

# The p110 $\gamma$ PI-3 kinase is required for EphA8-stimulated cell migration

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Received 11 November 2002; revised 6 January 2003; accepted 7 January 2003

First published online 11 March 2003

Edited by Beat Imhof

**Abstract** This study provides evidence that treatment with pre-clustered ephrin A5-Fc results in a substantial increase in the stability of the p110 $\gamma$  PI-3 kinase associated with EphA8, thereby enhancing PI-3 kinase activity and cell migration on a fibronectin substrate. In contrast, co-expression of a lipid kinase-inactive p110 $\gamma$  mutant together with EphA8 inhibits ligand-stimulated PI-3 kinase activity and cell migration on a fibronectin substrate, suggesting that the mutant has a dominant negative effect against the endogenous p110 $\gamma$  PI-3 kinase. Significantly, the tyrosine kinase activity of EphA8 is not important for either of these processes. Taken together, our results demonstrate that the stimulation of cell migration on a fibronectin substrate by the EphA8 receptor depends on the p110 $\gamma$  PI-3 kinase but is independent of a tyrosine kinase activity. © 2003 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Eph; Ephrin; p110 $\gamma$  PI-3 kinase

## 1. Introduction

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 [1–3]. 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 [4]. 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 [5,6]. More recently, an inhibition of Ras and Raf signaling has been shown for the EphB2-mediated signal transduction pathway that leads to axon collapse [7]. 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 [8]. 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 at-

tachment 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 is also implicated [9–11]. 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) [12,13]. EphA2 was also reported to regulate integrin function by causing the dephosphorylation of focal adhesion kinase (FAK) [14]. More recently, EphA8 has been shown to enhance integrin activity by a mechanism that requires the p110 $\gamma$  PI-3 kinase but which is independent of tyrosine kinase activity [15]. Other studies have also shown that the binding of EphA receptors to ephrin-A-expressing cells leads to  $\beta$ 1-integrin-dependent upregulation of the adhesiveness of fibroblast cells and that Fyn or another unidentified protein may play an important role in this process [16,17]. 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 phosphatidylinositol lipids at the D3 position of the inositol ring, producing lipid second messengers that are critical in the transduction of a variety of signals [18]. There are two 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 p110 $\gamma$  isotype through an interaction with G $\alpha$  or G $\beta\gamma$  subunits, which are released upon agonist activation of receptors linked to G proteins [19–22]. All studies of the p110 $\gamma$  isotype have focused on pathways that are triggered by G protein-coupled receptors. In this respect, the recent finding that p110 $\gamma$  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 p110 $\gamma$  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 p110 $\gamma$  isoform [23–27]. Possibly related, G protein-coupled receptors have been shown to induce actin rearrangement or cell migration through p110 $\gamma$  isoform [28]. The role of p110 $\gamma$  in cell migration has been studied primarily in neutrophils of p110 $\gamma$

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knock-out mice. For example, in p110 $\gamma$   $-/-$  mice phagocyte chemotaxis in response to fMet-Leu-Phe and C5a is reduced, and T lymphocyte development and activation are impaired [29]. However, the causal signaling components downstream of p110 $\gamma$  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 [30–32] and that integrin activation correlates well with cell migration [33–35]. We have previously demonstrated that the EphA8 receptor stimulates integrin activity through the intermediation of the p110 $\gamma$  PI-3 kinase isotype [15]. We therefore asked whether EphA8 activation could promote cell migration and if so, whether p110 $\gamma$  plays an essential role in this mechanism. In this report, we show that the ligand-stimulated EphA8 receptor promotes the stabilization of the p110 $\gamma$  protein through complex formation. Concomitantly, Eph receptor-associated lipid kinase activity is substantially elevated, and the ability of cells to migrate on fibronectin is also enhanced. In addition, the stimulation of cell migration depends on the lipid kinase activity of the p110 $\gamma$  PI-3 kinase. These data suggest a signaling pathway that begins with activation of the EphA8 receptor and culminates in cell migration, one that sequentially involves p110 $\gamma$  PI-3 kinase and integrin activation.

