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The p110γ PI-3 Kinase is Required for the Mechanism by Which the EphA8-induced Neurites are Modulated by Ephrin-A5 Engagement

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This study provides evidence that expression of EphA8 receptor in NG108-15 cells results in a substantial increase in the number of neurite-bearing cells. However, the EphA8-induced neurite outgrowth does not require either ephrin-A5 stimulation or ectopic expression of p110γ PI-3 kinase. In contrast, co-expression of a lipid kinase-inactive p110γ mutant together with EphA8 causes neurite retraction in the presence of ephrin-A5 stimulation. This effect was not observed in the absence of ephrin-A5 stimulation. Significantly, the tyrosine kinase activity of EphA8 is not important for either of these processes. Taken together, our results strongly suggest that p110γ PI-3 kinase is critically involved in the regulatory process by which ephrin-A5 exerts effects on the EphA8-induced neurite outgrowth.

The Eph family comprises at least 14 different receptors and nine ligands, all of which are widely expressed in the central and peripheral nervous systems during development and in the adult (Menzel et al., 2001). Individual members of the Eph family have been implicated in axon guidance as well as other processes such as cell migration, boundary formation through the restriction of cellular intermingling, and angiogenesis (Mellitzer and Wilkinson, 2000). Numerous reports indicate that members of the Eph family exert their function by a repulsive mechanism. Little is known about the Eph signaling mechanism that mediates repulsion, although activation of rho and rho kinase has been implicated in the collapse of growth cones in retinal ganglion axons (Wahl et al., 2000; Shamah et al., 2001). More recently, an inhibition of Ras and Raf signaling has been shown for the EphB2-mediated signal transduction pathway that leads to axon collapse (Elowe et al., 2001). However, in certain cases such as in the vomeronasal system, it appears that members of the EphA family play an attractive rather than repulsive role (Knoll et al., 2001). Identification of the signal transduction pathway that mediates this contradictory effect is likely essential for understanding the mechanistic basis of repulsion versus attraction. The signaling molecules involved in Eph receptor-mediated cell adhesion could be important for analyzing these mechanisms, but the interactions among these molecules are apparently quite complex. For example, the EphB1-promoted attachment of cells to fibronectin in a tyrosine kinase-dependent manner is an essential aspect of this signal transduction mechanism. Also, Nck or the low molecular-weight protein tyrosine phosphatase (LMW-PTP) is also implicated (Huynh-Do et al., 1999). EphB2 was shown to indirectly control integrin activity by inducing R-Ras tyrosine phosphorylation, possibly through the intermediary of the Src homology (SH2) domain-containing Eph receptor binding protein 1 (SHEP1) (Dodelet et al., 1999; Zou et al., 1999). EphA2 was also reported to regulate integrin function by causing the dephosphorylation of focal adhesion kinase (FAK) (Miao et al., 2000). More recently, EphA8 has been shown to enhance integrin activity by a mechanism that requires the p110γ PI-3 kinase but which is independent of tyrosine kinase activity (Gu and Park, 2001). Other studies have also shown that the binding of EphA receptors to ephrin-A-expressing cells leads to β1-integrin-dependent upregulation of the adhesiveness of fibroblast cells and that Fyn or another unidentified protein may play an important role in this process (Davy et al., 1999; Huai and Drescher, 2001). However, it has not been determined whether these signaling proteins are expressed and whether they play a pivotal role in axonal behavior and in the migration of physiologically relevant neurons that express Eph receptors or ephrin ligands.

PI-3 kinases phosphorylate phosphatidylinositide lipids at the D3 position of the inositol ring, producing lipid second messengers that are critical in the transduction of a variety of signals (Cantley, 2002). There are two

