24-well plate) on poly-l-ornithine coated coverslips, and then allowed to differentiate for 5 DIV in the presence of BrdUrd (10 μM) with or without 2.5 μg/ml (24-well plate) ephrin-A1-Fc immobilized on poly-l-lysine-coated coverslips, 170 ng/ml soluble human IgG-Fc, 500 ng/ml soluble ephrin-B2-Fc (Genzyme Technne), 500 ng/ml soluble ephrin-A1-Fc (Genzyme Technne), and 100 μg/ml P0098059 (Sigma). The ligand (human IgG-Fc, ephrin-B2-Fc, and ephrin-A1-Fc) multimers were generated by previously described (23), or for cytoplasmic lyses, the extracts were mixed with an equal volume of 2× SDS loading buffer.

Assay for ERK Activity—The neurospheres cultured for 3 days were rinsed with PBS by centrifuging at 400 rpm for 5 min, and the medium was replaced with the same volume of MHM without mitogens. Twenty-four hours later, the neurospheres were exposed to 340 ng/ml human IgG-Fc or 500 ng/ml ephrin-A1-Fc for 0 min, 1 min, 5 min, 30 min, or 1 h. Then the cells were lysed in lysis buffer (1% Nonidet P-40, 10% glycerol, 50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 2.5 mM MgCl₂, 2 mM sodium orthovanadate, 1 μg/ml leupeptin, 10 μg/ml trypsin inhibitor). Protein concentrations between the cell lines were equalized. Either lyses and Western blot were immunostained with antibodies as previ-ously described (23), or for cytoplasmic lyses, the extracts were mixed with an equal volume of 2× SDS loading buffer.

Ras Activity Assay—Ras activity was assessed by employing the Ras activation assay kit (Upstate Biotechnology, Inc.). Briefly, treated cells were lysed in Mg²⁺ lysis buffer (MLB buffer) containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM MgCl₂, 1 mM EDTA, 2% glycerol, 2 mM sodium orthovanadate, 1 mM ethylsulfonyl fluoride, and a protease inhibitor mixture tablet. The cell lyses were incubated with 3 μl of Raf-1 Ras binding domain-agarose conjugate for 30 min at 4°C. The beads were washed three times with MLB buffer and resuspended in 2× SDS sample buffer. Anti-Ras antibody (Upstate Biotechnology, Inc.) was used for the blotting.

Rap1 Activity Assay—Affinity precipitation of the activated Rap1 was performed with glutathione-S-transferase-Ral binding domain fusion protein precomplexed to glutathione-Sepharose (Amersham Bio- sciences). The Ral-binding domain ofRal GDS in pGEX vector was kindly provided by Dr. Johannes L. Bos. The activity of Rap1 was determined in immunoprecipitates by Western blot analysis using the anti-Rap1 antibody (BD Bioscence). To quantify the relative amounts of protein, we scanned x-ray film and analyzed the digital images using NIH Image.

RESULTS

EphA Receptors Are Expressed in Neuroepithelial Cells in the Embryonic Day 14.5 Telencephalon—To investigate whether Ephrin/Eph receptors are associated with neurogenesis during the developmental stage, the expression patterns of EphA recep-tors and their cognate ligands were examined. The antibody RAT401, which recognizes nestin, an intermediate filament specific to undifferentiated neural precursor cells (7), was used to identify the neural precursor cells (8). At E14.5, immunore-activities for EphA3 and 4 were observed in the ventricular zone and the adjacent mantle/intermediate zone (Fig. 1, A and B). Double immunostaining revealed that EphA3 and 4 were colocalized with nestin, which was expressed on the fibers, in the ventricular zone of the telencephalon. Colocalization of

GACGAGG-3’, 5’-CCCTGACAGGAGAGGACAG-3’, 616 bp); Pfx-3 (5’-CTGGCTGGTTGTCAGAAA-3’, 5’-GGCTGAGCACAAGTT- GTGAAAG, 616 bp), and -actin (5’-TCTCCTGGGAGAAGAGGACT-3’, 5’-TCTCCTGGGTGACTGACCAT, 383 bp). Reverse transcription-PCR products were analyzed in an agarose gel.

Slice Culture—The forebrains of mouse embryos at E14.5 were sag-ittally sliced manually on a glass plate. The slices were mounted in collagen gel matrices, (described above) and placed in an incubator. The slices were kept in MHM on ice until mounting. The culture medium was modified neurosphere culture containing BrdUrd (10 μM) and 80 ng/ml EGF plus 80 ng/ml bFGF with or without soluble 100 μg/ml EphA2-Fc or 100 μg/ml EphB2-Fc (Genzyme Technne). After a 5-DIV culturing in a well of a 6-well plate, the slices were embedded in Tissue Tek OCT compound, followed by cryosection at 14 μm, and sectioned with a vibratome slicing machine for immunohistochemistry.