## 2. Materials and methods

### 2.1. Cell culture and transfection

HEK 293 cells were cultured in alpha-MEM (Sigma) containing 10% heat-inactivated fetal bovine serum (BioWhittaker). Transient transfections were carried out using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's instructions. For treatment with preclustered ephrin A5-Fc proteins, purified ephrin A5-Fc (5  $\mu$ g/ml) [36] was aggregated using anti-human Fc (Jackson ImmunoResearch) for 30 min on ice and stimulations were for 30 min at 37°C.

### 2.2. Expression vectors

The murine wild-type and kinase-inactive (K666M) EphA8 cDNA tagged with the nine-amino-acid HA epitope (YPYDVPDYA) at its COOH termini have been described elsewhere [15]. The murine wild-type p110 $\gamma$  cDNA tagged with the eight-amino-acid FLAG epitope (DYKDDDDK) at its COOH terminus has been described elsewhere [15], together with its variants, including the lipid kinase-inactive K833R and the membrane-targeted Myr-p110 $\gamma$ .

### 2.3. Immunoprecipitation, Western blotting and PI-3 kinase assay

Immunoprecipitation and Western blot analysis were performed as previously described [37]. PI-3 kinase activity was measured as previously described with some modifications [38]. Briefly, proteins were immunoprecipitated by incubating cell extracts with an anti-HA antibody and protein A-Sepharose. The Sepharose beads were washed three times with HNTG buffer, once with 1% Nonidet P-40 in phosphate-buffered saline (PBS), once with 100 mM Tris-HCl (pH 7.5) containing 500 mM LiCl and once with 50 mM Tris-HCl buffer (pH 7.2) containing 150 mM NaCl. After the last wash, the beads were resuspended in kinase buffer (20 mM HEPES, pH 7.2, 50 mM NaCl and 1 mM EGTA) containing 4  $\mu$ g phosphatidylinositol (PI, Sigma), 10  $\mu$ M ATP, 5 mM MnCl<sub>2</sub>, and 10  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP and incubated for 20 min at 30°C. The reaction was stopped by the addition of 100  $\mu$ l 1 N HCl and 200  $\mu$ l of a 1:1 mixture of chloroform and methanol. The lipids were extracted and resolved on potassium oxalate-pre-treated thin layer chromatography (TLC) plates (EM Science) with 35 ml 2N acetic acid and 65 ml 1-propanol as the mobile phase. Dried plates were exposed to Kodak X-ray film for autoradiography.

### 2.4. Cell migration assay

Cell migration assays were performed using modified Boyden cham-

bers (tissue culture treated, 6.5 mm diameter, 10  $\mu$ m thickness, 8  $\mu$ m pores, Transwell Costar Corp., Cambridge, MA, USA) containing a polycarbonate membrane [39,40]. The underside of the upper chamber membrane was coated with 10  $\mu$ g/ml fibronectin for 2 h at 37°C, rinsed once with PBS, and placed into the lower chamber in 500  $\mu$ l tissue culture medium containing 10  $\mu$ g/ml fibronectin. Cells were removed from culture dishes by brief trypsinization, washed once with PBS and resuspended in culture medium. Fifty thousand cells were added to the top of each migration chamber and allowed to migrate to the underside of the top chamber for 2 h. Non-migratory cells on the upper membrane surface were removed with a cotton swab, and migratory cells attached to the bottom surface of the membrane were stained with 0.1% crystal violet in 0.1 M borate, pH 9.0, and 2% ethanol for 20 min at room temperature. The migratory cells on each membrane were counted with an inverted microscope. Each determination represents the average of three individual wells, and error bars represent S.E.

### 2.5. Antibodies

The polyclonal rabbit antibody specific for the juxtamembrane domain of EphA8 was described previously [37]. Polyclonal rabbit anti-HA antibody was obtained from Zymed. Monoclonal mouse anti-FLAG antibody was from Sigma. The HRP-conjugated secondary antibodies were from Amersham Pharmacia Biotech.