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distinct mechanisms by which receptors can trigger increases in PI-3 kinase activity and in the level of 3-OH phosphoinositides. The first involves the interaction of p110-p85 heterodimeric PI-3 kinases with tyrosine-phosphorylated receptors or their substrates, through the SH2 domains of the p85 adaptor subunits. The second involves direct activation of the p110γ isotype through an interaction with Gα or Gβγ subunits, which are released upon agonist activation of receptors linked to G proteins (Tang and Downes, 1997). All studies of the p110γ isotype have focused on pathways that are triggered by G-protein coupled receptors. In this respect, the recent finding that p110γ biochemically links the EphA8 receptor tyrosine kinase to integrin activation suggests that receptor triggered-signaling mechanisms could be more diverse than previously thought. More importantly, the signaling mechanism that is triggered by the EphA8 receptor through the p110γ PI-3 kinase does not require the EphA8 tyrosine kinase function. Although PI-3 kinase enzymes have been intensively studied with respect to the regulation of integrin activity, few reports have addressed the role of the p110γ isoform (Shimizu et al., 1995; Nielsen et al., 1996; Zell et al., 1996). Possibly related, G-protein coupled receptors have been shown to induce actin rearrangement or cell migration through p110γ isoform (Ma et al., 1998). The role of p110γ in cell migration has been studied primarily in neutrophils of p110γ knock-out mice. For example, in p110γ -/- mice phagocyte chemotaxis in response to fMet-Leu-Phe and C5a is reduced, and T lymphocyte development and activation are impaired (Sasaki et al., 2000). However, the causal signaling components downstream of p110γ in this process remain to be identified, although proteins containing phosphoinositide-interacting PH domains are good candidates.

It has been well documented that Eph receptors and their ligands are involved in the repulsive migration of spinal and branchial neural crest cells (Smith et al., 1997) and that integrin activation correlates well with cell migration (Holly et al., 2000). We have previously demonstrated that EphA8 activation could promote cell adhesion and cell migration and that p110γ plays an essential role in this mechanism (Gu and Park, 2001; Gu and Park, 2003). In this report, we show that the EphA8-induced neurites were retracted by ephrin-A5 treatment only when the p110γ PI-3 kinase function was inhibited by expression of its dominant negative mutant. These data suggest that p110γ PI-3 kinase is crucial in the ligand-mediated regulatory process of the EphA8-induced neurites in NG108-15 cells.

Materials and Methods

Cell culture, transfection, and reagents

NG108-15 cells (hybrid of mouse neuroblastoma and rat glioma) were cultured in DMEM (Sigma) containing 10% fetal bovine serum (BioWhittaker) and HAT (100 μM hypoxanthine, 1 μM aminopterin, and 16 μM thymidine) (Invitrogen), as described previously (Nelson et al., 1976). Transient transfection procedures were performed using LipofectAMINE (Invitrogen), according to the manufacturer's instructions. For treatment with preclustered ephrinA5-Fc proteins, purified ephrinA5-Fc (1 μg/ml) was aggregated with anti-human Fc (Jackson Immunoresearch) for 30 min on ice, and stimulated for 30 min or 1 h at 37°C (Gu and Park, 2001).

Neuronal differentiation

For analyzing neuronal differentiation, cells were plated (3×10^5 cells per 60 mm dish) and transiently transfected with individual EphA8 cDNAs subcloned into the pIRES2-EGFP vector (Clontech). At 24 h post-transfection, cells (2×10^4) were seeded onto coverslips coated with fibronectin. After 48 h, green fluorescent cells were observed under a fluorescence microscope (Olympus, IX71 model), and cells containing neurites at least three cell body diameters in length were scored as neurite-bearing cells. Transfected cells that grew neurites were normalized to the number of total green fluorescent cells, and presented as a percentage. For each transfectant, at least 500 transfected cells were counted. Data points are presented as the mean±S.E. of at least three independent experiments.

Results

EphA8-induced neurite outgrowth is not altered by either co-expression of wild type p110γ PI-3 kinase or ephrin-A5 stimulation in NG108-15 cells