The numbers of cells double-positive for BrdUrd and Tuj1 or micro-tubule-associated protein 2 were counted on sections taken from sections taken from 1000 ng/ml EGF plus 80 ng/ml bFGF with or without soluble 100 μg/ml EphA2-Fc-treated slice culture (six different culture series of ~10 slice cultures each). Within each sample slice culture, several sections were analyzed. For each section at least three different grid-fields (10000 μm²) in SVZ were randomly selected for analysis.

Immunoprecipitation—The neurospheres cultured for 3 days were rinsed with PBS by centrifuging at 400 rpm for 5 min, and the medium was replaced with the same volume of MHM with mitogens. Twenty-four hours later, the neurospheres were exposed to 340 ng/ml human IgG-Fc or 500 ng/ml ephrin-A1-Fc for 0 min, 1 min, 5 min, 30 min, or 1 h. Then the cells were lysed in lysis buffer (1% Nonidet P-40, 10% glycerol, 50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 2.5 mM MgCl₂, 2 mM sodium orthovanadate, 1 μg/ml leupeptin, 10 μg/ml trypsin inhibitor). Protein concentrations between the cell lines were equalized. Either lyses and Western blot were immunostained with antibodies as previ-ously described (23), or for cytoplasmic lyses, the extracts were mixed with an equal volume of 2× SDS loading buffer.
FIG. 1. Patterns of expression of EphA3, EphA4, and ephrin-A2 in the E14.5 telencephalon. A, immunofluorescence staining for EphA3 and nestin on transverse cryosections of the developing mouse neocortex at E14.5 (panel a). Representative single optical sections for EphA3, nestin and overlay images (Merge) are shown. Note the close association of these markers. VZ, ventricular zone; IZ, intermediate zone; CP, cortical plate; MZ, mantle zone. The specificity of anti-EphA3 antibody was examined by using the blocking peptide (panel b). B, immunofluorescence staining for EphA4 and nestin on transverse cryosections of the developing mouse neocortex at E14.5. Representative single optical sections for EphA4, nestin, and overlay images (Merge) are shown. C, immunofluorescence staining for ephrin-A1 and nestin on transverse cryosections of the developing mouse neocortex at E14.5. D, immunofluorescence staining for ephrin-A1 and EphA3 on transverse cryosections of the developing mouse neocortex at E14.5. The arrowheads indicate immunolabeling of ephrin-A1. E, binding of EphA2-Fc (panel a) or ephrin-A1-Fc (panel b) to the transverse cryosections of the developing mouse neocortex at E14.5.
other members, such as EphA2, with nestin was also found (data not shown). One of their cognate ligand, ephrin-A1, was weakly expressed in neuroepithelial cells at E14.5 (Fig. 1C). There is a little population that showed double-positive for Eph-A2, EphA3, EphA4, and nestin, and only a minority of the cells positive for these EphA immunoreactivities were colocalized with nestin in the neurospheres (Fig. 2A, panel c). Binding of Eph-A2-Fc or ephrin-A1-Fc fusion protein, the expression of ephrins and Eph receptors was verified (Fig. 2C). Binding of Eph-A2-Fc (Fig. 2C, panel a) or ephrin-A1-Fc (Fig. 2C, panel b) fusion protein to some of the nestin-positive cells was observed. One of the cognate ligands for EphA, ephrin-A1, was expressed weakly in a few cells in each sphere and was not colocalized with EphA2 (Fig. 2B, arrowheads). These results suggest the possibility that neural precursor cells expressing EphA receptor may be responsive to ephrin-A ligands.

Activation of EphA Receptors Did Not Cause Proliferation of Neural Precursor Cells—It was previously reported that infusion of EphB2 or ephrin-B2 into the lateral ventricle in the adult rat disrupted migration of neuroblasts and increased cell proliferation (5). The authors suggested that EphB/ephrin-B signaling is involved in the regulation of cell proliferation. In contrast, another paper showed nonmitogenic activity of ephrin-B (10). To determine whether EphA receptors maintained neural stem cells in an undifferentiated state, we stimulated EphA receptors by adding a fusion protein containing ephrin-A1 joined to human Fc to the cultures (8). Immunocytochemistry was performed using the primary spheres cultured for 7 days after dissociation. As expected, immunoreactivities for EphA3 and 4 were abundantly observed in the spheres and were colocalized with those for nestin (Fig. 2A, panels b and c). Although neurospheres are heterogeneous, and only a minority of the cells positive for these EphA immunoreactivities was nestin-positive, EphA2 was also found to be colocalized with nestin in the neurospheres (Fig. 2A, panel a). These immunoreactivities for EphA3 and 4 were abundantly observed in the spheres and were colocalized with those for nestin (Fig. 2A, panels b and c).