## 3. Results

### 3.1. The stability of p110 $\gamma$ complexed with wild-type EphA8 is increased by stimulation with ephrin A5

We have previously shown that the EphA8 receptor associates with the p110 $\gamma$  PI-3 kinase and that lipid kinase activity plays an essential role in the EphA8-stimulated adhesion of cells to fibronectin [15]. These results led us to postulate 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 p110 $\gamma$  protein. To test this experimentally, we created murine p110 $\gamma$  PI-3 kinase cDNA variants that encode the NH<sub>2</sub>-terminal myristoylation motif from c-Src [myr-p110 $\gamma$ ] [41,42], which is a membrane localization signal, or that are mutated for a residue predicted to be required for binding ATP [Lys-833 to Arg; p110 $\gamma$ -K833R] or both [myr-p110 $\gamma$ -K833R] [21]. To assess the ability of these p110 $\gamma$  PI-3 kinase variants to form a stable complex with the EphA8 receptor under conditions of ephrin A5 stimulation, we transiently co-expressed wild-type EphA8 with the p110 $\gamma$  PI-3 kinase variants in HEK293 cells. The transfected cells were stimulated with preclustered ephrin A5-Fc soluble ligand for 30 min or left untreated, and cell lysates were prepared. Ten percent of the whole cell lysate was directly assayed using the anti-FLAG antibody as a probe, which detects the murine p110 $\gamma$  PI-3 kinase tagged with the FLAG epitope at the carboxyl terminus (Fig. 1B). Interestingly, the steady state level of each p110 $\gamma$  variant, and of wild-type p110 $\gamma$ , was significantly higher in cells stimulated with ephrin A5 than in unstimulated cells (Fig. 1B, lanes 3, 5, 7 and 9). In the absence of EphA8, no increase in the level of p110 $\gamma$  protein in response to treatment with ephrin A5 was observed (data not shown). It also appeared that the level of expression of each of the variant p110 $\gamma$  proteins differed. For example, the membrane-targeted wild-type p110 $\gamma$  protein was consistently expressed at the highest level (Fig. 1B, lanes 5 and 6). To exclude the possibility that the different levels of p110 $\gamma$  are due to differences in transfection efficiency, another 10% of each whole cell lysate preparation was analyzed by immunoblotting using an anti-EphA8 antibody as a probe. As shown in Fig. 1C, the levels of expression of the EphA8 receptor were not signifi-