NG108-15 cells were transiently transfected with EGFP or EphA8-IRES-EGFP as described in experimental procedures. Cells were cultured on fibronectin-coated cover glasses for two additional days. Very intense green fluorescence was observed, not only in soma, but also neurites of transfected cells, indicating that EGFP is an excellent marker for visualization of neurites sprouting from NG108-15 cells (Fig. 1B). The transfection efficiency varied from 20% to 30% in both vector- and EphA8-transfected cells, based on the relative numbers of GFP-positive cells. We then examined the effects of EphA8 expression on neurite outgrowth. A neurite was defined as a process extending at least three cell diameters from the cell body. The percentage of neurite-bearing cells remained around 3% (n (GFP-positive cells)=500) in GFP-transfected cells (Fig. 1A, lane 1). Interestingly, the percentage of EphA8-transfected cells with neurites (37±3% (n=500)) was much higher than that in vector-transfected cells (Fig. 1A, lane 3). However, the percentage of cells with neurites was not further

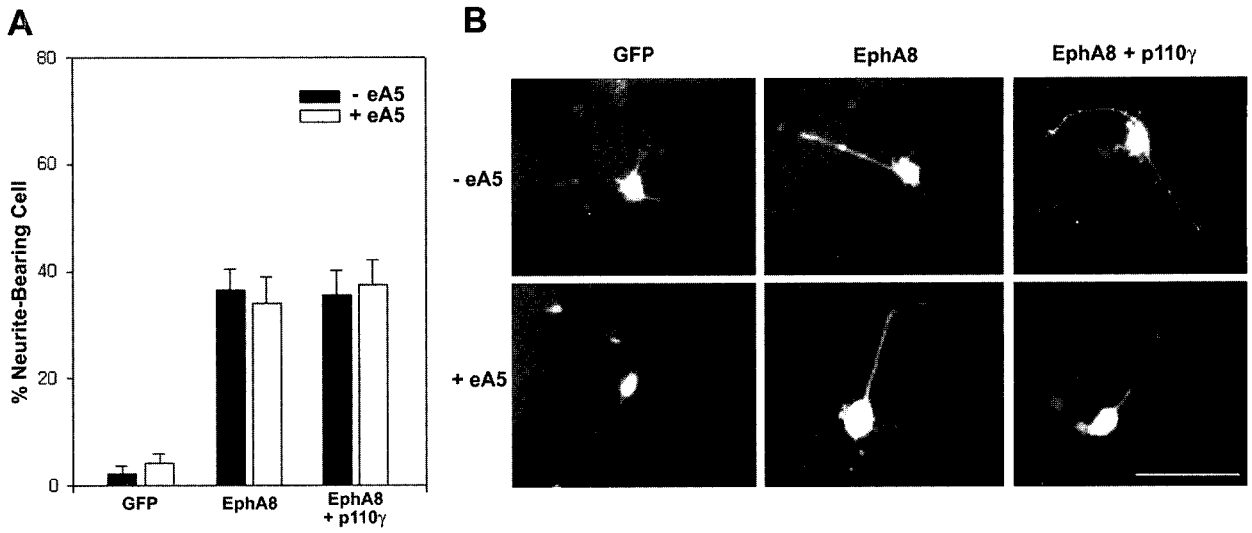

Fig. 1. Induction of neurite outgrowth by wild type EphA8 is not altered by the p110γ protein or ephrin-A5 treatment in NG108-15 cells. (A) EphA8-IRES-EGFP expression construct was transiently transfected with or without the p110γ expression construct into NG108-15 cells using Lipofectamine. The pIRES2-EGFP was used as a control vector without insert cDNA. At 24 h post-transfection, cells were cultured on fibronectin (FN)-coated coverslips. At 48 h post-transfection, cells were treated (+) with preclustered ephrin A5-Fc for 30 min or left untreated (–). Cells containing neurites exceeding three cell body diameters in length were counted as neurite-bearing cells. A total of at least 500 GFP-positive cells were scored in each condition. Data are presented as means±S.E. from at least three independent experiments. (B) Representative photographs of NG108-15 cells expressing EGFP without EphA8 (left panels), and EGFP plus EphA8 with (right panels) or without p110γ (middle panels), described in panel A. At 48 h post-transfection, cells were treated (+) with preclustered ephrin A5-Fc for 30 min or left untreated (–), and examined by fluorescence microscopy. Scale bar = 100 μm.

enhanced in cells co-transfected with both EphA8 and p110γ PI-3 kinase (36±5% (n= 500)) (Fig. 1A, lane 5).