Neural Precursor Cells from the E14.5 Telencephalon Express EphA Receptors—In vitro, single neural stem cells proliferate to form clonally derived floating sphere colonies, designated neurospheres, which contain cells that, upon dissociation into single cells, give rise to new sphere colonies. These cells can differentiate into neurons and glia proliferate in the presence of bFGF and EGF (9). Sphere-producing cells derived from E14.5 mouse brain were isolated by primary neurosphere formation, and their self-renewing capacity was demonstrated by assessing the number of secondary neurospheres formed (8).

Activation of EphA Receptors Did Not Cause Proliferation of Neural Precursor Cells—It was previously reported that infusion of EphB2 or ephrin-B2 into the lateral ventricle in the adult rat disrupted migration of neuroblasts and increased cell proliferation (5). The authors suggested that EphB/ephrin-B signaling is involved in the regulation of cell proliferation. In contrast, another paper showed nonmitogenic activity of ephrin-B (10). To determine whether EphA receptors maintained neural stem cells in an undifferentiated state, we stimulated EphA receptors by adding a fusion protein containing ephrin-A1 joined to human Fc to the cultures in vitro. Because the ephrin-Eph signaling system is able to elicit bi-directional signals, EphA can have dual effects: the inhibition of endogenous ephrin-Eph interactions and the unidirectional activation of their respective binding partners.
cells were transferred onto poly-l-ornithine-coated glass slides and cultured in the absence of EGF and bFGF to facilitate the differentiation. DAPI nuclear staining suggested that ephrin-A1-Fc did not increase the cell number and that cell death was apparently not induced by ephrin-A1-Fc (data not shown). Staining with the anti-nestin antibody revealed that the number of nestin-positive cells was gradually decreased between the 1-day and 5-day assay periods, demonstrating the differentiation of neural precursor cells under the condition we adopted (Fig. 3A). Cessation of cell proliferation was clear, from the total number of the cells stained by DAPI. The number of nestin-positive cells treated with ephrin-A1 for 1 or 5 days was not significantly changed compared with that without ephrin-A1 treatment (Fig. 3A), demonstrating that ephrin-A1 did not inhibit the differentiation of neural precursor cells under our experimental conditions. In contrast, ephrin-B treatment resulted in a significant increase in the number of nestin-positive cells, similar to a previous observation in vivo (5). On the other hand, ephrin-A or -B treatment had no effect on apoptosis (Fig. 3B). Thus, activation of EphA receptors may not contribute to proliferation or death of neural precursor cells derived from the E14.5 telencephalon.

**Ephrin-A-induced Switch in the Fate of Neural Precursor Cells**—Because activation of EphA receptors did not promote the differentiation of neural precursor cells in the undifferentiated condition that contained bFGF and EGF, we next determined whether ephrin-A has the potential to switch the fate of neural precursor cells. A thymidine analog, BrdUrd, was included in the culture medium, and the cells positive for BrdUrd, TuJ1, or GFAP were counted in 1- and 5-day cultures of the precursor cells in the differentiated condition, which did...
not contain bFGF or EGF. This method allowed the direct examination of the possibility that ephrin-A ligands affect the cell fate by specific killing of the differentiated glia in the neurospheres. BrdUrd-unlabeled neurons or glia may be present from the beginning of the culture, and the number of these neurons or glia was not significantly altered by the addition of ephrin-A1 (data not shown). As shown in Fig. 4 (A and B), the ratio of the cells double-positive for TuJ1, a marker for immature neuron, and BrdUrd to cells positive for BrdUrd was significantly higher in ephrin-A-Fc-treated cultures compared with untreated cultures. In addition, ephrin-A1 treatment increased the expression of neurofilament, a marker for mature neuron, positive cells. Next, we used immobilized ephrin-A1-Fc on poly-l-lysine-coated cover slips to mimic the cell-cell contact condition. Immobilized ephrin-A1-Fc induced neurogenesis to the same extent as soluble ephrin-A1-Fc (Fig. 4, A and B). As TuJ1-positive cells express the EphA3 receptor (Fig. 4C), these data suggest that ephrin-A promotes neurogenesis via an EphA-dependent mechanism. In contrast, ephrin-A1 did not increase the number of GFAP-positive cells (Fig. 4, D and E). The ratio of the cells positive for both neuronal and BrdUrd markers to GFAP and BrdUrd markers was significantly increased with ephrin-A-Fc treatment for 5 days (Fig. 4E). The majority of the cells negative for TuJ1 as well as GFAP may be immature precursor cells, as much of these cells were also negative for oligodendrocyte markers. Our data show that ephrin-A1 appears to direct the neuronal fate of proliferating precursors.