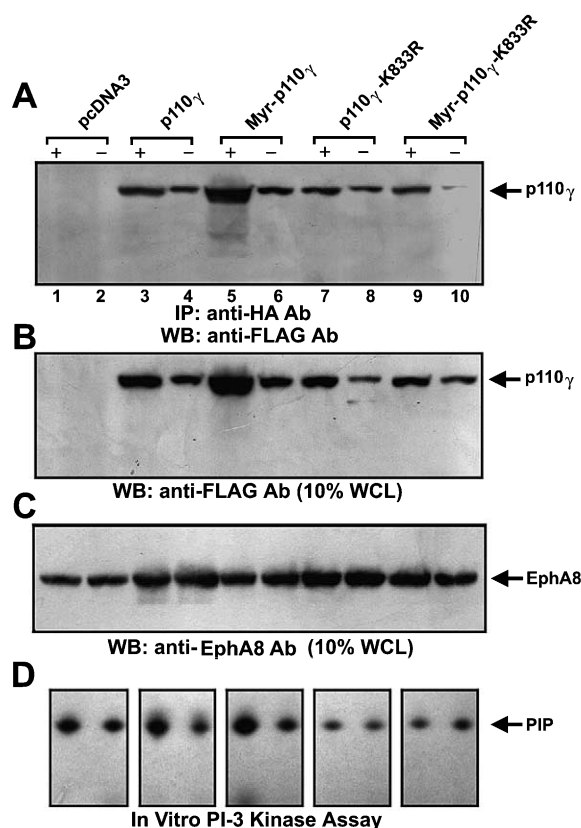


Fig. 1. The p110γ PI-3 kinase associates with the wild-type EphA8 receptor. A: Wild-type EphA8-HA construct and each of the individual p110γ cDNA variants were co-transfected into HEK293 cells in lanes 3–10, whereas pcDNA3 was transfected as a control in lanes 1 and 2. Forty-eight hour posttransfection, the cells were treated (+) with preclustered ephrin A5-Fc for 30 min at 37°C or were untreated (–). Proteins from cell lysates were immunoprecipitated with anti-HA antibody and then analyzed by immunoblotting with anti-FLAG antibody as a probe. B: 10% of the whole cell lysates (approximately 20 μg) described above were directly analyzed by immunoblotting with anti-FLAG antibody. C: 10% of the whole cell lysates (approximately 20 μg) described above were analyzed by immunoblotting with anti-EphA8 antibody. D: Proteins from cell lysates were immunoprecipitated with anti-HA antibody and then incubated with 4 μg PI and 10 μCi [ $\gamma$ -<sup>32</sup>P]ATP for 10 min, and the reaction was analyzed by TLC followed by autoradiography.

cantly altered by ephrin A5 stimulation nor did they correlate with the levels of expression of the individual p110γ variants. Together, these results suggest that the level of expression of each p110γ variant is specifically due to its respective alteration. In addition, our results suggest that stimulation by ephrin A5 stabilizes both wild-type and variant p110γ proteins, possibly by promoting stable complex formation with the EphA8 receptor.

To examine the ability of each of the p110γ variants to associate with the activated wild-type EphA8 receptor in our co-transfection system, we carried out co-immunoprecipitation experiments using anti-HA antibody to harvest protein complexes containing the EphA8 protein tagged with the HA epitope. The anti-HA immunocomplexes were subjected to stringent washing conditions and then to Western blot analysis using the anti-FLAG antibody as a probe. As shown in Fig. 1A, the level of each of the p110γ variants associated with EphA8 was substantially higher in cells stimulated by ephrin A5 (see lanes 3, 5, 7 and 9). Wild-type p110γ, p110γ-K833R

mutant, and myr-p110γ-K833R mutant were associated with EphA8 up to approximately 2.5-fold higher, whereas myr-p110γ protein was up to five-fold higher. A time course study with preclustered ephrin A5-Fc revealed that the steady state level of p110γ PI-3 kinase increased within 5–30 min of ligand stimulation (data not shown). The same immunoprecipitates were then assayed for their ability to phosphorylate PI in vitro (Fig. 1D). In cells expressing EphA8 with a vector control, PI-3 kinase activity, as indicated by the appearance of PI(3)P, was reproducibly increased by ephrin A5 stimulation (Fig. 1D, lane 1). This increased activity can be explained by our previous report that preclustered ephrin A5-Fc results in a substantial increase in the stability of the endogenous p110γ PI-3 kinase associated with EphA8. As expected from the observation that the association of p110γ protein with EphA8 is stabilized by ephrin A5 stimulation, PI-3 kinase activity was high in response to ephrin A5 stimulation in cells expressing EphA8 with either the wild-type p110γ (2.5-fold