To further investigate whether induction of neurite outgrowth by EphA8 is further enhanced by the ephrin-A5 stimulation, NG108-15 cells were transiently transfected with either EphA8 or p110γ PI-3 kinase expression constructs, followed by incubation with preclustered ephrin A5-Fc for additional 30 min. As shown in Fig. 1, ephrin-A5 stimulation exerted no modulatory effects on EphA8-induced neurite outgrowth

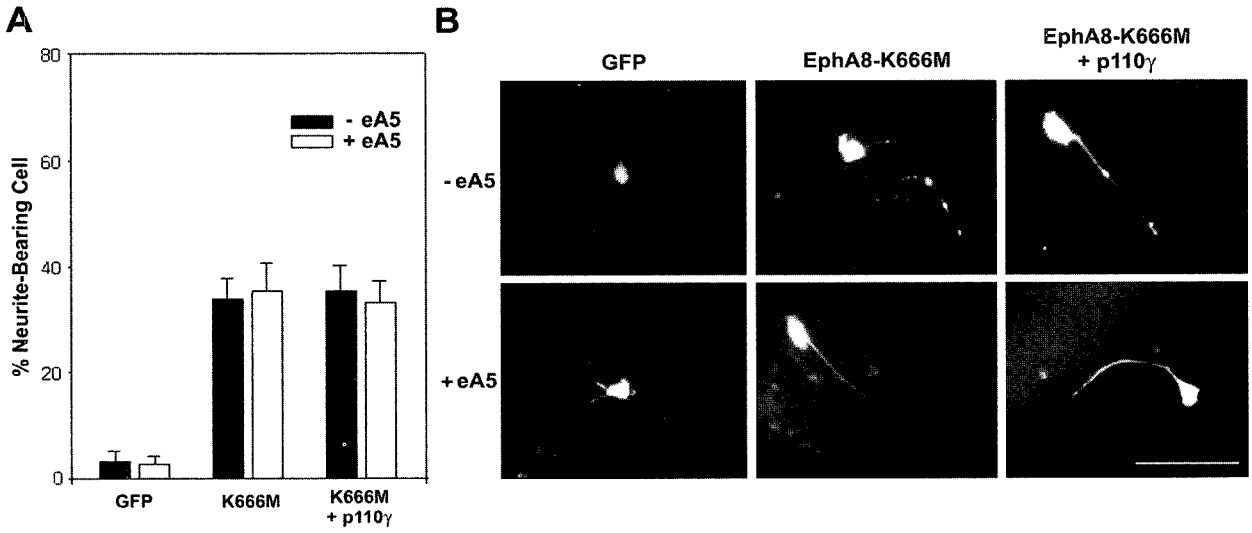

Fig. 2. Induction of neurite outgrowth by kinase-inactive EphA8 is not altered by the p110γ protein or ephrin-A5 treatment in NG108-15 cells. (A and B) *Experiments were performed as described in the legend to Fig. 1A, except that the wild type EphA8 was replaced by the kinase-inactive EphA8 mutant (K666M). A total of at least 500 GFP-positive cells was scored in each condition. Data are presented as means±S.E. from at least three independent experiments. Scale bar = 100 μm.*