Next, we examined whether ephrin-A induced specific neuronal lineages from the neuroepithelial cells contained in neurospheres. As in differentiation assay, immunocytochemical analysis of the characteristics of ephrin-A-induced neurons revealed that most of the neurons immunoreactive for TuJ1 express tyrosine hydroxylase (Fig. 5, A and B), although a significantly lower percentage of neurons in the control cultures were immunoreactive for tyrosine hydroxylase. We assessed expression of cholin acetyltransferase, dopamine β-hydroxylase, or glutamate decarboxylase and found that the values for these markers were much smaller in the ephrin-A-treated group than that for tyrosine hydroxylase (data not shown).

Reverse transcription-PCR analyses showed up-regulation of mRNAs for dopaminergic neurons, such as tyrosine hydroxylase, L-aromatic amino acid decarboxylase, and the midbrain dopaminergic neuron marker (Ptx-3) in 5-DIV ephrin-A1-treated cells, whereas the band for vesicular monoamine transporter mRNA was not found (Fig. 5C). These results show that the generation of tyrosine hydroxylase-positive neurons was facilitated through EphA pathways.

Neurogenesis through an EphA-dependent Pathway in a Slice Culture of Telencephalon—We assessed the effects of endogenous ephrin-A using forebrain slices from E14.5 mice. Mitotic cells were labeled by BrdUrd, and the neurogenesis was examined 5 days later. Extensive colocalization of BrdUrd with TuJ1, demonstrating the proper neurogenesis in the slices, was observed (Fig. 6A, arrowheads). Next, we employed the extracellular domain of EphA2 or EphB2 fused with Fc (EphA2-Fc and EphB2-Fc) to competitively block the endogenous interaction of EphA with ephrin-A or of EphB with ephrin-B. The number of the cells positive for TuJ1 was significantly decreased by the addition of excess EphA2-Fc (Fig. 6, arrowheads). However, EphA2-Fc had no effect on the differentiation to the GFAP-positive cells (Fig. 6B). In contrast, EphB2-Fc treatment did not result in decrease in the number of the cells positive for TuJ1 (Fig. 6A). These results also observed in clustered EphA2-Fc treatment (data not shown). Thus, EphA is required for only the differentiation of neural precursor cells into neurons in a slice preparation. These results strengthen our suggestion that is raised from the data using neurospheres.

Signaling Mechanisms of EphA-induced Neurogenesis—In some non-neuronal cell lines, such as pRNS-1-1, PC-3, or MEF cells, ephrin-A-Fc stimulation inhibits the Ras/MAPK pathway, leading to cessation of proliferation (6). These reports prompted us to examine whether the biological activities of ephrin-A on neural precursor cells were attributable to Ras-MAPK activities, because we observed no growth inhibition by ephrin-A (data not shown).
cal significance. *, p < 0.05 (Student’s t test). Inhibition of the basal activity of ERK was induced by ephrin-A1 (Fig. 7B). Next, we asked whether MAPK activation is sufficient for the effect. The activity of Ras, an upstream regulator of MAPK, was measured by a pull-down assay (10). Intriguingly, stimulation of neuroepithelial cells with ephrin-A1-Fc in vitro caused a mild decrease (5, 30, 60 min) in the amount of GTP-bound form of Ras compared with the basal activity (Fig. 8A). In contrast, ephrin-B-Fc as well as EGF activated Ras (data not shown). Our results are consistent with the previous report that shows the inactivation of Ras by ephrin-A in prostatic epithelial cells and endothelial cells (6).