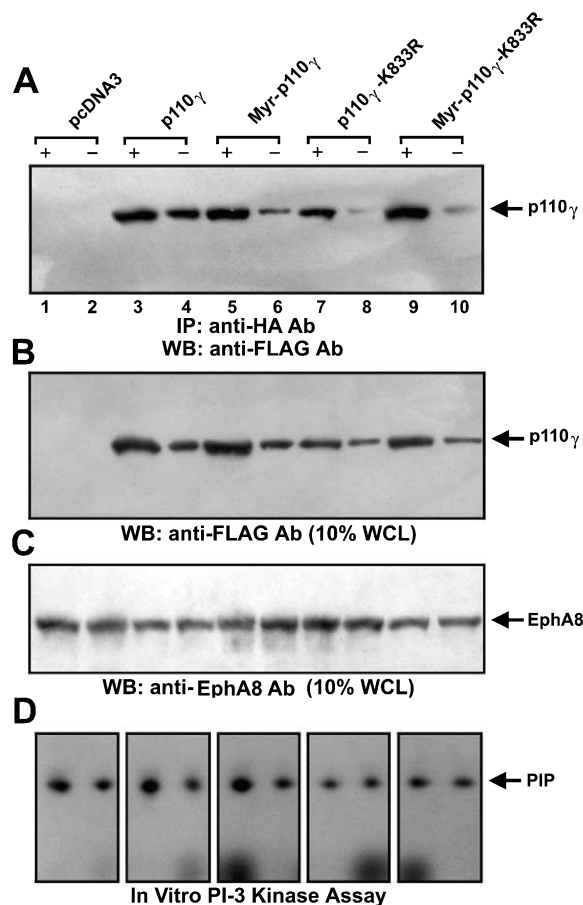


Fig. 2. The p110γ protein associates with the kinase-inactive EphA8 receptor. Experiments were performed essentially as described in the legend to Fig. 1, except that the kinase-inactive EphA8-K666M mutant was used instead of the wild-type EphA8. A: Proteins from cell lysates were immunoprecipitated with anti-HA antibody and then analyzed by immunoblotting with anti-FLAG antibody as a probe. B: 10% of the whole cell lysates (approximately 20 μg) were directly analyzed by immunoblotting with anti-FLAG antibody. C: 10% of the whole cell lysates (approximately 20 μg) were analyzed by immunoblotting with anti-EphA8 antibody. D: Proteins from cell lysates were immunoprecipitated with anti-HA antibody, and PI-3 kinase activity was measured as described in the legend to the previous figure.



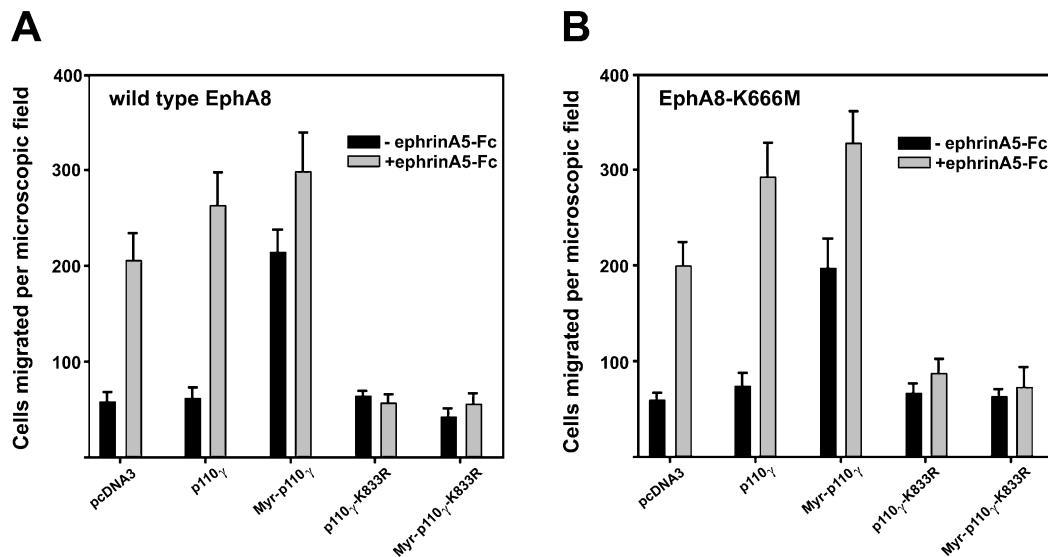


Fig. 3. A p110 $\gamma$  PI-3 kinase is required for cell migration induced by EphA8 expression and ephrin A5 stimulation. Cells were allowed to migrate for 2 h on a fibronectin substrate after exposure to preclustered ephrin A5-Fc fusion protein for 30 min. Migration studies were performed using Transwell migration chambers coated with fibronectin as described in Section 2. A: Wild-type EphA8 and the p110 $\gamma$  cDNA variants were co-transfected into HEK293 cells. Forty-eight hour posttransfection, the cells were treated with preclustered ephrin A5-Fc for 30 min at 37°C or were untreated. Cells were briefly treated with trypsin and subjected to cell migration assays. B: Experiments were performed essentially as described in panel A, except that the kinase-inactive K666M EphA8 mutant was used instead of the wild-type EphA8 cDNA. The results represent the mean  $\pm$  S.E. of three independent experiments. In each experiment, four random fields per well were scored.

increase) or the membrane-targeted p110 $\gamma$  PI-3 kinase (four-fold increase) (Fig. 1D, lanes 3 and 5). In contrast, the presence of the lipid kinase-inactive K833R mutants strongly inhibited PI-3 kinase activity stimulated by ephrin A5 regardless of the membrane localization signal (Fig. 1D, lanes 7 and 9). Taken together, these results reveal that treatment with ephrin A5 rapidly enhances the stability of the p110 $\gamma$  protein complexed with the EphA8 receptor, thereby promoting its lipid kinase activity.