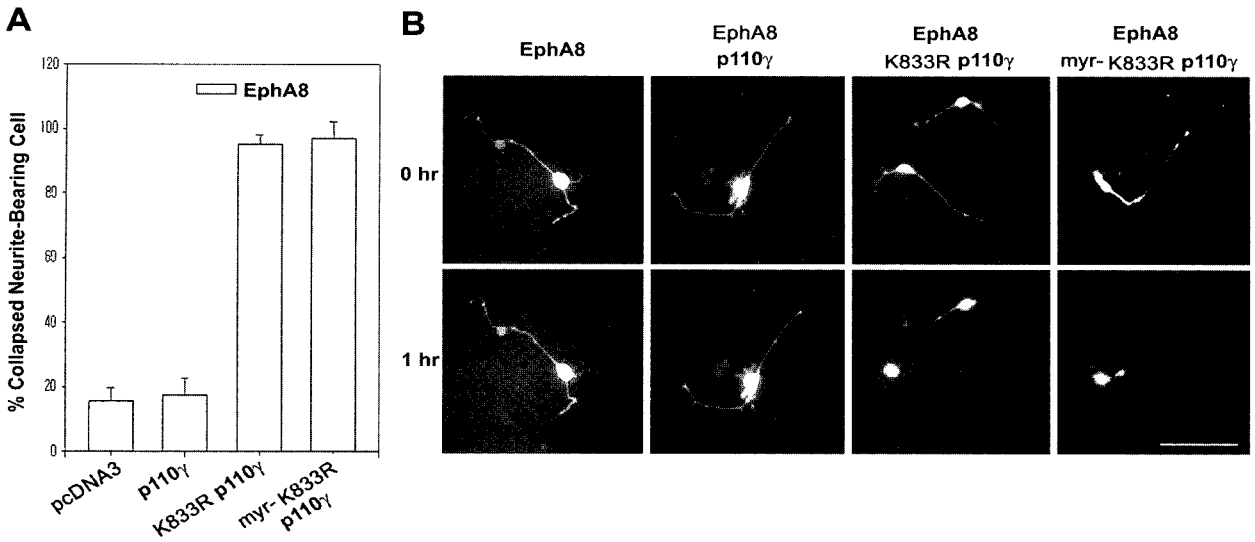

Fig. 3. Eprhin-A5 stimulation results in retraction of the EphA8-induced neurites when the p110γ protein is inhibited by its dominant negative mutants. EphA8-IRES-EGFP expression construct was transfected into NG108-15 cells, together with wild type p110γ or a dominant-negative mutant for p110γ (K833R or myr-K833R). (A) At least 50 GFP-labeled and neurite-elongated cells were observed to see whether their neurite retraction occurred for 1 h after ephrin-A5 treatment. Data are presented as means±S.E. from at least three independent experiments. (B) At 48 h post-transfection, cells were treated (+) with preclustered ephrin A5-Fc for 1 h or left untreated (–), and examined by fluorescence microscopy. Scale bar = 100 μm.

in NG108-15 cells (panel A, lanes 2, 4, and 6; panel B, lower panels).

Similar experiments were performed in transiently transfected NG108-15 cells to determine whether kinase-inactive EphA8 mutant is capable of inducing neurite outgrowth. The EphA8^{K666M} mutant used for this experiment is defective in tyrosine kinase activity due to the presence of Met in place of Lys 666, the putative ATP binding residue. As shown in Fig. 2 and analogous to data obtained with wild type EphA8 cDNA, the kinase-inactive EphA8 mutant induced neurite outgrowth in NG108-15 cells, irrespective of ephrin-A5 stimulation or co-expression of p110γ PI-3 kinase. Taken together, these data indicate that the tyrosine kinase activity of