Differentiation of PC12 cells in response to nerve growth factor is reported to be involved in two distinct pathways: Ras that induces the initial activation of MAPK and another small G protein Rap1 that induces sustained activation of MAPK (11). Because we observed sustained activation of ERK in the neuroepithelial cells, we hypothesized that Rap1 might be responsible for the activation of ERK. By employing a construct consisting of the Rap1-binding domain of Ral fused to glutathione-Sepharose, affinity precipitation was done to detect GTP-bound active Rap1 (12). The amount of the GTP-bound form of Rap1 was increased after the addition of ephrin-A1-Fc (5, 30, and 60 min) (Fig. 8B). Because of the similarity of temporal activity change of ERK and Rap1, it is suggested that the Rap1/MAPK pathway contributes to the neurogenesis-inducing activity of EphA on the neural precursor cells.

**DISCUSSION**

In this manuscript, we show that the presence of EphA receptors and its cognate ligand ephrin-A in the neural precursor cells in vitro and in vivo and that activation of EphA receptors alters the fate of neural precursor cells to a neuronal commitment. Blocking the signal by the extracellular domain of EphA in forebrain slices results in a decrease in neurogenesis, suggesting that endogenous ephrin-A signal is required for the differentiation of the neural precursor cells. Extracellular signal-regulated kinase is activated by the ligand binding to EphA receptors and is involved in the neurogenesis through EphA receptors. Rap1, but not Ras, is activated in response to ephrin-A1.

Ephrin-A5-induced repulsive guidance was suggested to be mediated by activation of Rho (13), which plays major roles in neurite growth and growth cone guidance (14). In fact, the group led by Greenberg (15) identified a guanine nucleotide exchange factor for Rho, as an EphA-interacting protein, elucidating a molecular link between EphA receptors and reorganization of the actin cytoskeleton. Independently, others reported that inhibition of cell proliferation in prostatic epithelial cells and endothelial cells, but not fibroblasts, was mediated by ephrin-A1, which inhibits the Ras/MAPK pathway (6). In contrast, prolonged activation of ERK-1 and ERK-2 in response to ephrin-A5 was reported to contribute to the changes in cell morphology in NIH3T3 cells (16). In the neuroepithelial cells used in the current study, activation of ERK is necessary for the neurogenesis induced by the activated EphA. These seemingly contradictory findings suggest that the ligand binding to EphA receptors elicits bi-directional signals. The missing link between the intracellular domain of EphA receptors and MAPK should be elucidated to clearly explain the molecular events underlying these observations.

It should be noted that, whereas our data demonstrate that activation of MAPK is a necessary component of neurogenesis induced by EphA receptors, it remains to be determined whether activation of MAPK is sufficient for the effect. The
rapid and potent activation of the MAPK cascade by EphA receptors is inconsistent with other receptor tyrosine kinases (17). Previous work has explored the influence of the diffusible factors that, by acting via receptor tyrosine kinases and recruiting the MAPK pathway, regulate the phenotypical potential of neural stem/precursor cells (1). For example, ciliary neurotrophic factor causes transient activation of MAPK that contributes to initiation of glial differentiation of the neural precursor cells from E14 rat (18). Taken together, EphA receptors might work on neural precursor cells through multiple signals that include MAPK to direct the differentiation of neural precursor cells.

Recently, the MAPK cascade was shown to play a role in axonal guidance. Netrin-1-mediated attraction of the growth cones requires activation of MAPK, which directly interacts with the receptor deleted in colorectal cancer (19). This finding may suggest a role for MAPK in growth cone guidance in general, because MAPK activation is known to stimulate cell motility by phosphorylating and activating myosin light chain kinase (20). In regard to the issue above mentioned, it would be interesting to assess whether netrin-1 regulates proliferation or differentiation of neural precursor cells. Although Ras seems not to be responsible for the activation of MAPK in ephrin-A-treated cells, Rap1 could be one of the factors that contribute to the activation of MAPK. It remains unclear yet whether Rap1 regulates ERKs (11) or not (21). Rap1 has been shown to be implicated in a particularly wide range of biological processes, from cell proliferation and differentiation to cell adhesion. During the course of the differentiation of PC12 cells induced by nerve growth factor, Rap1 as well as Ras is activated. Rap1 mediates B-Raf-mediated sustained ERK activation, whereas Ras elicits transient activation of ERK (11). Sustained activation of ERK, which is dependent on Rap, is reported to be the key for the very different outcomes: differentiation or proliferation. Therefore, the Rap1 signal may be an important factor for the new roles of Rap.