### 3.2. The stability of p110 $\gamma$ complexed with kinase-inactive EphA8 is also increased by ephrin A5 stimulation

It was previously shown that the association of the p110 $\gamma$  PI-3 kinase with EphA8 does not require the tyrosine kinase activity of EphA8 [15]. To further assess whether the expression of the kinase-inactive EphA8 affects the stability and activity of the p110 $\gamma$  variants, we carried out identical experiments except with the kinase-inactive K666M EphA8 mutant, which contains Met in place of Lys 666, the putative ATP binding residue. In co-transfected cells, the level of expression of each of the p110 $\gamma$  variants was elevated by ephrin A5 stimulation (Fig. 2B, lanes 3, 5, 7 and 9), whereas the expression of the kinase-inactive EphA8 receptor was not significantly altered by ephrin A5 stimulation or by the p110 $\gamma$  variant (Fig. 2C). In addition, ephrin A5 stimulation caused an increase in the steady state level and lipid kinase activity of both the wild-type and membrane-targeted p110 $\gamma$  associated with the kinase-inactive EphA8 (Fig. 2A,D, lanes 3 and 5). In contrast, by tightly binding to the EphA8 receptor, the lipid kinase-inactive p110 $\gamma$  mutant efficiently blocked PI-3 kinase activity stimulated by ephrin A5 (Fig. 2A,D, lanes 7 and 9). Taken together, these findings are consistent with our previous result that the mechanism by which ephrin A5 promotes the stable association of EphA8 and p110 $\gamma$  PI-3 kinase is independent of the EphA8 auto-kinase activity.

### 3.3. The lipid kinase activity of p110 $\gamma$ is essential for cell migration promoted by the ligand-stimulated EphA8 receptor

To determine whether the stability of the p110 $\gamma$  PI-3 kinase complexed with Eph receptor correlates with its ability to promote cell migration, transiently co-transfected cells were analyzed by a modified Boyden chamber assay [39,40] using fibronectin as an attractant. In the absence of exogenous p110 $\gamma$ , cells expressing either the wild-type EphA8 or kinase-inactive EphA8 receptor exhibited an increased level of migration in response to ephrin A5 stimulation, as compared with unstimulated cells (Fig. 3A,B, second bar of each panel). Likewise, the co-expression of either wild-type or membrane-targeted p110 $\gamma$  with the EphA8 receptor markedly promoted cell migration in response to ephrin A5 stimulation (Fig. 3A,B, fourth and sixth bars of each panel). These effects were independent of EphA8 kinase activity and were not observed in the absence of EphA8 expression (data not shown). In addition, the stimulation of cell migration correlated well with the quantitative level and *in vitro* lipid kinase activity of the p110 $\gamma$  PI-3 kinase associated with the EphA8 receptor (see Figs. 1 and 2, panels A and D, lanes 3 and 5). Interestingly, the membrane-targeted p110 $\gamma$  PI-3 kinase enhanced cell migration even without ephrin A5 stimulation, as compared with the wild-type p110 $\gamma$  (Fig. 3A,B, fifth bar of each panel), and the extent of cell migration was independent of the amount of protein associated with EphA8. This result suggests that the membrane localization of the p110 $\gamma$  PI-3 kinase has complex consequences for cell migration. As shown in Figs. 1 and 2, lipid kinase-inactive p110 $\gamma$  protein forms a tight complex with the EphA8 receptor in response to ephrin A5 stimulation, thereby inhibiting the endogenous p110 $\gamma$  protein in a dominant negative manner. Consistent with these results, expression of K833R or myr-K833R mutant p110 $\gamma$  proteins efficiently blocked ephrin A5-stimulated cell migration (Fig.