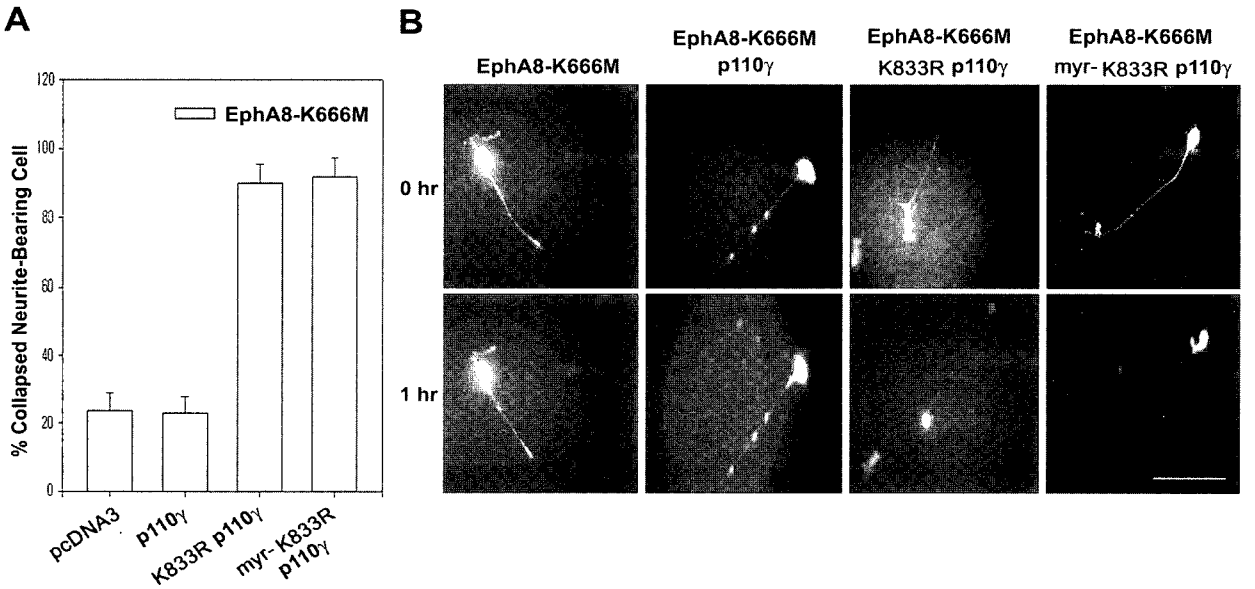

Fig. 4. Eprhin-A5 stimulation results in retraction of the kinase-inactive EphA8-induced neurites when the p110γ protein is inhibited by its dominant negative mutants. (A and B) Experiments were performed as described in the legend to Fig. 2, except that the wild type EphA8 was replaced by the kinase-inactive EphA8 mutant (K666M). A total of at least 50 GFP-positive and neurite-elongated cells were scored in each condition. Data are presented as means±S.E. from at least three independent experiments. Scale bar = 100 μm.

EphA8 is not required for its signaling function leading to neurite outgrowth in NG108-15 cells.

Inhibition of the p110γ protein induces neurite retraction in response to eprhin-A5 stimulation in EphA8-transfected NG108-15 cells

We have previously shown that the EphA8 receptor associates with the p110γ PI-3 kinase and that lipid kinase activity plays an essential role in the EphA8-stimulated adhesion of cells to fibronectin (Gu and Park, 2001; Gu and Park, 2003). Moreover, it was demonstrated that activation of the EphA8 receptor upon stimulation by ephrin-A5 may promote cell migration on fibronectin by increasing the lipid kinase activity of the p110γ protein. These studies suggest that p110γ PI-3 kinase may be involved in EphA8-mediated signaling pathway leading to the neurite elongation. To test this hypothesis, we investigated whether inhibition of p110γ PI-3 kinase using p110γ-K833R mutant causes any modulatory effects on the EphA8-mediated neurite outgrowth. The p110γ -K833R PI-3 kinase variant was mutated for a residue predicted to be required for ATP binding (Lys-833 to Arg) (Gu and Park, 2001). The myr-p110γ-K833R PI-3 kinase mutant is identical with K833R mutant, except that it contains the NH_2-terminal myristoylation motif from c-Src, which is a membrane localization signal (Gu and Park, 2001). As shown in Fig. 3B, co-expression of lipid kinase-inactive p110γ mutants did not cause any modulatory effects on the wild type EphA8-induced neurite outgrowth in the absence of ephrin-A5 stimulation (top panels). However, the neurites induced by EphA8 were retracted upon ephrin-A5 stimulation when the cells were co-transfected with EphA8 and lipid kinase-inactive p110γ (Fig. 3B, bottom panels). This effect was not observed in the cells cotransfected with EphA8 and wild type p110γ. Quantitative analysis revealed that neurite retraction in response to ephrin-A5 treatment occurred in about 95% of NG108-15 cells co-expressing both EphA8 and K833R p110γ mutant (Fig. 3A, lanes 3 and 4).

Similar experiments were performed using $EphA8^{K666M}$ mutant. As shown in Fig. 4 and analogous to data obtained with wild type EphA8 cDNA, the lipid kinase-inactive p110γ mutants induced neurite retraction in response to ephrin-A5 stimulation. Taken together, these data strongly suggest that the lipid kinase activity of p110γ PI-3 kinase is critically involved in a certain process in which ephrin-A5 modulates the EphA8-induced neurites, and that EphA8 tyrosine kinase activity *per se* is not crucial for this process.