Recently, it was demonstrated that ephrin-A3 of astrocytes could induce spine retraction by stimulating EphA4 of neurons (22). Conover et al. (5) reported that ephrin-B of astrocytes stimulated proliferation of neuroblast. These findings suggest a possible involvement of glial cells in controlling the differentiation of neural precursor cells. Although EphA receptors are shown to be involved in the promotion of neurogenesis in the developmental stages, other numerous factors should affect the fate of the neural precursor cells. Uncovering the signaling mechanisms of these molecules will answer the question what determines the fate of the precursor cells such that some of them differentiate into neurons while others become glial cells.

Further work is required to address clearly the molecular mechanisms of ehrinA-EphA. Our work identifies ephrin-A/EphA as possible regulators of the fate of neural precursor cells in the developing central nervous system.

REFERENCES

1. Gage, F. H. (2000) Science 287, 1433–1438
2. Song, H., and Poo, M. (2001) Nat. Cell Biol. 3, 81–88
3. Klein, R. (2001) Curr. Opin. Cell Biol. 13, 196–203
4. Katz, L.C., and Shatz, C. J. (1996) Science 274, 1133–1138
5. Conover, J. C., Doetsch, F., Garcia-Verdugo, J. M., Gale, N. W., Yanopoulos, G. D., and Alvarez-Buylla, A. (2000) Nat. Neurosci. 11, 1091–1097
6. Mias, H., Wei, B. R., Peehl, D. M., Li, G., Alexandrou, T., Schelling, J. R., Rhim, J. S., Sedor, J. R., Burnett, E., and Wang, B. (2001) Nat. Cell Biol. 3, 527–530
7. Lendahl, U., Zimmerman, L. B., and McKay, R. D. (1990) Cell 60, 585–595
8. Miyata, M., Finch, E. A., Khiroug, L., Hashimoto, K., Hayasaka, S., Oda, S., Inouye, M., Takagishi, Y., Augustin, G. J., and Kano, M. (2000) Neuron 28, 233–244
9. Reynolds, B. A., and Weiss, S. (1996) Dev. Biol. 175, 1–13
10. Posner, G., Weber, C. K., Rapp, U. R., and Feller, S. M. (1998) J. Biol. Chem. 273, 24297–24300
11. York, R. D., Yao, H., Dillon, T., Ellig, C. L., Eckert, S. P., McCleskey, E. W., and Stork, P. J. (1998) Nature 392, 622–626
12. Franke, B., Akkerman, J. W., and Bos, J. L. (1997) EMBO J. 16, 252–259
13. Wahl, S., Barth, H., Ciossek, T., Aktories, K., and Mueller, B. K. (2000) J. Cell Biol. 149, 263–270
14. Hall, A. (1998) Science 279, 509–514
15. Shamah, S. M., Lin, M. Z., Goldberg, J. L., Estrach, S., Sahin, M., Hu, L., Bazalakova, M., Neve, R. L., Corfas, G., Dehant, A., and Greenberg, M. E. (2001) Cell 105, 233–244
16. Day, A., and Robbins, S. M. (2000) EMBO J. 19, 5396–5405
17. Marshall, C. J. (1995) Cell 80, 179–185
18. Rajan, P., and McKay, R. D. (1998) J. Neurosci. 18, 3620–3629
19. Forcet, C., Stein, E., Fays, L., Coret, V., Llambi, F., Tessler-Lavigne, M., and Mehlen, P. (2002) Nature 417, 443–447
20. Klemke, R. L., Cai, S., Ghiselli, V., de Lanerolle, P., and Cheresh, D. A. (1997) J. Cell Biol. 137, 481–492
21. Ensinger, J. M., Christensen, A. E., de Rooij, J., van Triest, M., Schwede, F., Genieser, H. G., Doskeland, S. O., Blank, J. L., and Bos, J. L. (2002) Nat. Cell Biol. 4, 901–906
22. Murai, K. R., Nguyen, L. N., Irie, F., Yamaguchi, Y., and Pasquale, E. B. (2003) Nat. Neurosci. 6, 153–160
23. Kaplan, K. B., Bihbins, K. B., Swedlow, J. R., Arnaud, M., Morgan, D. O., and Varmus, H. E. (1994) EMBO J. 13, 4745–4756
EphA Receptors Direct the Differentiation of Mammalian Neural Precursor Cells through a Mitogen-activated Protein Kinase-dependent Pathway
Miwa Aoki, Toshihide Yamashita and Masaya Tohyama

J. Biol. Chem. 2004, 279:32643-32650.
doi: 10.1074/jbc.M313247200 originally published online May 15, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M313247200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 23 references, 9 of which can be accessed free at http://www.jbc.org/content/279/31/32643.full.html#ref-list-1