3A,B, eighth and tenth bars of each panel). Together, these results demonstrate that the lipid kinase activity of the p110 $\gamma$  PI-3 kinase is required for the ephrin A5-stimulated migration of cells on fibronectin and that this signal transduction pathway does not require the tyrosine kinase activity of the EphA8 receptor.

#### 4. Discussion

Previous studies of p110 $\gamma$  PI-3 kinase isotypes have focused on the heterotrimeric G protein-linked receptor signaling mechanism by which the  $\alpha$  or  $\beta\gamma$  subunit or both directly modulate the activity of p110 $\gamma$ . [19–22]. Interestingly, our recent studies revealed that the p110 $\gamma$  PI-3 kinase plays an essential role in the EphA8-promoted cell adhesion to fibronectin [15]. In the present study, we further showed that the p110 $\gamma$  PI-3 kinase also plays a crucial role in the EphA8-stimulated migration of cells on fibronectin. Taken together, the above findings delineate a pathway, leading from activation of the EphA8 receptor to an increase in cell migration, that sequentially involves EphA8, p110 $\gamma$  and integrin. In this signal transduction mechanism, the p110 $\gamma$ -EphA8 complex is stabilized, and thus possesses higher lipid kinase activity, when stimulated by ephrin A5. Strikingly, the tyrosine kinase function of the EphA8 receptor is not involved in this signal transduction mechanism. These observations raise several important issues, including (i) the mechanism by which the EphA8 receptor stabilizes p110 $\gamma$ , (ii) the mechanism by which p110 $\gamma$  stimulates cell migration, and (iii) the physiological relevance of the p110 $\gamma$  protein in the migration of neuronal cells expressing EphA8.

The first issue concerns how ephrin A5 stabilizes the p110 $\gamma$  protein–EphA8 receptor complex. We previously showed that the stable association of EphA8 and p110 $\gamma$  involves the juxtamembrane segment of EphA8 and that the level of expression of EphA8 does not affect p110 $\gamma$  mRNA levels [15]. The juxtamembrane region may be masked when the EphA8 extracellular domain is unbound by ligand but may be exposed to p110 $\gamma$  upon ligand binding. The multimeric form of the EphA8 receptor, which is induced by ephrin ligand binding, may protect the tightly associated p110 $\gamma$  PI-3 kinase from degradation. Preaggregation of ephrin A5-Fc was required for observing both p110 $\gamma$  PI-3 kinase stabilization and the cell migration effect, suggesting that only superaggregation of EphA8 plays an essential role in p110 $\gamma$  stability (data not shown). In this protein complex, lipid kinase activity may partially contribute to the stability of p110 $\gamma$ , as manifested by the lower level of expression of the K833R mutant (Figs. 1 and 2, panels A and B, lanes 8 and 10). In our previous report, we claimed that kinase-inactive p110 $\gamma$  does not associate with EphA8. This misleading conclusion most likely results from the lower sensitivity of the FLAG antibody used for p110 $\gamma$  immunoprecipitation and the relatively low expression level of kinase-inactive p110 $\gamma$ . In its active conformation, the p110 $\gamma$  lipid kinase may more readily form a stable complex with EphA8, although this conformation is not absolutely required for complex formation. The p101 regulatory subunit may play an important role in the heterotrimeric G protein-linked receptor signaling pathway that leads to the activation of p110 $\gamma$  [43,44]. However, we observed that co-expression of p101 together with p110 $\gamma$  has no effect on EphA8-stimulated cell migration (data not shown) or on

p110 $\gamma$  stability, suggesting that p101 is not required for the EphA8-mediated modulation of p110 $\gamma$  PI-3 kinase activity.