Discussion

In the present study, we provide evidence that p110γ PI-3 kinase may be involved in the EphA8-induced neurite outgrowth in NG108-15 cells. Strikingly, the tyrosine kinase function of the EphA8 receptor is not involved in this signal transduction mechanism. Several important issues remain to be determined, including (i) the reason why expression of wild type p110γ does not exert any effects on the EphA8-mediated neurite outgrowth, (ii) the mechanism by which EphA8 induces neurite outgrowth in ligand-independent manner, and (iii) the mechanism by which ephrin ligand stimulation induces neurite retraction only when p110γ lipid kinase activity is impaired.

The first issue concerns why co-transfection of p110γ PI-3 kinase does not influence the EphA8-mediated neurite outgrowth. It is evident that NG108-15 cells do express endogenous p110γ PI-3 kinase. This was confirmed by RT-PCR analysis using specific primers designed for amplifying the mouse p110γ cDNA (data not shown). This suggests that endogenous p110γ PI-3 kinase may be involved in the EphA8-mediated neurite outgrowth. In this respect, ectopic expression of p110γ PI-3 kinase would not have any further effects on the EphA8-mediated neurite outgrowth. However, in NG108-15 cells transfected with the lipid kinase-inactive p110γ, the EphA8-induced neurites were not retracted in the absence of ephrin-A5 stimulation. This result suggests that the p110γ PI-3 kinase is not directly involved in the EphA8-induced neurite outgrowth. Instead, p110γ PI-3 kinase may play a role in maintaining neurites in a way that it modulates F-actin polymerization.

The next question concerns the mechanism by which ephrin-A5 stimulation does not have any modulatory effects on the EphA8-mediated neurite outgrowth. Prevous studies demonstrated that ephrinA5-Fc binds to the EphA8 receptor with high nM affinity (Park and Sanchez, 1997), and that treatment with its aggregated forms stimulates cell migration on the fibronectin substrate (Gu and Park, 2003). These studies clearly indicate that ephrin-A5 is an authentic ligand for EphA8. Our finding that ephrin-A5 does not exert any modulatory effects on the EphA8-mediated neurite outgrowth suggests that the EphA8 receptor alone is sufficient and necessary in inducing the neurite outgrowth. One possibility is that the EphA8 receptor may contain unique structural features to spontaneously form a multimerized complex in the absence of the ligand. Ephrin-A5 is likely to regulate the behavior of growth cones rather than having effects on the neurite elongation, which remains to be determined using growth cone turning assay.

A final issue is how inhibition of p110γ lipid kinase activity leads to neurite retraction in a ligand-dependent manner. Our previous studies demonstrate that coexpression of a catalytically inactive p110γ variant has inhibitory effects on cell migration enhanced by ephrin-A5 (Gu and Park, 2001; Gu and Park, 2003). These studies indicate that a catalytically inactive p110γ variant works as a dominant negative mutant for the

endogenous p110γ protein. Strikingly, our data indicate that inhibition of p110γ lipid kinase does not have any effects on the EphA8-mediated neurite outgrowth in the absence of ligand stimulation. This result could be explained in two different ways.

Firstly, the endogenous p110γ PI-3 kinase is tightly associated with the EphA8 receptor in the absence of ligand so that its dominant negative mutants may not work efficiently. In the presence of the ligand, the structure of EphA8 is altered so that the dominant negative mutants efficiently block the endogenous p110γ PI-3 kinase function. Secondly, in the absence of the ligand, the EphA8 receptor may induce neurite outgrowth without requirement of p110γ PI-3 kinase function. In contrast, the ephrin ligand may induce the aggregated EphA8 complexes, which recruits the endogenous p110γ PI-3 kinase from the cytoplasm to the plasma membrane. In this case, local production of phosphoinositides in response to ephrin-A5 stimulation may be important in processes such as maintenance of the elongated neurites. Testing of this hypothesis would require biochemical experiments using a sensitive antibody against the murine p110γ PI-3 kinase, which is under development in our laboratory.

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