The next question concerns the mechanism by which p110 $\gamma$  promotes cell migration in response to stimulation by the ephrin A5 ligand. Our studies suggest a role for phosphoinositides in the inside-out signaling mechanism employed by EphA8 in integrin activation [15]. The production of phosphoinositides in response to ephrin A5 stimulation is crucial for promoting cell migration because treatment with pharmacologic inhibitors of PI-3 kinases (such as wortmannin or LY294002) (data not shown) or co-expression of a catalytically inactive p110 $\gamma$  variant have inhibitory effects on cell migration enhanced by ephrin A5. Phosphoinositides may act as second messengers by binding and modulating PH-domain-containing proteins such as Vav. Indeed, Vav, the Rac GEF, has been implicated as an important mediator of a signal transduction pathway leading from activation of the chemoattractant formyl peptide receptor to cytoskeletal reorganization, through the intermediation of p110 $\gamma$  PI-3 kinase and Rac [28]. However, we have not observed dramatic changes in actin polymerization in response to ephrin A5 stimulation (data not shown). Significant changes in the activity of Rho, Rac or the Cdc42 small GTP binding protein also have not been detected (data not shown). These results suggest that the EphA8 receptor signals the p110 $\gamma$  PI-3 kinase in a manner different from the stimulation of G protein-coupled receptors. Although it remains to be determined whether the activated EphA8 receptor can mediate cell migration through other PH-domain proteins, active phospholipid products may also directly promote integrin conformational changes, leading to an increase in cell migration. FAK has also been implicated in integrin-mediated cell migration [34,45], suggesting that it may be involved in EphA8-stimulated cell migration. However, ephrin A5 does not induce significant changes in FAK tyrosine phosphorylation under conditions that promote cell migration (data not shown). Furthermore, co-expression of the dominant negative FRNK [40,46–48], an N-terminally truncated form of FAK, does not inhibit EphA8-stimulated cell migration (data not shown), suggesting that FAK and EphA8 mediate cell migration by independent and parallel mechanisms. This may explain why the dominant negative forms of p110 $\gamma$  do not further inhibit the basal level of cell migration, which could be mediated by other signaling proteins such as FAK.

A final issue is the biological and physiological relevance of EphA8-stimulated p110 $\gamma$  for cell migration in vivo. Eph receptors are well documented in guiding the migration of spinal or branchial neural crest cells, and several reports have shown that ephrin ligands induce the repulsive migration of neural crest cells [30–32]. In addition, cell migration was also stimulated when cells were plated for 30 min on substrate-immobilized ephrin A5-Fc protein, confirming the physiological importance of the results obtained with preaggregated ephrin A5-Fc ligand (data not shown). We also observed that under conditions that promote cell migration, the coating of ephrin A5 ligands, together with fibronectin, on Transwell membranes efficiently prevents cells from migrating towards the bottom well (data not shown). These results suggest that the activation of Eph receptors by ephrin ligand stimulates cell migration along the extracellular matrix but not toward the ligand-expressing region. In this mechanism, the p110 $\gamma$  PI-3 kinase may represent an important transducer in a pathway

leading from Eph receptor activation to cell migration. We also observed that the EphA4 receptor associates with p110 $\gamma$  irrespective of its tyrosine kinase activity, suggesting that the integrin activation property of p110 $\gamma$  is shared by other members of the EphA family. The EphA8 gene is expressed only in a subpopulation of midbrain neurons during murine embryonic development, and it has been suggested that the EphA8 receptor plays an important role in axonal pathfinding in a subset of tectal commissural axons [49]. We could detect the p110 $\gamma$  mRNA in tectal neurons by RT-PCR (reverse transcription-polymerase chain reaction) but not in whole mount embryos by in situ RNA hybridization (data not shown). This observation suggests that the p110 $\gamma$  protein is present at low levels or is unstable and that it may be increased by stimulation with ephrin ligand. A test of this hypothesis would require a sensitive antibody against the murine p110 $\gamma$  PI-3 kinase, which is under development in our laboratory. In addition, to elucidate the biological relevance of our current findings, we have initiated studies of the p110 $\gamma$  variants expressed under the control of the EphA8 promoter in transgenic mice.

**Acknowledgements:** We are indebted to Sungbo Shim, Jongdae Sin, and Jiyeon Kim for their excellent technical assistances. The authors wish to acknowledge the financial support of the Korea Research Foundation made in the Program Year 2001 (DP0433).

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